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FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

MOLECULAR IDENTIFICATION AND INFECTION
MONITORING OF *MONILINIA* SPP. CAUSING
POSTHARVEST BROWN ROT IN STONE FRUIT

TIPO TESI: Sperimentale

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ABSTRACT

Brown rot of stone fruit caused by *Monilinia* species results in major preharvest and postharvest losses in Italy and in the rest of the world. In the summer of 2018 and 2019 a study was conducted to investigate *Monilinia* species distribution in two peach orchards and also monitor infection incidence after application of commercial synthetic fungicides and alternative compounds. Species identification was carried out through use of multiplex polymerase chain reaction (PCR). *Monilinia laxa* was the most abundant species found in both orchards while *Monilinia fructigena* was not as widespread. *Monilinia fructicola* was detected for the first time in high numbers on one orchard in 2019, while in previous season no isolates had tested positive for the species, whereas on another orchard only seven samples tested positive for *M. fructicola*. . Additionally, 3 samples were positive for mixed infection of *M. fructigena* and *M. fructicola*. The effectiveness against brown rot of two fungicides with active ingredients fluopyram (17.7%) and tebuconazolo (17.7%) and fenpyrazamine (50%) as well as alternative compounds sulphur, chitosan and potassium bicarbonate, sprayed at pit hardening and 20 days before harvest, were observed during multiple samplings. Latent infection incidence was overall low, in any case chemical treatments had the most effective control, and sulphur exhibited the highest infection incidence. Chitosan was more effective in reducing field infection incidence amongst the alternative compounds. On the contrary, potassium bicarbonate had the highest incidence in the field. None of the alternative compounds were effective in controlling postharvest infection, unlike chemicals treatments. Generally, chemicals were not as effective in controlling brown rot infection as expected to be, thus further studies should be conducted to investigate fungicide resistance of these species to commercial fungicide products used in Italy. Furthermore, improved and intensive management strategies must be implemented urgently to limit further spread of *M. fructicola* within the orchards and nearby areas where it has been detected.

Keywords: Alternative compounds, Disease incidence, Latent infections, *Monilinia*, Stone fruit

RIASSUNTO

Il marciume bruno delle drupacee causato dalle specie *Monilinia* provoca gravi perdite di produzione in preraccolta e postraccolta in Italia e nel resto del mondo. Nell'estate del 2018 e del 2019 è stato condotto uno studio per valutare la distribuzione delle specie di *Monilinia* in due frutteti e monitorare la diffusione della malattia dopo l'applicazione di fungicidi sintetici commerciali e composti alternativi. L'identificazione delle specie è stata effettuata tramite multiplex PCR. *Monilinia laxa* è risultata la specie più frequente in entrambi i frutteti mentre *Monilinia fructigena* la meno diffusa. *Monilinia fructicola* è stata rilevata per la prima volta nel 2019 in gran numero in uno dei due frutteti, mentre nella stagione precedente nessun isolato è risultato positivo per questa specie. Nell'altro frutteto solo sette campioni sono risultati positivi a *M. fructicola*. Inoltre, 3 campioni sono risultati positivi sia a *M. fructigena* che a *M. fructicola*. Numerosi campionamenti sono stati effettuati per valutare l'efficacia, nei confronti del marciume bruno, di due fungicidi a base di fluopyram (17,7%) e tebuconazolo (17,7%), e fenpirazamina (50%), nonché composti alternativi a base di zolfo, chitosano e bicarbonato di potassio, applicati nella fase di indurimento del nocciolo, 20 giorni prima della raccolta. L'incidenza delle infezioni latenti è stata nel complesso bassa, in ogni caso i trattamenti chimici sono stati più efficaci e lo zolfo ha mostrato la più alta incidenza di infezione. Il chitosano si è rivelato il più efficace tra i composti alternativi nel ridurre l'incidenza delle infezioni in campo. Al contrario, il bicarbonato di potassio ha avuto la più alta incidenza in campo. Nessuno prodotto alternativo è risultato efficace nel controllo dell'incidenza della malattia in post-raccolta, a differenza dei trattamenti chimici. In generale, i prodotti chimici non sono stati particolarmente efficaci nel controllo delle infezioni da marciume bruno come previsto, quindi ulteriori studi dovrebbero essere condotti per studiare la resistenza di queste specie ai fungicidi commerciali usati in Italia. Inoltre, è necessario attuare con urgenza strategie di gestione innovative per limitare l'ulteriore diffusione di *M. fructicola* nei frutteti e nelle aree limitrofe.

Keywords: Composti alternativi, Drupacee, Incidenza di malattia, Infezioni latenti, *Monilinia*

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DEDICATION

To My Lovely Mother **Rebecca Lefentswe Maphela,**

Thank you for instilling strength and courage in me to endure hardships so I can fully enjoy my victories. You will forever have my love and respect.

To **Zelalem Bekele Mengistu,**

You were great and brilliant. Rest in Peace.

LIST OF PRESENTATIONS

Romanazzi, G., Mancini, V., Murolo, S., Landi L., and **Makau, S.M.**, 2019. Fitoplasmosi e moniliosi nel frutteto. Innovazione strategica in Valdaso, Marina di Altidona, 4 April 2019.

Mancini, V., **Makau, S.M.**, Landi, L., and Romanazzi, G., 2019. Survey on *Monilinia* affecting stone fruits in the Marche region, Central-eastern Italy. V International Symposium on Postharvest Pathology, Liegi, Belgium, 19-24 May 2019. Acta Horticulturae (in press).

Romanazzi, G., Mancini, V., Landi L., **Makau, S.M.**, Kavari, M., Quarticelli, L., Mazzoni, S., and Nardi, S., 2019. Gestione della moniliosi delle drupacee: dal campo al laboratorio e viceversa. Atti Postraccolta dei prodotti ortoflorofrutticoli, Milano, 28-29 October 2019, 23.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
°C	Degree Celsius
µl	Microlitre
µm	Micrometre
BCA	Biological control agents
Bp	Base pairs
Cv.	Cultivar
DMI	Demethylation inhibitor
DNA	Deoxyribonucleic acid
EPPO	European and Mediterranean Plant Protection
EU	European Union
H	Hour
Ha	Hectare
Kg	Kilogram
L	Litre
Mm	Millimetre
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
QoI	Quinone outside inhibitor
S	Seconds
SDHI	Succinate dehydrogenase inhibitor
Spp.	Species
β	Beta

1. INTRODUCTION

1.1. BROWN ROT OF STONE FRUIT

Brown rot is the most prominent fungal disease in stone fruit causing decay in postharvest stages and blossom blight and stem cankers during preharvest stages. It contributes to fast deterioration of fruit quality. Brown rot is responsible for major losses in stone and pome production areas across the world under favourable weather conditions such as high humidity, warm temperatures and abundant rainfall prior to harvest. *Monilinia* spp. occur in all major stone, and pome, fruit production areas worldwide but their distribution differs due to occurrence of some species only in certain parts of the world while being absent in other parts (Hrustić *et al.*, 2015). Introduction of new species into a territory is inevitable, due to trade amongst countries being a common factor thus making constant surveillance of produce an important prevention step.

Brown rot can infect fruit in the orchard and remain dormant until the fruit reaches maturity when it starts to show symptoms and could easily spread to other fruits. Ripening processes such as fruit softening increases susceptibility of the fruit to brown rot pathogens. Infection can also be facilitated by wounds or injuries on the fruit inflicted during mechanical and handling practices such as pruning, picking, sorting, and transportation. This decay is of economic importance in all stone fruit production areas worldwide though it is caused by different fungal species depending on the area where it occurs. Under favourable environmental conditions for pathogen growth, brown rot incidence can cause up to 80% fruit loss after harvest with an estimated 1.7 billion euros worldwide annual loss. Significant losses also occur in preharvest stage where fungi infect flowers and twigs causing blossom blight and canker respectively. In some cases resulting in death of the plant part, commonly referred to as necrosis (Obi *et al.*, 2018).

Stone fruit include peach and nectarine (*Prunus persica* (L.) Batsch), plum (*P. domestica* L.), apricot (*P. armeniaca* L.), sweet (*P. avium* L.) and sour (*P. cerasus* L.) cherry. They are consumed worldwide due, in part, to their nutritional composition mostly constituted by carotenoids including β -carotene, lutein and β -cryptoxanthin (Konopacka *et al.*, 2010). Stone fruits are also rich in anthocyanins and phenolic acids (Byrne *et al.*, 2007) as well as vitamins, minerals, and excellent source of dietary fibres (Barret *et al.*, 2004). Consumption of stone fruit, along with other fruits and vegetables, has been associated with decrease in oxidative stress, reduced risk of some forms of cancer, heart disease, stroke and

other chronic diseases. Most noticeably is the benefit of improved health due to their content of phytochemicals (Barret *et al.*, 2004).

In Europe, peach production is distributed between Spain, Italy, Greece and France by rank of highest producer (Konopacka *et al.*, 2010; Reig *et al.*, 2013). Availability of this commodity on the fresh produce market depends on growth season. An important factor to be taken into account when dealing with peach is that it is a climacteric fruit which undergoes rapid ripening making it a highly perishable produce and for this reason has a short shelf life (Khademi *et al.*, 2013). Although shelf life has a great deal of importance is it worth noting that other factors such as disease infection caused by fungi have the same, if not more importance, in determining fruit quality and should be given as much attention. Such diseases have the ability to cause losses due to spoilage and reduced yield.

Peach fruit is a drupe with a hard endocarp forming the stone while mesocarp is fleshy (soft or juicy) and the exocarp is a thin outer flesh. Fruit is characterized by a hairy layer cover; no waxy cover is present, and is perishable and sensitive to mechanical injuries. Peach fruit is climacteric and the increase in ethylene production is associated with the increase in respiration rate at ripening. Ripening is characterized with colour appearance of the cultivar, decrease in firmness, increase of sugars and the development of the characteristic flavour (Yahia *et al.*, 2008).

1.2. CAUSAL AGENTS

The genus *Monilinia* [family Sclerotiniaceae, order *Helotiales*, class *Leotiomycetes* and phylum *Ascomycota*; (Martini and Mari, 2014)] contains the most economically important pathogens in stone fruit production which consists of 35 species; the most common species include *Monilinia fructicola* (G Winter) Honey, *Monilinia fructigena* (Aderhold & Ruhland) Honey and *Monilinia laxa* (Aderhold & Ruhland) Honey. *M. fructicola* is found in North America and Australasia while *M. fructigena* and *M. laxa* are mainly in Europe and South Africa; the three species do not occur simultaneously in production areas (Carstens *et al.*, 2010; Rungjindamai *et al.*, 2014). The species have been studied extensively and widely across the continents for many years as the causal agents of brown rot of stone fruit considered to be the most important preharvest and postharvest fungal disease in the fruit industry (Carstens *et al.*, 2010). While in Asia different species of *Monilinia* have also been reported, this includes *M. polystroma*, *M. mumecola*, and *M. yunnanensis* (Papavasileiou *et al.*, 2016). However, Poniatowska *et al.* (2016) conducted a study in which *M. polystroma* was positively isolated from sweet and sour cherries in Poland.

M. laxa is the most prevalent of the species in Europe accounting for 85-90% (Di Francesco and Mari, 2018). Whereas introduction of *M. fructicola* in Europe started in peach orchards in France in the year 2001 and it has since spread to other European countries including Italy, Poland, Germany, Spain and Hungary to name a few countries (Rungjindamai *et al.*, 2014). According to EPPO (2018) *M. fructicola* is still classified as a quarantine pest on the A2 list; a quarantine pest present in an area but not widely distributed there and being officially controlled. The pathogen has been reported in several regions of Italy including Emilia Romagna, Lazio, Piemonte and Puglia on stone and pome fruits. In recent years, *M. fructicola* was discovered in Marche region on cherry fruits (Landi *et al.*, 2016). Landi *et al.* (2016) reported that *M. laxa* is the most widespread of the species in Italy while *M. fructigena* is not so commonly distributed and tends to occur more frequently on pome fruit and to a lesser extent on stone fruit (Di Francesco *et al.*, 2018). *M. laxa* and *M. fructicola* can co-exist in the same peach orchard. Currently, very little is known about the severity of distribution of these pathogens in Italy on stone fruit, particularly on peaches.

Monilinia species typically gain entry into their host through wounds or natural openings such as the stomata on leaves but they also attack healthy fruits. *M. laxa* and *M. fructicola* can infect both healthy and wounded fruit while *M. fructigena* can only infect the latter (Rungjindamai *et al.*, 2014). Although it is very difficult to differentiate species of *Monilinia* certain morphological characteristics can be used for differentiation using cultures; such as looking at growth rate, growth pattern, colour and morphological data. This is not always reliable because misidentification of species can occur (Martini *et al.*, 2014).

1.2.1. *Monilinia laxa*

The disease caused by this ascomycete fungus is usually referred to as European brown rot because it has been reported in almost all European countries (Holb, 2008a). The disease is also commonly present in Asia, America, Africa and Australia. In China and North America *M. laxa* is considered a quarantine pathogen. Peach, apricot, plum, sweet cherry and sometimes apple are the principal hosts. *M. laxa* shows on potato dextrose agar (PDA) at 22°C a lower growth rate (2-11 mm/24h) than *M. fructicola*. The colony margin is lobed, and sporulation is sparse, black rings and arcs associated with the formed rosette are visible in the bottom of the petri dish (**Figure 1.1**). The stromata are greyish or hazel in colour. Conidial germination occurs at temperatures of 25°C within 4 hours if moist conditions are maintained (Obi *et al.*, 2018). Size of conidia is 11.5-17 x 8-11 µm, while germ tubes are short and branching near the margins (Martini *et al.*, 2014; Rungjindamai *et al.*, 2014). On fruits, lesion

development caused by *M. laxa* occurs at 10°C, this means that the species is better adapted to low temperatures (Obi *et al.*, 2018). Landi *et al.* (2019) studied and compiled a complete genome sequence of *M. laxa* strain obtained from a cherry fruit; such studies enable further research to better understand mechanisms of its pathogenicity and thus development of highly effective management tools and systems.

