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PhD thesis

Brown Rot disease development in peach (*P. persica* L. Batsch): from fungal biology to high-throughput on-field phenotyping

A Dissertation
by

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Thesis Abstract

Brown rot (BR) disease, caused by *Monilinia* spp., causes significant pre-and post-harvest losses in stone fruit production, especially in humid and warm temperatures. In this thesis, we tried to tackle the subject with three complementary approaches. First, the recent progress in BR resistance in peach fruit was reviewed. Then we highlighted best practices in phenotyping BR susceptibility/resistance procedures in field and *in vitro*. We concluded that the main factors contributing to disease development are *Monilinia* inocula availability, environmental conditions, cultivars, fruit stage and management practices.

Secondly, we investigated the anti-fungal effect of some phenolics such as chlorogenic and ferulic acids and triterpenoids such as oleanolic, betulinic, and ursolic acids. Furthermore, fruit surface compound (FSC) extracts of peach fruit at two developmental stages on *Monilinia fructicola* and *M. laxa* characteristics during *in vitro* growth were studied. A new procedure for assaying anti-fungal activity of triterpenoids, which are notoriously difficult to assess *in vitro* because of their hydrophobicity, has been developed. Also, a follow-up of this study revealed that certain phenolics and triterpenoids showed modest anti-fungal activity while dramatically modulating *M. fructicola* gene expression. *MfRGAE1* gene was overexpressed by chlorogenic and ferulic acids and *MfCUT1* by betulinic acid at 4- and 7-days post-inoculation.

The third objective was to investigate the genetic background responsible for disease resistance in peach by detecting Quantitative Trait Loci (QTL) and attempts to identify molecular markers for assisted selection (MAS) in peach. For this, three F2 progenies, derived from three selfied F1 selections obtained from "Contender" (C, resistant) × "Elegant Lady" (El, susceptible), were investigated for two seasons (2019 and 2021). The whole progeny was genotyped by Single-Primer Enriched Technology (SPET) and a recently developed 18K SNP array. The genome-wide QTL analysis showed intriguing areas relevant to disease resistance, mainly the QTLs on chromosomes 2 and 4, which may be candidates for future MAS

applications. Several new QTLs were detected for other fruit quality traits, including maturity date, soluble solid content and fruit weight.

ACKNOWLEDGMENT

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BA	Betulinic acid
BC1	Backcrossing
BLAST	Basic local alignment search tool
BLUP	Best linear unbiased prediction
BR	Brown rot
CAZymes	carbohydrate-active enzymes
cDNA	Complementary DNA
CGA	Chlorogenic acid
Chr	Chromosome
CTAB	Cetyltrimethyl ammonium bromide
CWDEs	cell wall degrading enzymes
DNA	Deoxyribonucleic acid
dpi	days post-inoculation
ESTs	Expressed sequence tags
EtOH	Ethanol
FA	Ferulic acid
FarmCPU	Fixed and random model Circulating Probability Unification
FSC	Fruit surface compounds
FW	Fruit Weight
GLM	General linear model
GWAS	Genome-Wide Association Study
IAD	index of absorbance difference
LG	linkage group
LMM	Linear mixed model
LOD	logarithm of odds
MAF	minor allele frequency
MAS	Marker-Assisted Selection
mbp	megabase pair
MD	Maturity date
NCBI	National Center for Biotechnology Information
OA	oleanolic acid
PCA	Principal component analysis
PDA	Potato dextrose agar
qRT-PCR	Quantitative real-time PCR
QTL	Quantitative trait locus

RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
SPET	Single-Primer Enriched Technology
SSC	soluble solid concentration
UA	ursolic acid
V8A	Vegetable 8 juice agar

CHAPTER 1. General introduction

1.1. Peach: Taxonomy and Brief history

Peaches and nectarines are members of the Rosaceae family, subfamily Prunoideae, in the genus *Prunus*, subgenus Amygdalus, section Euamygdalus. Commercial peach and nectarine cultivars belong to the species *Prunus persica* (L.) Batsch. (Bassi & Monet, 2008). Peaches including the smooth-skinned peaches known as nectarines are grown throughout the warmer temperate regions; although the peach's major production zones are between 30° and 45° degrees latitude in both hemispheres, it is also grown in the subtropics and tropical regions (Bassi & Monet, 2008; Byrne et al., 2000). The recessive allele of the MYB gene *PpeMYB25,* is a candidate gene for the skin trichome formation, thus responsible for fuzzless fruit skin, nectarines play a vital role in the peach industry (Vendramin et al., 2014). Peach fruit is a drupe and the stone maybe freestone, with mature flesh that readily separates, or clingstone, with flesh that sticks firmly to the stone.

The cultivated peach is a diploid species (2n = 2x = 16); the size of the *Prunus* genome is one of the smallest among cultivated species, with an estimated length of 290 Mbp in peach. Peach has a self-compatible mating behavior, (unlike most of its congeneric species, which have a gametophytic self-incompatibility system), causing bottlenecks in its breeding history, resulting in a lower level of genetic variability compared to other *Prunus* crops (Abbott et al., 2008).

Peach diversity is concentrated in China, where it was, most likely, domesticated some 3,000 years ago. Peaches were introduced from China to all temperate and subtropical areas on the Asian continent and then spread to Persia (modern Iran) along the Silk Road more than 2,000 years ago, and from there to the Mediterranean basin. It was then carried to the Americas by Spanish and Portuguese explorers from Europe since the 14th century (Byrne et al., 2012).

1.2.   Economic Importance of peaches

After apples and pears, the peach is the third most significant temperate tree fruit species. Since the last 15 years, the global output of peaches (including nectarines) has grown marginally, with most of the growth attributed to Asia, mainly China, while production in Europe has remained stable (Table 1.1). The world commercial value was estimated at ~$7.23 trillion in 2018, with ~$0.6 trillion being produced in Italy alone (FAOSTAT, 2021).

Table 1.1. World peaches and nectarines production (1000 metric tons: MT*) from 2005 to 2020, average of 4 years (FAOSTAT, 2021).

Region	2005-2008	2009-2012	2013-2016	2017-2020
Africa	869.8	867.6	899.6	1055.3
Americas	2479.8	2465.9	2171.7	1809.4
Asia	11049.7	13157.9	15677.7	17473.5
Europe	4374.2	4257.7	4037.5	4049.3
Oceania	125.7	104.2	83.5	83.5
World	18899.1	20853.2	22869.8	24470.9

* Metric tons = 1,000 kilograms.

Peaches are primarily produced in Asia, accounting for roughly 70% of total production, followed by Europe, accounting for 16%; in other continents is still a minor fruit (Figure 1.1). Italy is the second-largest European producer, after Spain. However, in the case of Italy, yearly oscillations and a tendency to reduce the production of the peach industry can be seen, ranging from 1.35 to 1.0 million MT. On the other hand, Greece has seen consistent growth over the past five years (Figure 1.2).

World production of peaches and nectarines by region
FAOSTAT, average 2015-2020