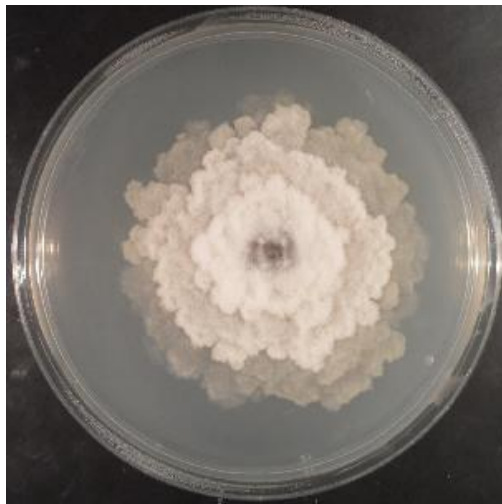


Figure 1.1 *Monilinia laxa* on potato dextrose agar (PDA) (Di Francesco *et al.*, 2018)

1.2.2. *Monilinia fructigena*

On PDA *M. fructigena* produces yellowish or creamy colonies (**Figure 1.2**) at a low to moderate rate with mycelium on distinct tufts. Colonies could produce concentric rings with entire margins. The pathogen can produce conidia at temperatures of 15-20°C with dimensions of 17.5-20.5 × 10.5-12.5 μm. Only two long, straight germ tubes with length of 600-900 μm develop per conidia. Sporulation is sparse (Rungjindamai *et al.*, 2014; Obi *et al.*, 2018). *M. fructigena* is mostly known to affect pome fruit, apple being the principal host (Di Francesco *et al.*, 2018). This species is less virulent and aggressive compared to *M. laxa* and *M. fructicola* (Cox *et al.*, 2018), however, it still causes the most important disease of stone and pome fruits with fruit rots at preharvest and postharvest stages being the most prevalent symptoms (Landi *et al.*, 2018). The pathogen is widely spread in Europe, Asia, and North Africa as well as some parts of South America whereas in USA, Canada, Australia and New Zealand it is a quarantine pathogen. Landi *et al.* (2018) reported a complete genome sequence of *M. fructigena* which is a step further in understanding epidemiology of the pathogen, its interactions with host, and ultimately finding the most effective brown rot management systems.

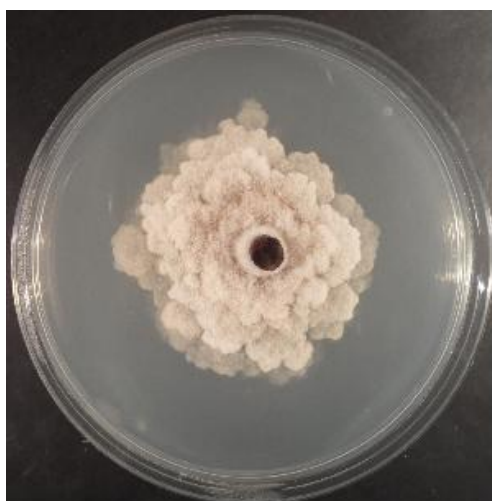


Figure 1.2 *Monilinia fructigena* on potato dextrose agar (PDA) (Di Francesco *et al.*, 2018)

1.2.3. *Monilinia fructicola*

M. fructicola is commonly referred to as the American brown rot fungus given its wide spread presence in the North and South America (O'Brien *et al.*, 2019). In Europe it is considered a quarantine pathogen by EPPO. On PDA conidia dimensions are observed at $12.5-14.5 \times 8-10 \mu\text{m}$ with only one germ tube developing per conidia. Like *M. fructigena*, this pathogen also produces conidia at temperatures between $15-20^\circ\text{C}$ (Rungjindamai *et al.*, 2014; Obi *et al.*, 2018). Germ tube is long, straight and larger than *M. laxa* and *M. fructigena*; it has a length between $750-900 \mu\text{m}$. Colonies appear as greenish-brown with entire margins and absence of rosette (**Figure 1.3**). Colonies also develop faster compared to other *Monilinia* species. Sporulation is often quick, intense and abundant. On fruits, lesion development caused by *M. fructicola* occurs at 30°C , this means that the species is better adapted to warmer temperatures unlike *M. laxa* (Obi *et al.*, 2018). Young and immature fruits of peach are highly susceptible to this pathogen (Di Francesco *et al.*, 2018). *M. fructicola* is the most destructive pathogen compared to other species of *Monilinia* genus (De Miccolis Angelini *et al.*, 2019). De Miccolis Angelini *et al.* (2019) reported a complete genome sequence of *M. fructicola* which is central to understanding how this pathogen is able to rapidly diffuse, adapt to new environments and thrive far more better compared to other species.

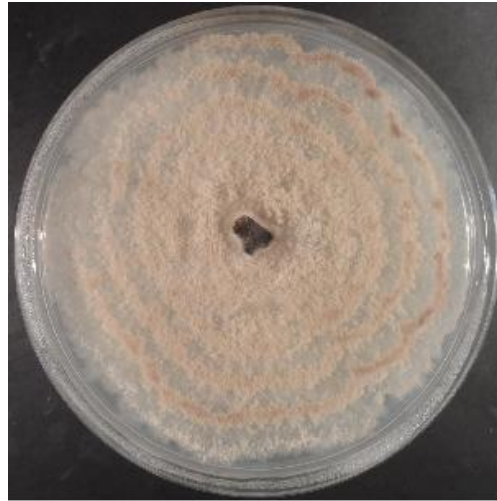


Figure 1.3 *Monilinia fructicola* on potato dextrose agar (PDA) (Di Francesco *et al.*, 2018)

1.3. LIFE CYCLE OF *MONILINIA* SPP.

The life cycle of brown rot disease occurs in a continuous cycle from season to season that follows a three phase sequence, (i) blossom blight and twig canker in early spring, (ii) brown rot in late spring and summer, and (iii) mummified fruits on trees and soil (Di Francesco *et al.*, 2018). Fungal pathogens of *Monilinia* spp. overwinters as mycelium on mummified fruits, in canopy, fruit peduncles, cankers on twigs, spurs and branches found either on the soil or still hanging on the tree (**Figure 1.4**). These materials will serve as primary source of inoculum. In early spring copious amounts of conidia grow from mummified fruits or ascospores from apothecia during warm and humid period with sporulation occurring right before flowering; conidial spores are released and dispersed to neighbouring trees and/or fruits in the orchard by wind, water or insects where they infect susceptible tissue when moisture and temperature favours their growth (Adaskaveg *et al.*, 2000).

Conidia will infect flowers and spread through blossoms to fruit pedicel and young fruits. Infection also occurs on young leaves, blossoms, twigs, buds, shoots, and cankers may develop on the stems. This will later constitute secondary source of inoculum in the orchards (Obi *et al.*, 2018, Willets and Bullock, 2019). Infected immature fruits show symptoms as maturity progresses; hence it is called a latent disease. Latent or “quiescent” infections are visible and non-visible infections that are established when environmental conditions or host physiology are conducive for penetration, but not for active growth of the pathogen (Holb, 2008b). Once favourable conditions are established and fruit begins to soften the pathogen starts to actively grow resulting in fruit decay (Adaskaveg *et al.*, 2000). Susceptibility to *Monilinia* spp. infection is highest during the early stages of fruit development which then

decreases during green fruit stage, also known as pit hardening, and again increases during the ripening period (Obi *et al.*, 2018).

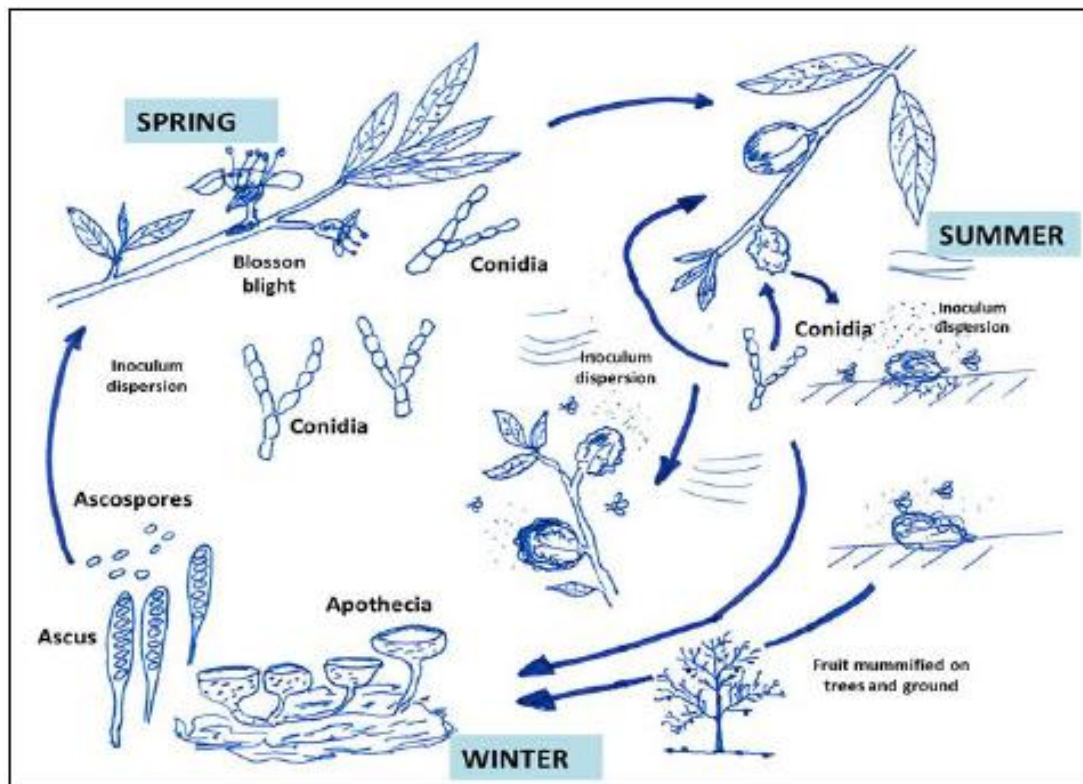


Figure 1.4 Disease cycle of brown rot of stone fruits (Obi *et al.*, 2018)

1.4. SYMPTOMS OF *MONILINIA* INFECTION

Monilinia species affect different aerial parts of a stone fruit plant; these include flowers, branches, twigs and leaves. Symptoms can manifest as blights, cankers and fruit rot which is mostly predominant in the postharvest stages where fruits are more susceptible to the pathogen due to several factors creating conducive development conditions. Of all the diseases caused by *Monilinia*, brown rot is the most important because this disease affects multiple orchard plants and causes major economic losses annually; up to 80% yield losses have been recorded. Any of the infected tissue can serve as source of inoculum if left to develop and germinate in the orchard (Byrde and Willetts, 1977; Aiello *et al.*, 2019).

1.4.1. Blossom blight

Early signs of brown rot infection in an orchard are observed as blossom blight which occurs in early spring; blossoms are most susceptible from bud formation to petal fall (Holb, 2008b).

Fungal macroconidia or ascospores penetrate flowers of host plants of which any part of the flower can serve as the first site of infection; this include the stamen, stigma, petals and sepals. Under continued moist and warm conditions infection will cause blight of flowers resulting in reduced fruit set and latent infection of fruitlets. Infected tissue turns dark brown and the discolouration may spread throughout all the flower parts, down the pedicel and into the young fruits. The flower will begin to wither and may fall to the ground, in some cases the flowers remain attached to the plant (**Figure 1.5a**). It will then serve as a source of inoculum such that when there are moist conditions the *Monilinia* pathogen within the infected, withered flower will produce conidiophores and conidia that can infect other trees within the orchard (Byrde *et al.*, 1977; Di Francesco *et al.*, 2018). Blossom blight of stone fruit is usually caused by *M. laxa* (Hrustić *et al.*, 2012).

1.4.2. Twig and leaf blight

Twig blight is essentially an extension of blossom blight in which fungus that had previously infected flowers starts growing through the peduncle into the twigs. The infected tissue will appear as brown, collapsed areas along the twig. Finally, gum will accumulate on the surface of infected surface. As observed previously, leaf blight is reported to start from twig blight due to that it is uncommon for spores to directly penetrate leaves. Once infection has been established leaves begin to get brown discolouration which spreads throughout making the leaves shrivel and eventually die whereas in localized infection symptoms appear as a shot-holing effect marking the drying out of the diseased area (**Figure 1.5b**) (Byrde *et al.*, 1977; Di Francesco *et al.*, 2018). Like blossom blight, this infection is caused particularly by *M. laxa* (Hrustić *et al.*, 2012).

1.4.3. Stem canker

Stem canker is characterised by necrosis of bark around the area of infection followed by discolouration of tissue found directly beneath the bark giving rise to an open wound on the stem. The infected area will then get covered by gum coming out from within the stem, and callus tissue forms around the area to prevent further growth of infection and to promote healing of the tissue (**Figure 1.5f**). Continued development of the wound be a source of inoculum and can lead to dieback of the tree. Stem canker does not develop through direct penetration of conidia; rather they are a result of mycelium growth from blighted twigs or fruits growing into the stem (Byrde *et al.*, 1977; Di Francesco *et al.*, 2018).

1.4.4. Fruit rot

Infection of fruit by *Monilinia* species may occur from early fruit set if blossoms are already infected by spores, or through direct penetration of fruits from wounds and lesions, and through natural stomata opening (Emery *et al.*, 2000). The disease remains dormant until conditions become favourable for it to develop and grow; this is due to that defence mechanisms of the fruit are still strong when fruits are young and immature (Obi *et al.*, 2018). Fruits become more susceptible to brown rot towards maturity when the sugar content increases and fruits defence mechanisms are weakened. Symptoms first appear as a small, superficial, brown, circular spot which gradually expands to the rest of the fruit (**Figure 1.5c**) (Liberato and Miles, 2006). Under high temperatures and humidity lesions can completely invade tissues in a matter of few days followed by appearance of grayish-brown masses of spores on the fruits (**Figure 1.5d**). Fruit loses moisture and begins to shrivel and become hard, the result of which is a mummified structure (**Figure 1.5e**) (Di Francesco *et al.*, 2018). Fruit rot of stone fruit is often caused by the species *M. fructicola* (Hrustić *et al.*, 2012).



(a) Blossom blight



(b) Twig blight



(c) Initial fruit rot



(d) Gray-brown masses of spores on fruit



(e) Mummified fruits



(f) Stem canker

Figure 1.5 Brown rot disease symptoms on peach fruit and tree (a) Blossom blight, (b) Twig blight, (c) Initial fruit rot, (d) Gray-brown masses of spores on fruit, (e) Mummified fruits, (f) Stem canker (Ritchie, 2000; Ellis, 2008)

1.5. AGRONOMIC AND ENVIRONMENTAL FACTORS FAVOURABLE FOR *MONILINIA* INFECTION

Extensive yield losses can occur at any point of the production season with peak infections occurring directly after blossom emergence through the postharvest stages under favourable conditions. Several agronomic and environmental factors have been shown to have an influence on infection, sporulation and incubation period of pathogenic *Monilinia* species in stone fruit producing orchards throughout the world. While there is no way of completely

eliminating the influence of these factors, knowing how they exert their influence and when the risk of infection is most high is a valuable tool for preventing disease outbreak (Biggs and Northover, 1988).

1.5.1. Agronomic factors

According to Mari *et al.* (2003) susceptibility to *Monilinia* species occurs in three different stages during fruit development. Susceptibility to infection was highest, 100%, 6th week from full blossom and decreased to 50% on the 8th week following full blossom which coincided with pit hardening. Again susceptibility increased 4 to 5 weeks before full ripeness. Natural infections of peach fruits generally appear at the end of the growing season when fruits have reached maturity. Holmes *et al.* (2011) also noted that fruit close to maturity were highly susceptible to brown rot and levels of brown rot were lowest on fruit at the post pit hardening stage.

Presence or absence of inoculum determines whether or not disease occurs in an orchard (Keane *et al.*, 1997). Aerial parts of tree infected by *Monilinia* species serve as sources of inoculum thus mummified fruits left on the trees or on the ground increase the spore load in the environment and thus become primary source of inoculum in the growing season (Villarino *et al.*, 2010).

Thick, dense tree canopy can cause humid microclimate within the tree resulting in less air circulation. Light penetration will also be limited to the outer areas of the canopy depriving inner parts of the most needed source for photosynthesis. This creates conducive conditions for pathogenic species to thrive; germination and sporulation of conidia will occur at a high rate and infection will occur at high rates too (Broembsen and Pratt, 2005; Bellingeri *et al.*, 2018).

Although insect pests have not been reported as vectors of brown rot disease they have been closely associated with development of infection in orchards. They are mainly involved in transportation of conidia from inoculum source to aerial parts of the tree. In an experiment conducted by Lack (1989) on artificially wounded apples more brown rot development occurred on tissues visited by insects than those which were not visited. *Carpophilus* species beetles (*Nitiduladae*) are one of the pests which inflict high levels of damage on stone fruit, particularly on susceptible varieties (Hossain *et al.*, 2013). Thus control of insect pests is crucial in preventing fruit injury since wounding increases fruit susceptibility to *Monilinia* species. Oriental fruit moth larvae penetrate small green fruits at the stem, creating infection courts and thus promoting infection by conidia present on

blighted twigs and blossoms (Tate and Ogawa, 1975). Tate *et al.*, (1975) concluded that nitidulid beetles have the potential to become important disseminating and inoculating agents of brown rot.

1.5.2. Environmental factors

Fluctuation in temperature predisposes the plant to infection by reducing the plant's level of partial resistance while simultaneously supporting rapid germination, host penetration, reproduction, invasion of the host, and sporulation of conidia (Agrios, 2005). Pathogens of brown rot disease remain dormant in the winter months and the quantity of inoculum that survives on mummified fruits, blighted twigs and cankerous lesions is relatively low due to delayed germination. Optimum germination temperature for *M. fructicola* is 20-25°C while temperatures above and below 0-30°C delays germination (Biggs *et al.*, 1988; Luo *et al.*, 2001). Under high temperatures, development of blossom blight occurs 2 or more days after infection (Luo *et al.*, 2001). According to Villarino *et al.* (2012) temperature is one of the two most important weather factors that contribute to incidence of latent infection caused by *Monilinia* species. Temperatures range between 15 and 25°C favour the development of the disease, although infection may also occur under more extreme conditions between 5–30°C.

The second most important weather factor influencing infection by *Monilinia* species is relative humidity (Villarino *et al.*, 2012). Rainfall, occurring during harvest time when fruits are mature, increases incidence and severity of brown rot decay (Adaskaveg *et al.*, 2005). Prevailing rainfall and continued wetness duration longer than 4 hours increased risk of blossom blight (Luo *et al.*, 2001). The ability of a pathogen to cause destructive disease epidemics depends on its ability to disperse rapidly over long distances (Keane *et al.*, 1997); rain and wind are dispersal modes in which inoculum can be carried from place to place. A rainy environment favours conidial dispersion (Fitt *et al.*, 1989). Hailstorms are natural events that occur every so often yet have detrimental effects on crops resulting in high losses either due to complete destruction of produce or wounding which increases susceptibility to infections fungal pathogens (Petoumenou *et al.*, 2019).

1.6. MANAGEMENT OF BROWN ROT

The relative abundance of *Monilinia* species in an orchard impacts the effectiveness of control (Holmes *et al.*, 2011). Chemical sprays, cultural practices, biological controls, and other control measures reduce or eliminate the possibility of an epidemic. However, certain

controls, such as the use of a certain chemical or planting of a certain variety, may lead to selection of virulent strains of the pathogen that either are resistant to the chemical or can overcome the resistance of the variety and thus lead to epidemics (Agrios, 2005). Detection of the disease is a rather intricate process given the nature of infections which only become visible either during late ripening, transit or at the consumer's home. This poses a threat of introducing the pathogen into new areas where the disease has not been reported before. Nonetheless, rapid, accurate and reliable detection of *Monilinia* latent infections is recommended to prevent and control dispersion of these pathogens (Obi *et al.*, 2018). Also, infection prediction forecasting can be made based on accurate monitoring of weather variables and knowledge of source of inoculum, disease cycles, susceptibility of stone fruit cultivars, and efficacy of chemical compounds to enable timely and effective implementation of control measures (Holmes *et al.*, 2011).

1.6.1. Cultural practices

The best first line of defence in orchard production is planting of disease-free certified trees with resistant rootstocks or cultivars (Barrett *et al.*, 2004). During production season good management practices must be implemented such as ensuring tree canopies are well pruned to allow sufficient circulation of air to promote faster drying of foliage and penetration of light, which helps in optimizing plant health. Routine thinning of fruits that come into contact with each other is mandatory because contact is an easy way to spread the infection (Broembsen *et al.*, 2005). Bellingeri *et al.* (2018) also noted that in moderately thinned trees brown rot disease incidence during the growing season was highest compared to null thinning in which slow growing fruits are less susceptible to brown rot. This is due to that low intensity thinning yields faster growing fruits which might be more prone to cracks but have less probability of infection given that the distance between fruits is much longer whereas in moderately thinned trees fruits are closer together increasing probability of spreading infection. Bellingeri *et al.* (2018) further elucidated that the probability of a fruit to become infected, and infectious, is a joint probability of being susceptible to the infection, which depends on the fruit growth rate, and to be exposed to the pathogen, which depend on the abundance and proximity of infectious fruits.

Irrigation water must be supplied through drip systems such that water does not come into direct contact with flowers, leaves and fruits. Pests inflict wounds on leaves and fruits as they feed, leaving them prone to infection. Control of pests by application of pesticides during development of fruit is necessary (Barrett *et al.*, 2004).

Sanitation practices such as removal of fallen fruits and twigs, particularly those with symptoms of infection, from orchard floor serve an important role reducing build-up of inoculum in the orchard (Ellis *et al.*, 2008). Villarino *et al.* (2010) stressed that an important way to manage brown rot in orchards is to lower disease pressure by minimizing the spore load in the environment, especially in the spring, by reducing the number of sources of primary inoculum. According to Barkai-Golan (2001) farm apparatus can harbour spores that can later infect fruits, therefore cleaning and disinfection of all apparatus coming into direct contact with fruits either in the fields or at packinghouses is crucial. This includes cleaning pruning shears, harvesting shears and boxes, and disinfecting packing line. Chemicals such as formaldehyde, isopropyl alcohol, quadronic ammonium compounds, captan or other chemicals can be used for disinfection of packinghouses and equipment.

In postharvest wounding of fruits during picking, storage and transit creates entry points for pathogens; therefore diligent handling of fruits is paramount to prevent infection outbreaks. Wounded fruits, even without infection symptoms, must be separated from intact, healthy fruits. Minimizing wounding through careful harvesting, sorting, packaging and transportation as well as preventing fruits from falling significantly reduces the risk of infection (Barkai-Golan, 2001). Furthermore, storage temperature and humidity must be maintained at correct levels of 0°C and 90-95% respectively (Yahia *et al.*, 2008) to prevent physiological injuries which predispose fruit to attack by wound pathogens (Barkai-Golan, 2001).

1.6.2. Chemical control

Monilinia species cause major postharvest losses in stone fruit and they are mostly associated with latent infection occurring preharvest manifesting when fruits become ripe; therefore preharvest treatments can be fundamental management of disease epidemiology. Thus it is an important tool of controlling latent infections in order to prevent brown rot disease epidemic in postharvest stage (Luo and Michailides, 2003). In Europe, two to three fungicide sprays around flowering, followed by one to two sprays between the beginning of ripening and preharvest are applied (EFSA, 2011; Sisquella *et al.*, 2013a; Sisquella *et al.*, 2013b). However, in the European Union application of postharvest fungicides is prohibited due to consumer demand of pesticide free fruits, while no use could lead to 59% postharvest losses (Mari *et al.*, 2012; Khumalo *et al.*, 2017). Development of fungicide resistance is another major factor contributing to strict restrictions put on use of fungicides (Karabulut *et al.*, 2010). Such restrictions make marketing of decay free fruits hard and also affect countries

that rely heavily on export of fresh produce to sustain their economy (Larena *et al.*, 2005), as well as increasing the risk of introduction of new species of *Monilinia* into a territory (Cox *et al.*, 2018).

Chlorothalonil is a multi-site protectant fungicide that is highly effective against brown rot. It is applied from bloom to petal fall and shuck fall to prevent blossom blight, shoot blight and immature fruits infections. The limitation of this fungicide is the short prescribed application window; application is prohibited after shuck split when green fruits are fully exposed. Iprodione (Rovral®) is a single-site preharvest fungicide for prevention of brown rot infection that is sprayed 1 day before harvest (Sholberg and Conway, 2004; Cox *et al.*, 2018). For prevention of fungicide resistance anilinopyrimidine fungicides, cyprodinil and pyrimethanil, are used in rotation to the above-mentioned fungicides. *M. laxa* pathogen have higher sensitivity to demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides. Single-site fungicides belonging to the DMI, QoI, and succinate dehydrogenase inhibitor (SDHI) classes are applied in rotation on 10- to 14-day intervals towards the start of harvest when fruits are more susceptible to brown rot. Additionally, premixed or single fungicide products containing the DMI fungicide difenoconazole, the QoI fungicide pyraclostrobin, and the SDHI fungicides fluopyram, penthiopyrad, and fluxapyroxad have been shown to be highly effective against *M. fructicola*. Although not permitted in Europe, fungicides such as fludioxonil or pyrimethanil are used in postharvest treatment of fruits in drenches in the United States. Sulphur and captan can be used to a lesser extent as multi-site protectant fungicides due to injury sensitivity of some species of stone fruits (Cox *et al.*, 2018). Moreover, application of insecticides during growing period to prevent wound damage on fruits caused by insect pests has been shown to lessen brown rot infection (Tate *et al.*, 1975).

1.6.3. Organic control

Psota *et al.* (2013) reported on the effective use of organic products with different active ingredients in controlling brown rot and blossom blight of apricot. The treatments included Alginure (sea algae extract 24%, plant aminoacids 7%, phosphates 20%), Kocide 2000 (53.8% of pure copper), Kumulus WG (80% of pure sulphur), VitiSan (potassium hydrogen bicarbonate 100%), and POLISENIO (lime sulphur, 380 g/l). The treatment efficacy against blossom blight was recorded as follows; 63.34%, 65.82%, 56.21%, 63.34% and 38.48% respectively. Combination of Alginure and Kocide 2000 showed the highest efficacy with 100% infection prevention.

Chitosan, a naturally occurring polysaccharide, has been used on fruits as preharvest and postharvest treatment. It is a suitable alternative to synthetic fungicides due to its non-toxic, biodegradable properties and antimicrobial activities (Jiao *et al.*, 2019). According to Romanazzi *et al.* (2017) chitosan coating works by forming a semi-permeable film which reduces growth of decay-causing fungi while simultaneously inducing resistance responses of the host tissue in postharvest treatment. Jiao *et al.*, (2019) added that conjugated chitosan-chlorogenic acids had increased antioxidant activity suggesting that its use as an edible coating could have significant impact in maintaining quality of peach fruits. Fruits treated with the conjugate and stored at 20°C for 8 days maintained firmness, reduced weight loss, inhibited decay and rate of respiration amongst other things. Thus maintaining quality of fruits and prolonging shelf life (Romanazzi *et al.*, 2017; Jiao *et al.*, 2019).

1.6.4. Biological control

Biological control agents (BCA) are mostly used in conjunction with fungicides. Use of these agents originated from the need to reduce amounts of fungicides used in agriculture (Hrustić *et al.*, 2012). The purpose of preharvest application of microbial antagonistic culture is to pre-colonize the fruit surface with an antagonist immediately before harvest so that wounds inflicted during harvesting can be colonized by the antagonist before colonization by a pathogen. Practically, postharvest application of antagonists has been proven to be a more useful and effective method of controlling postharvest diseases (Sharma *et al.*, 2009). BCA work by producing antimicrobial compounds which have an antagonistic effect against fungal pathogens. Antagonists possess the ability to rapidly increase their population after colonizing fruit surface such that they overcome pathogenic species population and produce toxins that inhibit their development. ‘Biosave’ 10LP and 110 based on *Pseudomonas syringae* (strain 10LP and 110) is one of the few biofungicides active against *Monilinia* species. Another biofungicide, ‘Serenade’, based on *Bacillus subtilis* has been commercialised but not yet registered in Europe for control of brown rot could serve as a good postharvest treatment of stone fruit (Di Francesco *et al.*, 2017).

1.6.5. Physical control

Pre-cooling of fruits shortly after harvest prevents moisture loss thus maintaining fruit firmness and subsequently prolonging storage life; this is due to elimination of field heat. Hydro-cooling is the most frequently used method of cooling for peach fruit. During hydro-

cooling treatment calcium, found in middle lamella and primary cell walls of plant tissue with the function of binding cell materials, infiltrates cell walls resulting in increased levels of the ion and thus promote stability and protects the fruit against fungal infection (Kalbasi-Ashtari, 2004). Heat treatments directly inhibit or retard germ tube elongation and stimulating host defence mechanisms at wound site detrimental to pathogen development (Casals *et al.*, 2010). Hot water treatment at 55°C for 2-3 minutes is an effective control measure (Yahia *et al.*, 2008). Karabulut *et al.* (2010) noted that brief immersion of stone fruit in hot water at 55°C for 60 s or 60°C for 30 or 60 s significantly reduced incidence and severity of brown rot. In plum fruit brown rot incidence was reduced from more than 80 to less than 2% after treatment with 60°C for 60 s, while in nectarine stored at 20°C incidence was reduced from 100 to less than 5% and from 73 to 28% on cold-stored fruit. This treatment could be most effective for the organic fruit industry. Washing fruit with chlorine water will keep inoculum build up under control (El-Ramady *et al.*, 2015). Casals *et al.* (2010) reported that curing treatment at 50°C for 2 h and 95-99% relative humidity effectively prevented brown rot development in peach and nectarine fruit without affecting internal and external appearance of fruit. Latent infection severity can be reduced by use of controlled atmosphere storage with low levels of oxygen and high levels of carbon dioxide (CO₂). CO₂ levels can be between 10 to 40% depending on the stone fruit crop and *Monilinia* species.

1.7. OBJECTIVES OF THE STUDY

The aim of this study is to locate the species distribution of fungal pathogens belonging to genus *Monilinia* in Marche region responsible for brown rot decay. This will contribute to improve management practices of brown rot in preharvest and postharvest stages.

The main objective is:

The identification of *Monilinia* species causing brown rot decay in stone fruit in two orchards using traditional and molecular diagnostic techniques. The required steps are listed below:

- collection of samples with visible infection by *Monilinia* spp. and of apparently healthy fruits;
- isolation of *Monilinia* spp. from infected tissue of symptomatic fruits;

- extraction and quantification of genomic DNA from fungal colonies resembling *Monilinia* spp.;
- molecular identification of *M. laxa*, *M. fructicola* and *M. fructigena* by multiplex polymerase chain reaction (PCR);
- visualization of the amplified fragments by agarose gel electrophoresis.

Another objective was the evaluation of some alternative compounds in preventing infection of stone fruit brown rot in the orchard of Mazzoni Company. The steps are listed below:

- application of three alternative compounds, two synthetic fungicides as chemical controls and an untreated control;
- sampling and incubation of the immature fruits to evaluate the presence of latent infection of *Monilinia* spp.;
- sampling of mature fruits to evaluate presence of field and postharvest infection of *Monilinia* spp.

2. MATERIALS AND METHODS

2.1. CHARACTERISTICS OF ORCHARDS

This study on molecular identification and latent infection monitoring of *Monilinia* spp. was carried out on two peach and nectarine producing orchards located in the province of Ascoli Piceno (AP) and Fermo (FM) as shown in Figure 2.2 and 2.3. Each orchard was allocated a reference code, Acciarri as AC and Mazzoni as MA (Figure 2.1). Additionally, main features of each orchard are reported in Table 2.1 and 2.2.



Figure 2.1 Geographical locations of companies (Acciarri, AC; Mazzoni, MA)

Table 2.1 Main characteristics of orchards of Acciarri Company

Location	Ortezzano	
Province	Fermo	
Proprietary	Acciarri Società Agricola SRL	
Latitude	43°01'51.8"N	
Longitude	13°37'19.4"E	
Altitudine	301 m a.s.l	
Species	Peach	Nectarine
Variety	Sweet Lady Elegant Lady Sweet Red Fairtime Summer Royal Extreme 486	BigTop

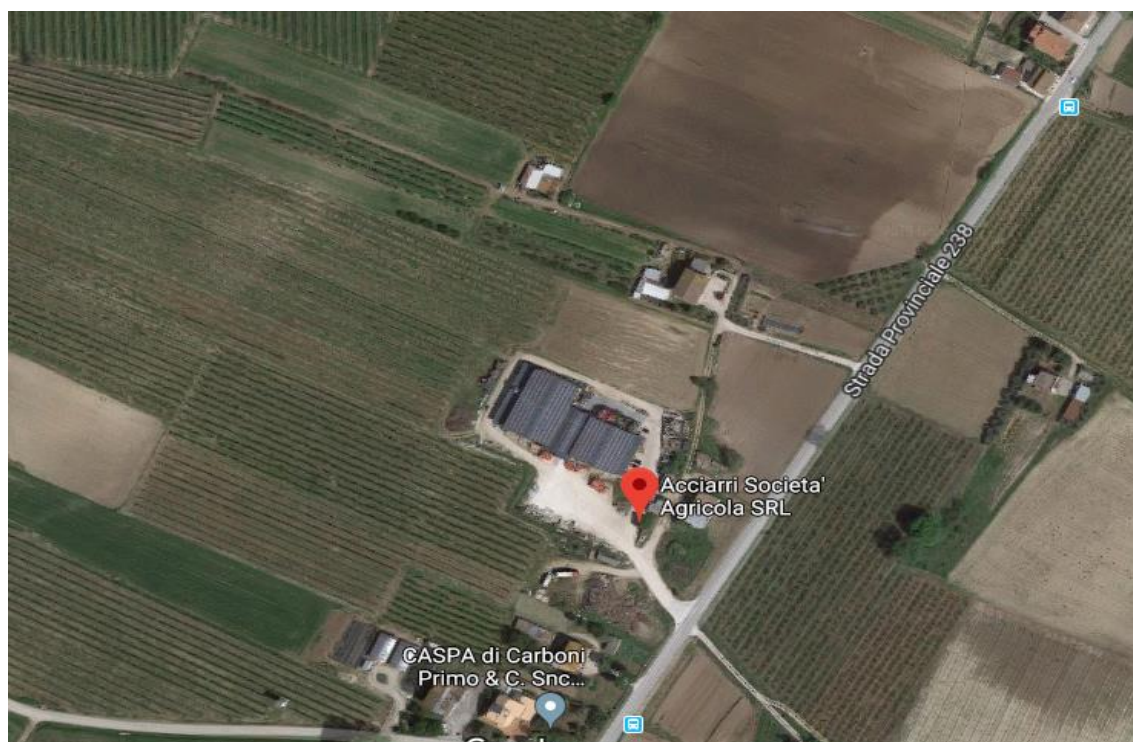


Figure 2.2 Geographic location of Acciarri Company

Table 2.2 Main characteristics of orchards of Mazzoni Company

Location	Montedinove
Province	Ascoli Piceno
Proprietary	Azienda Mazzoni
Latitude	42°59'15.6"N
Longitude	13°33'24.4"E
Altitude	249 m a.s.l
Species	Nectarine
Variety	Honey Royal



Figure 2.3 Geographic location of Mazzoni Company

2.2. IDENTIFICATION OF *MONILINIA* SPP. IN TWO PEACH ORCHARDS

2.2.1. Sample collection

In summer 2018 samples were collected from Acciarri (AC) Company and they had undergone standard management practices with regular spray program, irrigation, and other cultural practices carried out during the production season. Peach and nectarine fruits at the maturity stage were sampled by picking those with visible symptoms of brown rot from the harvesting lugs. During 2018 several samples were collected from five different cultivars as follows: 16 fruits for 'Sweet Lady', 16 fruits for 'Big Top', 12 fruits for 'Elegant Lady', 8 fruits for 'Sweet Red', and 5 fruits for 'Fairtime' giving a total of 59 subsamples collected (**Appendix 1**).

In summer 2019 samples of healthy fruits cv. Honey Royal were collected at harvest maturity at Mazzoni (MA) and Acciarri (AC) Companies, placed in sterilised plastic containers and covered with black plastic bag for incubation at room temperature. During incubation, a period of 5 days from the harvest fruits with apparent symptoms of brown rot was collected (**Appendix 2**).

2.2.2. Isolation of *Monilinia* spp.

For both orchards isolation of samples was performed in the same manner. Isolation of *Monilinia* spp. was performed by aseptic transfer of symptomatic tissue of naturally infected peach and nectarine fruits. Using a sterile scalpel, approximately 5 mm pieces of infected tissue were cut from each fruit and placed on petri dishes containing potato dextrose agar (PDA; 40 g L⁻¹) amended with ampicillin (100 mg L⁻¹) and streptomycin sulphate (100 mg L⁻¹). Each petri dish was then sealed with parafilm and labelled before incubation in a thermostat at 23 ± 1 °C; incubation was carried out for few days. After initial incubation growth of colonies was observed on the cultures. Subsequently, a portion of growth media with actively growing colony at the margins of the petri dish was transferred to a fresh culture media and incubated again in order to obtain a pure culture.

2.2.3. DNA extraction from fungal colonies

Each pure colony, resembling *Monilinia* spp. due to its morphology, was transferred to Potato Dextrose Broth (PDB), amended with the same antibiotics as mentioned above. After a period of 7-10 days of incubation, the mycelium growth was suitable for the next step. To

begin procedure of genomic DNA extraction, fungal mycelium was collected from PDB and put in 1.5 mL micro tubes which were then dipped in liquid nitrogen in order to freeze cells allowing cell wall breakage and deactivation of cell processes. Frozen mycelia were then ground followed by addition of 900 µL of 3% cetyltrimethylammonium bromide (CTAB) extraction buffer in order to promote cell lysis through denaturation of proteins, immobilization of carbohydrates and proteins, and release of nucleic acids in solution, as well as addition of 20 mg of sodium metabisulfite completed with vortex. Tubes were incubated in distilled bath water at 65 °C for 20 minutes with periodic stirring. A centrifuge at 8000 rpm was carried out for 7 minutes to separate the solid part from the liquid (supernatant) part.

After which 900 µl of supernatant were taken out and placed in new 2.0 mL micro tubes and 900 µl of chloroform was added under aseptic conditions for purification of DNA. Another centrifugation cycle was carried out at 12 000 rpm for 10 minutes in order to separate DNA from other components such as proteins and polysaccharides. Approximately 650 µl of supernatant was collected and placed in another micro tube together with 650 µl of 2-propanol for precipitation of DNA and a centrifuge cycle at 12 000 rpm for 15 minutes was performed. The result of which is a DNA pellet at the bottom of the tube and liquid at the top of the tube; liquid is thus discarded. Subsequently 300 µl of 70% cold ethanol were and final centrifuge cycle performed at 12 000 rpm for 7 minutes. Finally, ethanol was discarded and pellets left to dry completely, before being dissolved in 50 µl of sterile distilled water and stored for further processing (Doyle and Doyle, 1990).

A spectrophotometer (BioPhotometer plus Eppendorf Inc., USA) was used to analyse the quantity and quality of DNA. For each sample 2 µl of DNA and 118 µl of distilled water were placed in a cuvette and into the spectrophotometer. After which, the quantity in ng/ml and quality was recorded using the 260/280 ratio for each sample analysed.

2.2.4. Multiplex polymerase chain reaction (PCR)

Following extraction and quantification of DNA a multiplex polymerase chain reaction (PCR) was performed according to the protocol developed by Cotê *et al.* (2004). Three species specific forward primers and one identical reverse primer were used (**Table 2.3**). Accordingly, MO 368-8R to amplify *M. fructigena*, MO 368-10R for *M. fructicola*, Laxa-R2 for *M. laxa*, and MO 368-5 for all the three species. The analyses were performed with the thermal cycling iCycler PCR machine (Bio-Rad, USA). Reaction mix of 20 µL final volume for each sample consisted of: 1× Mastermix with Dye 2x (Lucigen Corporation, Wisconsin,

USA), 1 µL of each primer (0.5 M) and 1 µL of extracted DNA (20-40 ng/µL). The reaction conditions are as follows: initial denaturation 2 min at 95°C, followed by 35 cycles of 15 s at 95°C, 15 s at 60°C, and 1 min at 72°C. Final elongation was 3 min at 72°C.

Table 2.3 Primers used for amplification of *Monilinia* species with their sequences and number of base pairs

Primer	Sequence 5' to 3' (F, forward; R, reverse)	Species	Bp
MO 368-5	(R) GCAAGGTGTCAAACTT CCA	<i>M. fructigena</i> , <i>M. fructicola</i> , <i>M. laxa</i>	-
MO 368-8R	(F) AGATCAAACATCGTCCATCT	<i>M. fructigena</i> ,	402
MO 368-10R	(F) AAGATTGTCACCATGGTTGA	<i>M. fructicola</i> ,	535
Laxa-R2	(F) TGCACATCATATCCCTCGAC	<i>M. laxa</i>	351

2.2.5. Agarose gel electrophoresis

Aliquots of 10 µl of the amplification product were loaded onto a 1.5% agarose gel (Molecular Biology Certified Agarose, Bio-Rad Laboratories) and run in TAE buffer (45 mM Tris-borate; 1 mM EDTA, pH 8.0) at 100 Volts for 2 hours in an electrophoretic chamber submerged horizontal gel (Bio-Rad Laboratories). To estimate the size of the amplification products, 100 bp DNA Ladder (100bp Plus Opti-DNA Marker) was used as standard, which gives rise to 13 bands of sizes from 100 to 2,000 bp. The gels, pre-colored with GelRed 10,000 × (Biotium), were visualized and acquired using Gel Doc 1000 (Bio-Rad Laboratories).

2.3. EFFECTIVENESS OF ALTERNATIVE COMPOUNDS IN PREVENTING *MONILINIA* SPP. INFECTIONS ON STONE FRUITS

2.3.1. Orchard treatments

At Mazzoni (MA) Company three alternative products were tested compared with two chemical controls and an untreated control to evaluate their effectiveness in the protection of nectarine fruits cv. 'Honey Royal', a highly susceptible cv. to *Monilinia* species. A randomized block design with three replicates was used, and the treatments were assigned to

plots using a random number generator (Excel;Microsoft Corp., Redmond,WA). For the trial, three peach rows were considered and each plot consisted of six nectarine trees along a row. The treatments were as follows: two chemical controls with Luna experience (Fluopyram 17.7%; Tebuconazolo 17.7%) and Prolectus (Fenpyrazamine 50%) as first and second treatment respectively, Thiopron (Sulphur 57.3%), Chito Plant (Chitosan 99%), Karma 85 (Potassium bicarbonate 85%), and an untreated control (**Table 2.4**). A knapsack sprayer was used to apply the treatments on plants. Two applications for each treatment were done during the trial: the first one in the middle of June at hardening stage and the second one in the 2nd half of July, 20 days before harvest. Only in the second chemical treatment the application was done at the end of July, 10 days before harvest (**Table 2.5**).

Table 2.4 Synthetic and alternative formulations with relative active ingredients and application rate used at Mazzoni Company

Commercial products	Active ingredients	Application rate
Luna experience	Fluopyram (17.7%); Tebuconazolo (17.7%)	0.75 L/ha
Prolectus	Fenpyrazamine (50%)	1.2 kg/ha
Thiopron	Sulphur (57.3%)	10 L/ha
Chito Plant	Chitosan (99%)	1 kg/ha
Karma 85	Potassium bicarbonate (85%)	5 kg/ha
	Untreated control	

Table 2.5 Treatments and relative application date at Mazzoni Company

Treatment	Application dates
Chemical 10d	14 th June, 30 th July
Chemical 20d	15 th June, 19 th July
Thiopron	16 th June, 19 th July
Chito Plant	20 th June, 21 th July
Karma 85	18 th June, 21 th July
Untreated control	

2.3.2. Latent infection monitoring

To determine the incidence of latent infection on peach fruit 5 samplings were carried out every week starting from the end of June to the end of July. Every time 21 immature fruits were collected from each plants of the trail for a total of 104 samples. The overnight freezing incubation technique was employed (Luo and Michailides, 2003). For each sample, fruit were surface sterilized in a chlorine solution (32 ml of 0.525% sodium hypochlorite, 32 ml of 95% ethyl alcohol, and 0.01 ml surfactant Tween-20 in 2 litres of water) for approximately 15 to 20 minutes. Then fruits were washed with sterile water 3 times, and set aside to completely dry off after which samples were transferred to new plastic bags and placed in a freezer at -20°C for 10 hours. Later the fruit samples were put to incubate in sterilized plastic containers, considering three replicates for each sample, at room temperature for 5 days. Every day during the incubation period surveys were done to verify the presence of *Monilinia* spp. sporulation on the fruits. The data were collected and incidence, as percentage of brown rot of fruits, was calculated. The data were submitted to analysis of variance according to a randomized block design, and the means were separated by Tukey's honestly significant difference tests at $p < 0.05$ (Statsoft, Tulsa, OK).

2.3.3. Field and postharvest infection monitoring

To determine the incidence of *Monilinia* spp. in the field and postharvest infection on peach fruit, a single sampling was carried out at the end of July. About 21 mature fruits were collected from each plants of the trail for a total of 104 samples. The fruits were put in sterilized plastic containers, considering three replicates for each sample, at room temperature and surveys were conducted for a period of 5 days to verify the presence of *Monilinia* spp. sporulation on the fruits. The first 2 days were considered as field infection while the remaining 3 days were considered as postharvest infection. The data were collected and incidence, as percentage of brown rot of fruits, was calculated. The data were submitted to analysis of variance according to a randomized block design, and the means were separated by Tukey's honestly significant difference tests at $p < 0.05$ (Statsoft, Tulsa, OK).

3. RESULTS

3.1. IDENTIFICATION OF *MONILINIA* SPP. IN BOTH PEACH ORCHARDS

3.1.1. Morphological identification of *Monilinia* spp. from fungal colonies

From the 69 isolations carried out on symptomatic fruit tissue from Acciarri Company, only 44 colonies were synonymous with *Monilinia* species based on morphological characteristics of each isolate. Of the 44 colonies identified, 36 were viable for DNA extraction (**Appendix 1**).

A total of 346 isolates were collected from infected fruits obtained during postharvest survey of fruits from Mazzoni Company. Based on morphology of the colonies 312 isolates were identified as *Monilinia* spp., and DNA was extracted from 104 isolates (**Appendix 2**).

M. laxa colonies exhibited the characteristic lobes at the margin (**Figure 3.1**). *M. fructigena* presented itself in concentric ring pattern with entire margins (**Figure 3.2**). Lastly, *M. fructicola* has entire margins with sparse sporulation and absence of black arcs on colony rosette (**Figure 3.3**).

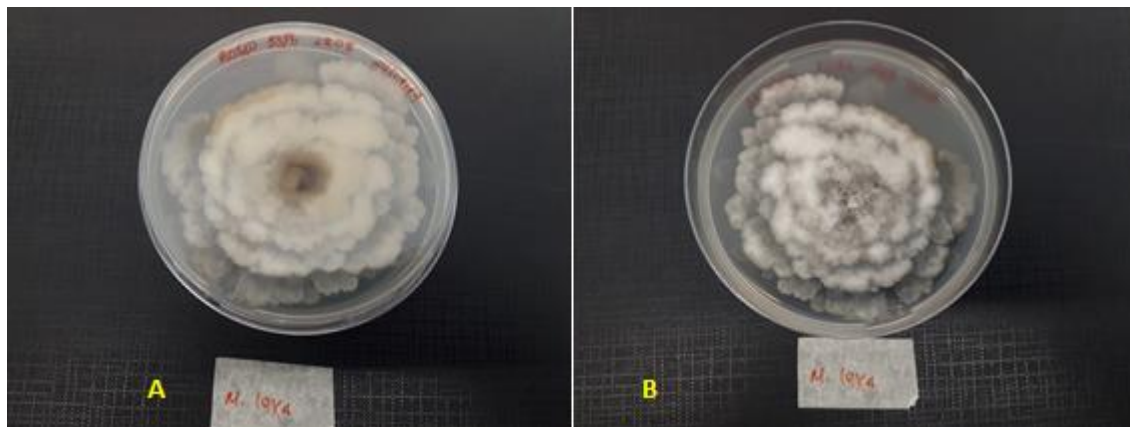


Figure 3.1 *Monilinia laxa* confirmed with multiplex PCR (A bottom view; B top view)

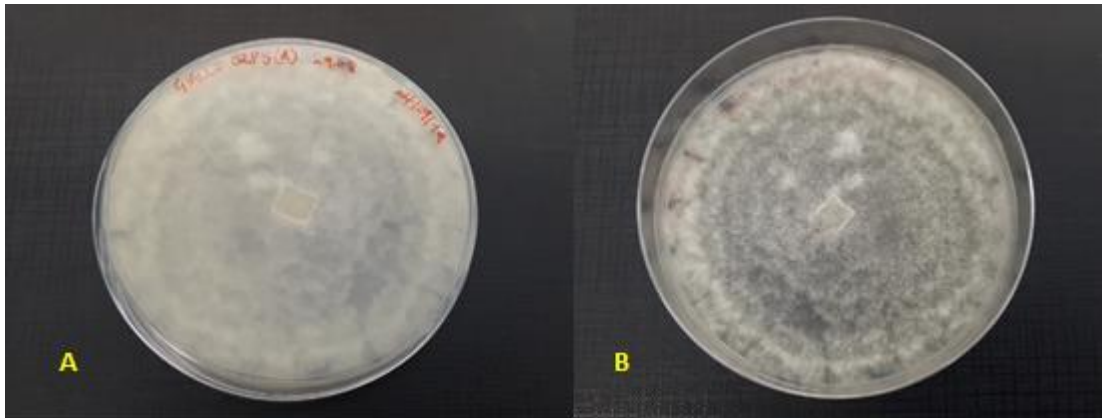


Figure 3.2 *Monilinia fructigena* confirmed with multiplex PCR (A bottom view; B top view)

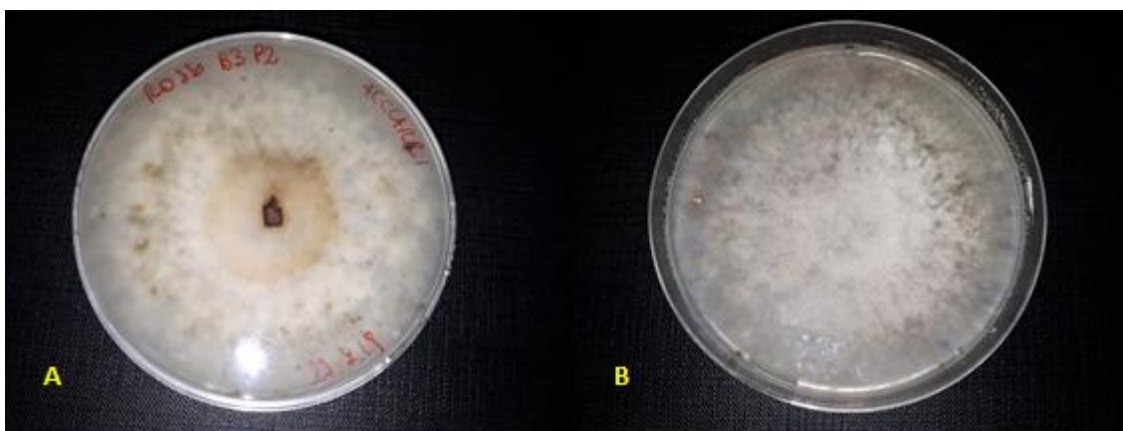


Figure 3.3 *Monilinia fructicola* confirmed with multiplex PCR (A bottom view; B top view)

3.1.2. Molecular identification of *Monilinia* spp. using multiplex polymerase chain reaction (PCR)

DNA amplification by multiplex PCR with the species specific primers Laxa-R2, MO-368-8R, MO 368-10R and common reserve primer MO 368-5, yielded 21 and 84 positive samples for Acciarri and Mazzoni orchards respectively. In detail, for Acciarri orchard 15 samples were positive for *M. laxa* (Figure 3.4-3.9) and 4 samples positive for *M. fructicola* (Figure 3.9, 3.12 and 3.13). Additionally, 3 samples were positive for mixed infection of *M. fructigena* and *M. fructicola* (Figure 3.5 and 3.7). Whereas for Mazzoni orchard 49 samples were positive for *M. laxa* (Figure 3.10-3.14), 7 samples were positive for *M. fructigena* (Figure 3.12 and 3.13), and 25 samples were positive for *M. fructicola* (Figure 3.12-3.14).

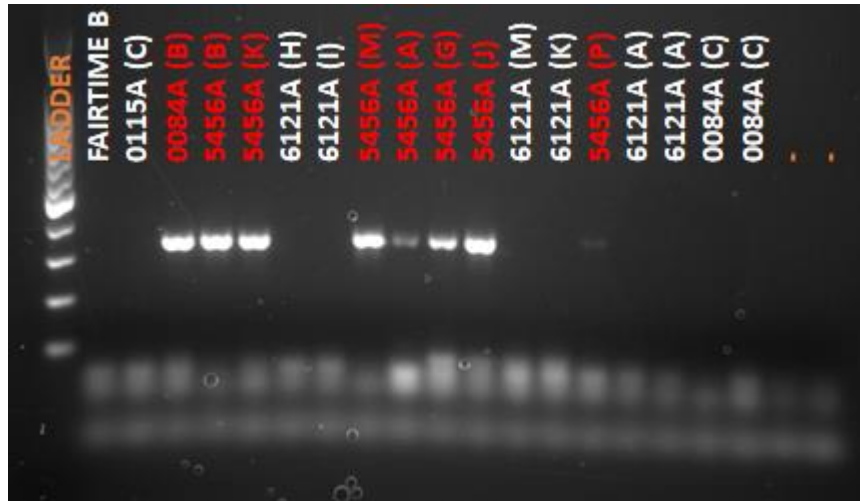


Figure 3.4 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** 0084A (B), 5456A (B), 5456A (K), 5456A (M), 5456A (A), 5456A (G), 5456A (J) and 5456A (P). **Negative samples:** FAIRTIME B, 0115A (C), 6121A (H), 6121A (I), 6121A (M), 6121A (K), 6121A (A), 6121A (A), 0084A (C) and 0084A (C). **Negative controls:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

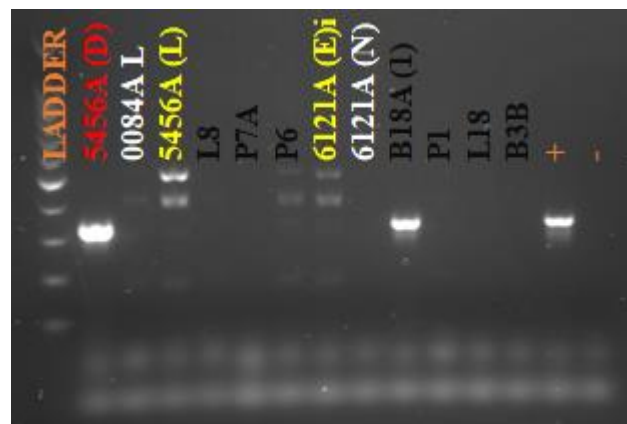


Figure 3.5 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** 5456A (D), 5456A (L), and 6121A (E). **Negative samples:** 0084A L and 6121A (N). **Positive control:** *Monilinia laxa*. **Negative control:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker



Figure 3.6 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** 5456A (C), 5456A (M) and 5456A (F). **Negative sample:** 6121A (N). **Negative controls:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

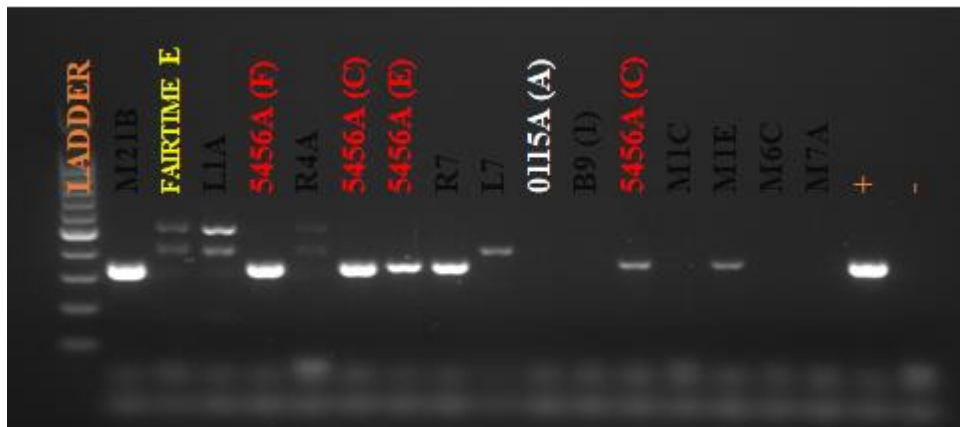


Figure 3.7 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** FAIRTIME E, 5456A (F), 5456A (C), 5456A (E), and 5456A (C). **Negative sample:** 0115A (A). **Positive control:** *Monilinia laxa*. **Negative control:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

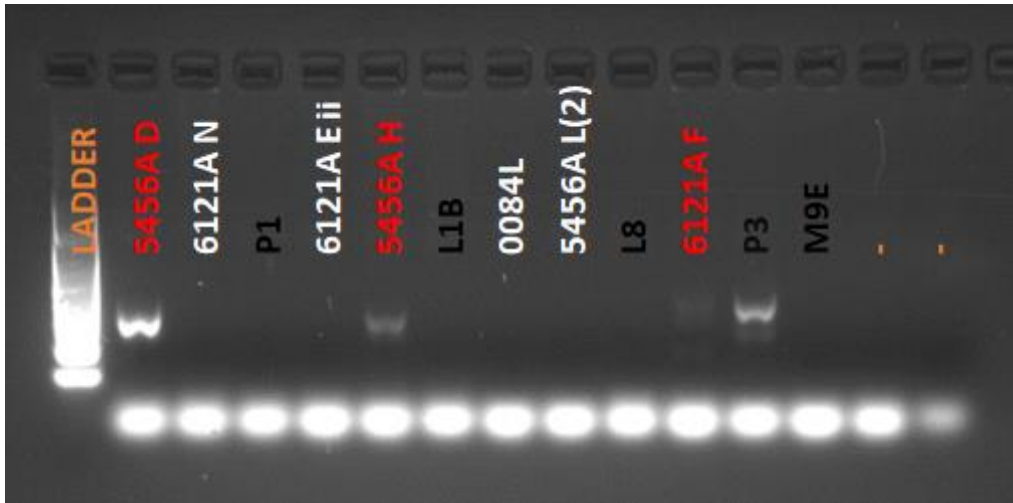


Figure 3.8 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** 5456A D, 5456A H, and 6121A F. **Negative samples:** 6121A N, 6121A E (ii), 0084A L, and 5456A L (2). **Negative controls:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

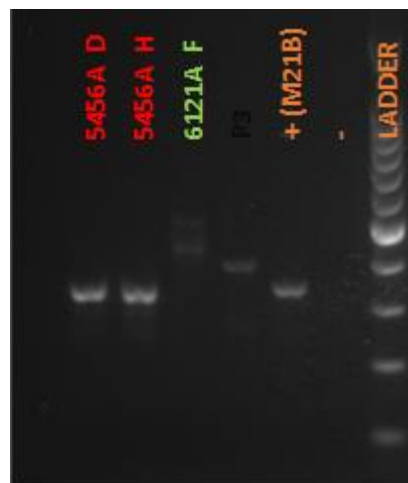


Figure 3.9 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** 5456A D, 5456A H and 6121A F. **Positive control:** *Monilinia laxa* (M21B). **Negative control:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

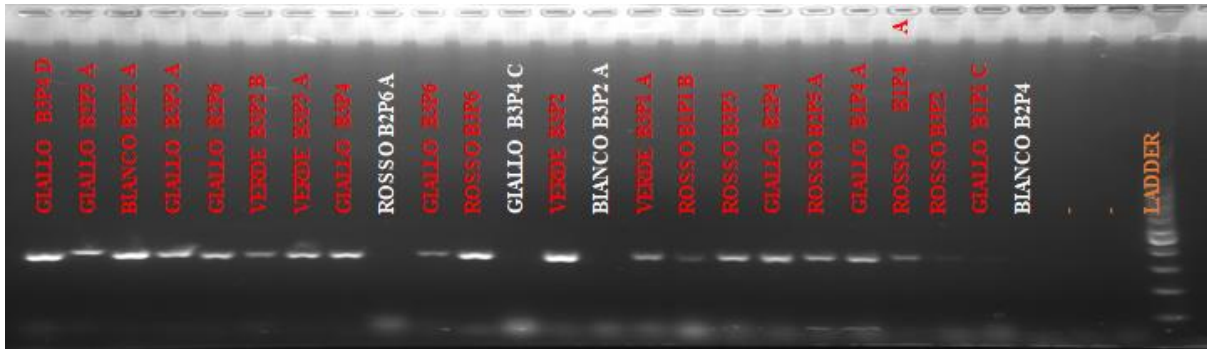


Figure 3.10 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** GIALLO B3P4 D, GIALLO B2P3 A, BIANCO B2P1 A, GIALLO B3P3 A, GIALLO B2P6, VERDE B3P2 B, VERDE B3P3 A, GIALLO B3P4, GIALLO B3P6, ROSSO B3P6, VERDE B3P2, VERDE B3P1 A, ROSSO B1P1 B, ROSSO B3P3, GIALLO B2P4, ROSSO B2P5 A, GIALLO B1P4 A, ROSSO B1P4 A, ROSSO B3P2, and GIALLO B1P1 C. **Negative samples:** ROSSO B2P6 A, GIALLO B3P4 C, BIANCO B3P2 A, and BIANCO B2P4. **Negative controls:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

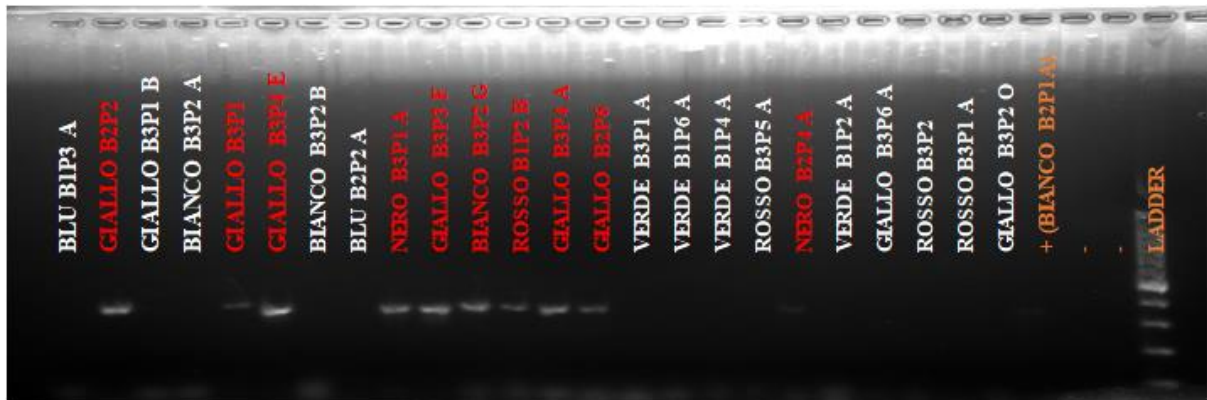


Figure 3.11 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** GIALLO B2P2, GIALLO B3P1, GIALLO B3P4 E, NERO B3P1 A, GIALLO B3P3 F, BIANCO B3P2 G, ROSSO B1P2 B, GIALLO B3P4 A, GIALLO B2P6, and NERO B2P4 A. **Negative samples:** BLU B1P3 A, GIALLO B3P1 B, BIANCO B3P2 A, BIANCO B3P2 B, BLU B2P2 A, VERDE B3P1 A, VERDE B1P6 A, VERDE B1P4A, ROSSO B3P5A, VERDE B1P2 A, GIALLO B3P6 A, ROSSO B3P2, ROSSO B3P1 A, and GIALLO B3P2 O. **Positive control:** *Monilinia laxa* (BIANCO B2P1 A). **Negative controls:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

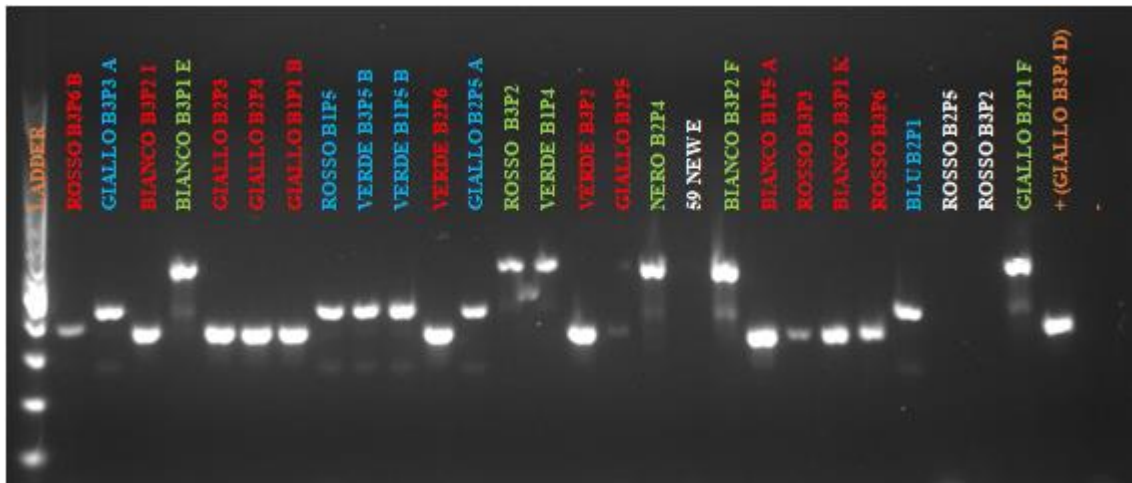


Figure 3.12 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** ROSSO B3P6 B, GIALLO B3P3 A, BIANCO B3P2 I, BIANCO B3P1 E, GIALLO B2P3, GIALLO B2P4, GIALLO B1P1 B, ROSSO B1P5, VERDE B3P5 B, VERDE B1P5 B, VERDE B2P6, GIALLO B2P5 A, ROSSO B3P2, VERDE B1P4, VERDE B3P2, GIALLO B2P5, NERO B2P4, BIANCO B3P2 F, BIANCO B1P5 A, ROSSO B3P3, BIANCO B3P1 K, ROSSO B3P6, BLU B2P1, and GIALLO B2P1 F. **Negative samples:** 59 NEW E, ROSSO B2P5 and ROSSO B3P2. **Positive control:** *Monilinia laxa* (GIALLO B3P4 D). **Negative control:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

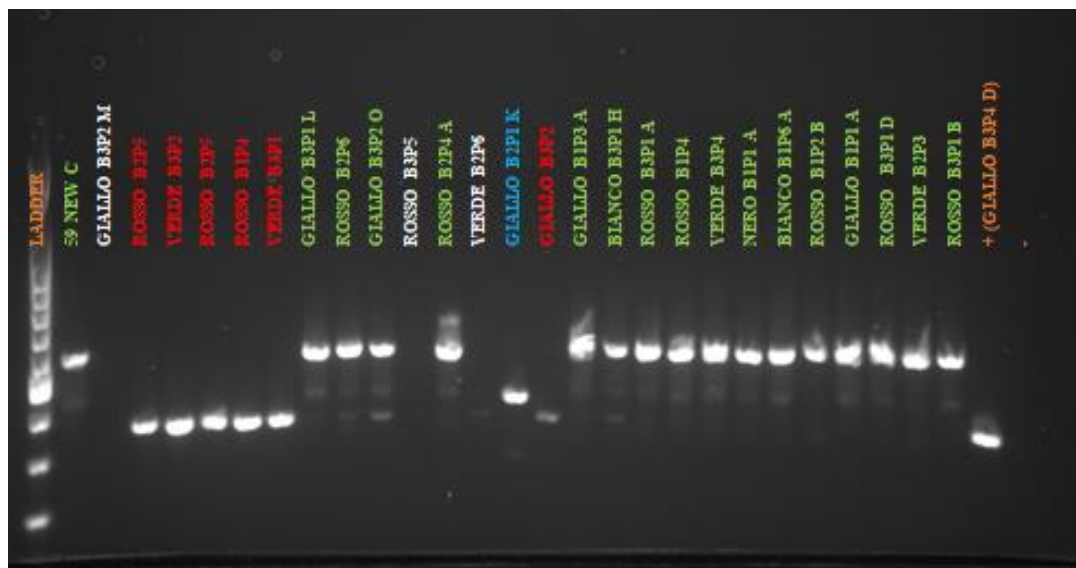


Figure 3.13 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** 59 NEW C, ROSSO B2P5, VERDE B3P2, ROSSO B2P5, ROSSO B1P4, VERDE B3P1, GIALLO B3P1 L, ROSSO B2P6, GIALLO B3P2 O, ROSSO B2P4 A, GIALLO B2P1 K, GIALLO B3P2, GIALLO B1P3 A, BIANCO B3P1 H, ROSSO B3P1 A,

ROSSO B1P4, VERDE B3P4, NERO B1P1 A, BIANCO B1P6 A, ROSSO B1P2 B, GIALLO B1P1 A, ROSSO B3P1 D, VERDE B2P3, and ROSSO B3P1 B. **Negative samples:** GIALLO B3P2 M, ROSSO B3P5, and VERDE B2P6. **Positive control:** *Monilinia laxa* (GIALLO B3P4 D). **Negative control:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

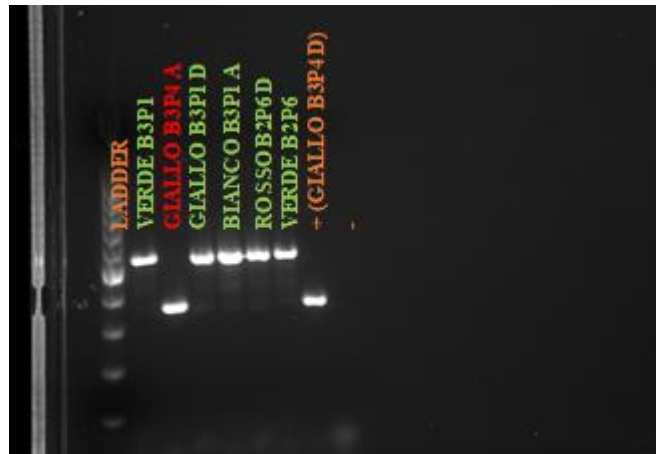


Figure 3.14 Results following amplification of DNA with multiplex PCR of fruit samples. **Positive samples:** VERDE B3P1, GIALLO B3P4 A, GIALLO B3P1 D, BIANCO B3P1 A, ROSSO B2P6 D and VERDE B2P6. **Positive control:** *Monilinia laxa* (GIALLO B3P4 D). **Negative control:** H₂O. **Ladder:** 100bp Plus Opti-DNA Marker

3.2. EFFECTIVENESS OF ALTERNATIVE COMPOUNDS IN PREVENTING *MONILINIA* SPP. INFECTIONS

3.2.1. Effects of alternative compounds on latent infection incidence caused by *Monilinia* spp.

The results obtained from this study indicate that nectarine cultivar, Honey Royal, subjected to commercial fungicides as well as alternative compounds and an untreated control showed varying degrees of latent infection incidence. The results are presented as an average for each sampling of a survey conducted over period of 3 days. On the first sampling treatments with chemical (10 gg), chemical (20 gg), chitosan, potassium bicarbonate, and untreated control determined an absence of latent infection compared to sulphur treatment which had a 0.11% infection incidence (**Table 3.1**). On the second sampling only potassium bicarbonate exhibited an incidence of 0.56%. On the third sampling there was total absence of brown rot

incidence. Fourth sampling showed an increase in infection incidence with the highest incidence of 4.76% recorded for sulphur treatment followed by chitosan with 3.17%, chemical (10 gg) and untreated control both at 2.12%, potassium bicarbonate at 1.68% and the lowest incidence at 0.89% for chemical (20 gg) treatment. On average, it is evident from the results that fruits treated with chemical (20 gg) exhibited the lowest percentage of incidence at 0.18% compared to fruits treated with sulphur, which amassed 0.97% (**Figure 3.15**).). Despite this, no significant differences were recorded among the various treatments performed. Figure 3.15 shows a representation of aggregated mean incidence per treatment.

Table 3.1 Mean latent infection incidence caused by *Monilinia* spp. during five samplings

Treatment	Incidence (%)				
	1 st sampling	2 nd sampling	3 rd sampling	4 th sampling	5 th sampling
Chemical (10 gg)	0	0	0	2.12	0
Chemical (20 gg)	0	0	0	0.89	0
Sulphur	0.11	0	0	4.76	0
Chitosan	0	0	0	3.17	0
Potassium bicarbonate	0	0.56	0	1.68	0
Untreated control	0	0	0	2.12	0

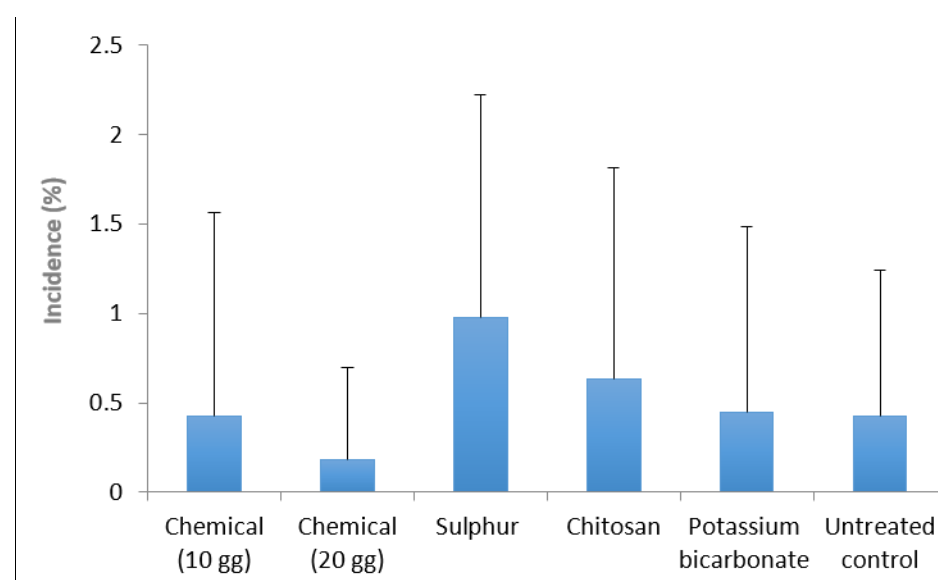


Figure 3.15 The effects of commercial fungicides and alternative compounds on latent infection incidence of *Monilinia* spp. on nectarine fruit

3.2.2. Effects of alternative compounds on field infection incidence caused by *Monilinia* spp.

The brown rot incidence values recorded in the field show that no treatment, including the chemical ones, had significantly reduced incidence compared to the untreated control. In any case, lower values were recorded in fruits treated with both the chemical strategies and among the alternative products chitosan showed the best control. Fruits treated with sulphur and potassium bicarbonate showed higher incidence values compared to the untreated control (Figure 3.16).

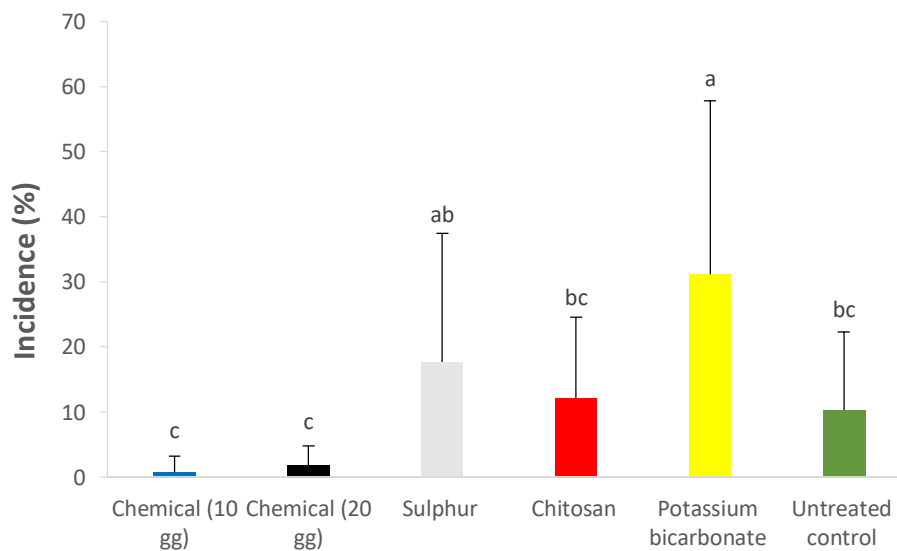


Figure 3.16 The effects of commercial fungicides and alternative compounds on field infection incidence of *Monilinia* spp. on nectarine fruit

3.2.3. Effects of alternative compounds on postharvest disease incidence caused by *Monilinia* spp.

Fruit treated with preharvest fungicides and alternative compounds, stored at room temperature, were used to study postharvest brown rot incidence caused by *Monilinia* spp. On day 1 of the survey chemical (10 gg) treatment did not show any incidence. On the same day for all other treatments brown rot infection were identified: the incidence was higher for chitosan at 3.70%, followed closely by potassium bicarbonate at 3.64%, sulphur at 1.96%, chemical (20 gg) at 1.19%, and control with the lowest incidence of 1.06% (Figure 3.17).

On day 2 there was a strong increase of brown rot incidence in all the treatments considered, especially those treated with alternative compounds. Chemical (10 gg) and chemical (20 gg) showed an incidence equal to 2.38% and 0.89% respectively. Sulphur and untreated control had dramatically sharp increased incidence to 7% and 6.61% respectively. Potassium bicarbonate experienced a slightly increased incidence of 3.92% while chitosan steadily reached 5.56% (**Figure 3.17**).

On day 3 fruit showed a lower incidence: chemical (20 gg), sulphur and potassium bicarbonate presented incidence less than 1%, while chemical (10 gg) had incidence of 1.06% and lastly, chitosan and control had incidence more than 2% (**Figure 3.17**).

Considering the sum of the incidence values obtained in the three surveys, a significant difference compared to the control was observed in the fruit treated with both chemicals, with incidence values significantly lower. All alternative compound treatments showed significantly equal values to the untreated control (**Figure 3.18**).

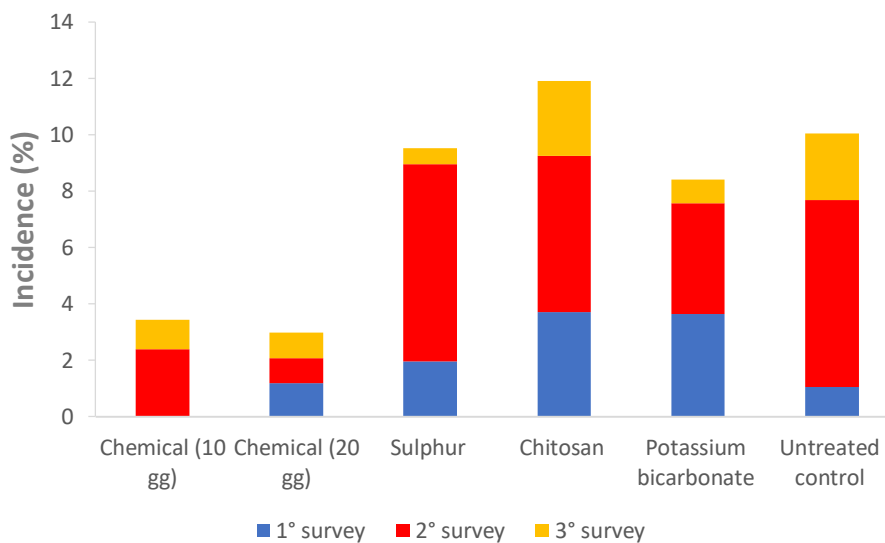


Figure 3.17 The effects of commercial fungicides and alternative compounds on postharvest disease incidence of *Monilinia* spp. on nectarine fruit

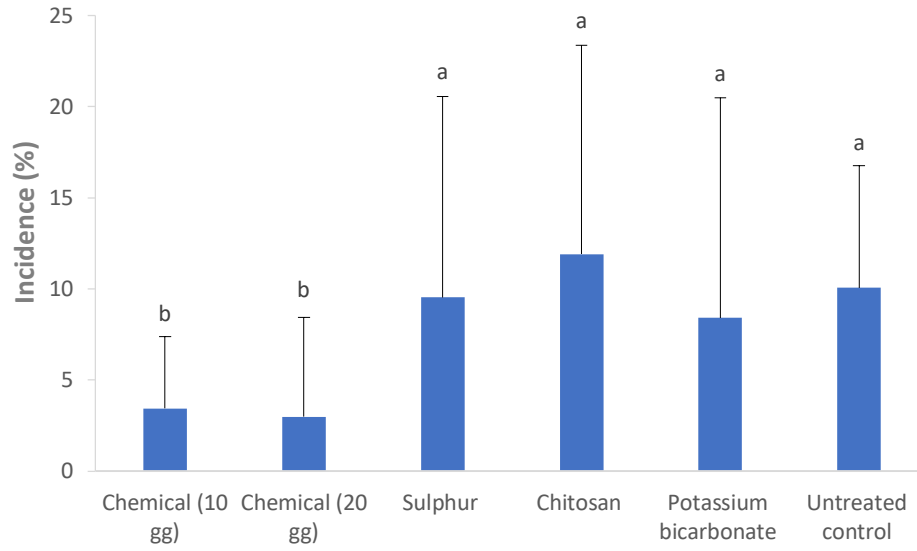


Figure 3.18 The effects of commercial fungicides and alternative compounds on postharvest disease incidence of *Monilinia* spp. on nectarine fruit (Means with different letters are significantly different at $p < 0.05$)

4. DISCUSSION AND CONCLUSIONS

One of the biggest challenges in stone and pome fruit production is brown rot caused by *Monilinia* species. The disease causes severe losses in preharvest and postharvest stages. Distribution of the pathogenic fungi is widespread through the continents, with more than one species occurring at a time in most affected areas but rarely with the same dominance. Although, some species are completely absent in some places other species have recently been introduced into new places.

M. laxa is the most common brown rot pathogen which occurs in many places worldwide and is known as causal pathogen of European brown rot (Holb, 2008a; Abate *et al.*, 2018). In Italy, *M. laxa* is the most widespread species found on stone fruit (Pellegrino *et al.*, 2009; Landi *et al.*, 2016). In Spain until the year 2006 the species was the most prevalent, causing 85-90% brown rot incidence, on peach fruit (Villarino *et al.*, 2016). In South Africa brown rot is solely caused by *M. laxa*. Carstens *et al.* (2010) confirmed the absence of *M. fructicola* using molecular techniques with species specific primers. *M. laxa* grows well at optimum temperature of 23°C (Abate *et al.*, 2018); lower than *M. fructicola* (Obi *et al.*, 2018). According to Obi *et al.* (2018) *M. laxa* can germinate in the absence of free water (a_w) in the host which makes this species as virulent as *M. fructicola*.

M. fructigena is known to mainly affect pome fruit than stone fruit. Vasić *et al.*, (2018) reported *M. fructigena* and *M. polystroma*, the former being the most predominant species, as the causal agents of brown rot on apple fruit in Serbia. Occurrence of the species was observed in the apple orchards as well as during storage. Infections by *M. laxa* and *M. fructicola* were also recorded though not as severe as the previous two species in the same apple orchards (Vasić *et al.*, 2018). *M. fructigena* grows well at optimum temperature of 20°C (Abate *et al.*, 2018).

M. fructicola is known as causal pathogen of American brown rot due to its wide spread in North and South America, and also in Japan, Australia, New Caledonia and New Zealand (Abate *et al.*, 2018). In Europe *M. fructicola* is classified as a quarantine pest (EPPO, 2018). In recent years *M. fructicola* has been spreading in Europe since 2001; it was first introduced in peach orchards in France and has since spread to other European countries including Italy, Poland, Germany, Spain, and Hungary (Rungjindamai *et al.*, 2014). The species was first introduced in Spain peach orchards in 2006, and since 2010 it has displaced *M. fructigena* and co-exists with *M. laxa*. This relationship works because each species causes infections at different times during fruit growth stages; *M. fructicola* frequency

increases on stored fruit, latent infections, pruned branches, blighted shoots and mummified fruit whereas frequency of *M. laxa* decreases progressively (Villarino *et al.*, 2016). The pathogen has been reported in several regions of Italy including Emilia Romagna, Lazio, Piemonte and Puglia on stone and pome fruits. In recent years, *M. fructicola* was discovered in Marche region on cherry fruits (Landi *et al.*, 2016). According to Abate *et al.* (2018) *M. fructicola* is replacing *M. laxa* and *M. fructigena* species on stone fruit in southern Italy; this is due to its well adaptation to climatic conditions of the area. The species grows well at optimum temperature of 26°C (Abate *et al.*, 2018) and warmer climate with extended periods of wetness (Angeli *et al.*, 2017, Obi *et al.*, 2018). Angeli *et al.* (2017) added that at high temperature lesions caused by *M. fructicola* produce more conidia in comparison to *M. laxa*.

In both orchards used for the study all three species of *Monilinia* were identified through morphological characteristics which were later confirmed by multiplex polymerase chain reaction (PCR) using three species specific primers. The most abundant and widely spread species was *M. laxa*, a result confirmed in multiple studies where this species represent the main causal agent of European brown rot. A total of 64 isolates tested positive for *M. laxa* between the two orchards. Only 7 isolates were positive for *M. fructigena*. This species is not as diffused as *M. laxa* as it is found more often on pome fruits. In summer 2019 *M. fructicola* was detected for the first time from 25 isolates in the orchard located in Ascoli Piceno, provided that in previous season no isolates had tested positive for the species. Whereas in the orchard located in Fermo 4 isolates tested positive for the species during surveys conducted in the summers of 2018 and 2019, one and three isolates respectively. The results also included 3 isolates which were positive for mixed infection of *M. fructigena* and *M. fructicola* from Fermo orchard collected in 2018.

Compared to previous results obtained in 2018, it is evident that *M. fructicola* is spreading rapidly in the area, and thus has the potential to displace *M. laxa* and *M. fructigena*. This can be due to several factors such as higher fitness in the hot and dry climate of the area, higher sporulation, and virulence (Abate *et al.*, 2018). The study area of Marche region falls within the Mediterranean climatic zone having temperate climate with dry and warm summers (De Pascale *et al.*, 2018). Villarino *et al.* (2016) added that *M. fructicola* has the lowest incubation and latency period on fruit at postharvest, and under favourable conditions the pathogen can cause up to 90% losses (Landi *et al.*, 2016). This explains the rapid spread in the environment. Within a period of less than five year from its first introduction in Spain *M. fructicola* co-existed with *M. laxa* and had completely displaced *M. fructigena*. A shift in occurrence of *Monilinia* species in a given area can cause a change in disease behaviour

(Angeli *et al.*, 2017), and therefore necessitates a change in phytosanitary strategies to effectively control the threat.

In recent years concerns have increased over the use of synthetic fungicides in fruit orchards due the dangers associated with fungicide use on human health and environmental pollution. Moreover, fungicides are prohibited by law for use in postharvest phase (Zhang *et al.*, 2019). In this study no alternative treatments were effective in controlling brown rot, and sometimes chemicals didn't seem to be effective in reducing latent infection and infections in the field. Despite this, chemical treatments were still more effective in comparison to alternative treatments. In fact, chemical treatments applied 10 days before harvest recorded the lowest value of field infection whereas chemical treatments applied 20 days before harvest recorded the lowest latent infection value. As a precaution, fungicides must be rotated using those having different active ingredients to prevent development of fungicide resistance. Villarino *et al.* (2016) reported high sensitivity of *M. fructicola* to cyflufenamid while Burnett *et al.* (2010) reported resistance to DMI fungicides by the same species.

In this study, the ONFIT method (Luo *et al.*, 2013) was used to detect latent brown rot infection on nectarine, cv. Honey Royal, caused by *Monilinia* species. During monitoring latent infection incidence was recorded at generally low values; nonetheless sulphur was the worst in controlling infection. While for infection in the field chitosan was better at controlling infection than other alternative compounds. *In vitro* and postharvest trials on peaches showed the effectiveness of chitosan in the control of *M. fructicola* (Yang *et al.*, 2012; Ma *et al.*, 2013). To increase the antifungal effectiveness, a combination of chitosan with other alternative compounds could be effective in reducing infection incidence of fruit at all production stages (Al-Eryani-Raqeeb *et al.*, 2009). Feliziani *et al.* (2013) also reported effective control of brown rot of sweet cherry using chitosan as preharvest and postharvest treatment. Furthermore, in this research at postharvest chemicals were most effective in controlling *Monilinia* spp. infection as there were significant differences observed compared to untreated control. A positive correlation between latent infection incidence and postharvest disease across the treatments was also observed, being that incidence increased at postharvest stage than it was at immaturity stage, as was also noted by Emery *et al.* (2000) and Gell *et al.* (2008).

In conclusion, accurate identification and distribution of the different *Monilinia* species occurring in an area is crucial for the development of correct and effective disease management strategies, thus minimising production loss due to ineffective chemical use, cost

of chemicals, and production yield loss. We found that chemicals used in this study were not as effective in controlling infection caused by *Monilinia* species as expected, thus further studies should be conducted to investigate fungicide resistance of these species to commercial fungicide products used in Italy. Also, more studies into chitosan amended with other alternative compounds could increase its effectiveness in controlling field infection, either on the same nectarine cultivar or on different cultivars. Lastly, if there is a chance for limiting *M. fructicola* spread, improved and intensive management strategies, including both cultural practices and chemical control methods, must be implemented urgently before it spreads any further than it already has within the orchards and nearby areas where it has been detected. The recent sequencing of main agents of brown rot on stone fruit, *M. laxa* (Landi *et al.*, 2019), *M. fructicola* (De Miccolis Angelini *et al.*, 2019) and *M. fructigena* (Landi *et al.*, 2018) can provide new information useful to clarify host-pathogen interactions, then contribute to better understand epidemiology and plan appropriate management strategies.

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APPENDICES

Appendix 1: Peach and nectarine isolates and *Monilinia* species identified by multiplex PCR.

	Isolates	Host	Cultivar	Vegetal tissue	DNA extraction	<i>Monilinia</i> spp.
1	5456A(A)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
2	5456A(B)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
3	5456A(C)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
4	5456A(D)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
5	5456A(E)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
6	5456A(F)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
7	5456A(G)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
8	5456A(H)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
9	5456A(I)	Peach	Sweet Lady	Fruit	-	-
10	5456A(J)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
11	5456A(K)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
12	5456A(L)	Peach	Sweet Lady	Fruit	+	<i>M. fructigena</i> <i>M. fructicola</i>
13	5456A(L) 2	Peach	Sweet Lady	Fruit	+	-
14	5456A(M)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
15	5456A(N)	Peach	Sweet Lady	Fruit	-	-
16	5456A(O)	Peach	Sweet Lady	Fruit	-	-
17	5456A(P)	Peach	Sweet Lady	Fruit	+	<i>M. laxa</i>
18	0084A(A)	Peach	Elegant Lady	Fruit	-	-
19	0084A(B)	Peach	Elegant Lady	Fruit	+	<i>M. laxa</i>
20	0084A(C)	Peach	Elegant Lady	Fruit	+	-
21	0084A(D)	Peach	Elegant Lady	Fruit	-	-
22	0084A(E)	Peach	Elegant Lady	Fruit	-	-
23	0084A(F)	Peach	Elegant Lady	Fruit	-	-
24	0084A(G)	Peach	Elegant Lady	Fruit	-	-
25	0084A(H)	Peach	Elegant Lady	Fruit	-	-
26	0084A(I)	Peach	Elegant Lady	Fruit	-	-

27	0084A(J)	Peach	Elegant Lady	Fruit	-	-
28	0084A(K)	Peach	Elegant Lady	Fruit	-	-
29	0084A(L)	Peach	Elegant Lady	Fruit	+	<i>M. laxa</i>
30	0115A(A)	Peach	Sweet Red	Fruit	+	-
31	0115A(B)	Peach	Sweet Red	Fruit	-	-
32	0115A(C)	Peach	Sweet Red	Fruit	+	-
33	0115A(D)	Peach	Sweet Red	Fruit	-	-
34	0115A(E)	Peach	Sweet Red	Fruit	-	-
35	0115A(F)	Peach	Sweet Red	Fruit	-	-
36	0115A(G)	Peach	Sweet Red	Fruit	-	-
37	0115A(H)	Peach	Sweet Red	Fruit	-	-
38	6121A(A)	Nectarine	Big Top	Fruit	+	-
39	6121A(B)	Nectarine	Big Top	Fruit	+	-
40	6121A(C)	Nectarine	Big Top	Fruit	-	-
41	6121A(D)	Nectarine	Big Top	Fruit	-	-
42	6121A(E) i	Nectarine	Big Top	Fruit	+	<i>M. fructigena</i> <i>M. fructicola</i>
43	6121A(E) ii	Nectarine	Big Top	Fruit	+	-
44	6121A(F)	Nectarine	Big Top	Fruit	+	<i>M. laxa</i>
45	6121A(F)	Nectarine	Big Top	Fruit	+	<i>M. fructicola</i>
46	6121A(G)	Nectarine	Big Top	Fruit	-	-
47	6121A(H)	Nectarine	Big Top	Fruit	+	-
48	6121A(I)	Nectarine	Big Top	Fruit	+	-
49	6121A(J)	Nectarine	Big Top	Fruit	-	-
50	6121A(K)	Nectarine	Big Top	Fruit	+	-
51	6121A(L)	Nectarine	Big Top	Fruit	-	-
52	6121A(M)	Nectarine	Big Top	Fruit	+	-
53	6121A(N)	Nectarine	Big Top	Fruit	+	-
54	6121A(O)	Nectarine	Big Top	Fruit	+	-
55	6121A(P)	Nectarine	Big Top	Fruit	-	-
56	Fairtime(A)	Peach	Fairtime	Fruit	-	-
57	Fairtime(B)	Peach	Fairtime	Fruit	+	-

58	Fairtime(C)	Peach	Fairtime	Fruit	-	-
59	Fairtime(D)	Peach	Fairtime	Fruit	-	-
60	Fairtime(E)	Peach	Fairtime	Fruit	+	<i>M. fructigena</i> <i>M. fructicola</i>
61	58 NEW A	Peach	Extreme 486	Fruit	-	-
62	59 NEW A	Peach	Extreme 486	Fruit	-	-
63	59 NEW B	Peach	Extreme 486	Fruit	-	-
64	59 NEW C	Peach	Extreme 486	Fruit	+	<i>M. fructicola</i>
65	59 NEW D	Peach	Extreme 486	Fruit	-	-
66	59 NEW E	Peach	Extreme 486	Fruit	+	-
67	VERDE B1P4	Peach	Summer Royal	Fruit	+	<i>M. fructicola</i>
68	ROSSO B3P2	Peach	Summer Royal	Fruit	+	<i>M. fructicola</i>
69	ROSSO B2P5	Peach	Summer Royal	Fruit	-	-
70	BLU B3P5	Peach	Summer Royal	Fruit	-	-

Appendix 2: Nectarine isolates and *Monilinia* species identified by multiplex PCR.

	Isolates	Host	Cultivar	Vegetal tissue	DNA extraction	<i>Monilinia</i> spp.
1	BLU B1P3 A	Nectarine	Honey Royal	Fruit	+	-
2	BLU B2P2 A	Nectarine	Honey Royal	Fruit	+	-
3	BLU B2P1	Nectarine	Honey Royal	Fruit	+	<i>M. fructigena</i>
4	NERO B3P1 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
5	NERO B2P4 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
6	NERO B2P4	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
7	NERO B1P1 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
8	BIANCO B2P1 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
9	BIANCO B3P2 A	Nectarine	Honey Royal	Fruit	+	-
10	BIANCO B2P4	Nectarine	Honey Royal	Fruit	+	-
11	BIANCO B3P2 A	Nectarine	Honey Royal	Fruit	+	-
12	BIANCO B3P2 B	Nectarine	Honey Royal	Fruit	+	-
13	BIANCO B3P2 G	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>

14	BIANCO B3P2 I	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
15	BIANCO B3P1 E	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
16	BIANCO B3P2 F	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
17	BIANCO B1P5 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
18	BIANCO B3P1 K	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
19	BIANCO B3P1 H	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
20	BIANCO B1P6 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
21	BIANCO B3P1 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
22	ROSSO B2P6 A	Nectarine	Honey Royal	Fruit	+	-
23	ROSSO B3P6	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
24	ROSSO B1P1 B	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
25	ROSSO B3P3	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
26	ROSSO B2P5 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
27	ROSSO B1P4 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
28	ROSSO B3P2	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
29	ROSSO B1P2 B	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
30	ROSSO B3P5 A	Nectarine	Honey Royal	Fruit	+	-
31	ROSSO B3P2	Nectarine	Honey Royal	Fruit	+	-
32	ROSSO B3P1 A	Nectarine	Honey Royal	Fruit	+	-
33	ROSSO B3P6 B	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
34	ROSSO B1P5	Nectarine	Honey Royal	Fruit	+	<i>M. fructigena</i>
35	ROSSO B3P3	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
36	ROSSO B3P6	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
37	ROSSO B2P5	Nectarine	Honey Royal	Fruit	+	-
38	ROSSO B3P2	Nectarine	Honey Royal	Fruit	+	-
39	ROSSO B2P5	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
40	ROSSO B2P5	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
41	ROSSO B1P4	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
42	ROSSO B2P6	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
43	ROSSO B3P5	Nectarine	Honey Royal	Fruit	+	-
44	ROSSO B2P4 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
45	ROSSO B3P1 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>

46	ROSSO B1P4	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
47	ROSSO B1P2 B	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
48	ROSSO B3P1 D	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
49	ROSSO B3P1 B	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
50	ROSSO B2P6 D	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
51	VERDE B3P2 B	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
52	VERDE B3P3	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
53	VERDE B3P2	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
54	VERDE B3P1 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
55	VERDE B3P1 A	Nectarine	Honey Royal	Fruit	+	-
56	VERDE B1P6 A	Nectarine	Honey Royal	Fruit	+	-
57	VERDE B1P4 A	Nectarine	Honey Royal	Fruit	+	-
58	VERDE B1P2 A	Nectarine	Honey Royal	Fruit	+	-
59	VERDE B3P5 B	Nectarine	Honey Royal	Fruit	+	<i>M. fructigena</i>
60	VERDE B1P5 B	Nectarine	Honey Royal	Fruit	+	<i>M. fructigena</i>
61	VERDE B2P6	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
62	VERDE B3P2	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
63	VERDE B3P2	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
64	VERDE B3P1	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
65	VERDE B2P6	Nectarine	Honey Royal	Fruit	+	-
66	VERDE B3P4	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
67	VERDE B2P3	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
68	VERDE B3P1	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
69	VERDE B2P6	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
70	GIALLO B3P4 D	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
71	GIALLO B2P3 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
72	GIALLO B3P3 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
73	GIALLO B2P6	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
74	GIALLO B3P4	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
75	GIALLO B3P6	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
76	GIALLO B3P4 C	Nectarine	Honey Royal	Fruit	+	-
77	GIALLO B2P4	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>

78	GIALLO B1P4 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
79	GIALLO B1P1 C	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
80	GIALLO B2P2	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
81	GIALLO B3P1 B	Nectarine	Honey Royal	Fruit	+	-
82	GIALLO B3P1M	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
83	GIALLO B3P4 E	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
84	GIALLO B3P3 F	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
85	GIALLO B3P4 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
86	GIALLO B2P6	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
87	GIALLO B3P6 A	Nectarine	Honey Royal	Fruit	+	-
88	GIALLO B3P2 O	Nectarine	Honey Royal	Fruit	+	-
89	GIALLO B3P3 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructigena</i>
90	GIALLO B2P3	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
91	GIALLO B2P4	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
92	GIALLO B1P1 B	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
93	GIALLO B2P5 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructigena</i>
94	GIALLO B2P5	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
95	GIALLO B2P1 F	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
96	GIALLO B3P2 M	Nectarine	Honey Royal	Fruit	+	-
97	GIALLO B3P1 L	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
98	GIALLO B3P2 O	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
99	GIALLO B2P1 K	Nectarine	Honey Royal	Fruit	+	<i>M. fructigena</i>
100	GIALLO B3P2	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
101	GIALLO B1P3 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
102	GIALLO B1P1 A	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>
103	GIALLO B3P4 A	Nectarine	Honey Royal	Fruit	+	<i>M. laxa</i>
104	GIALLO B3P1 D	Nectarine	Honey Royal	Fruit	+	<i>M. fructicola</i>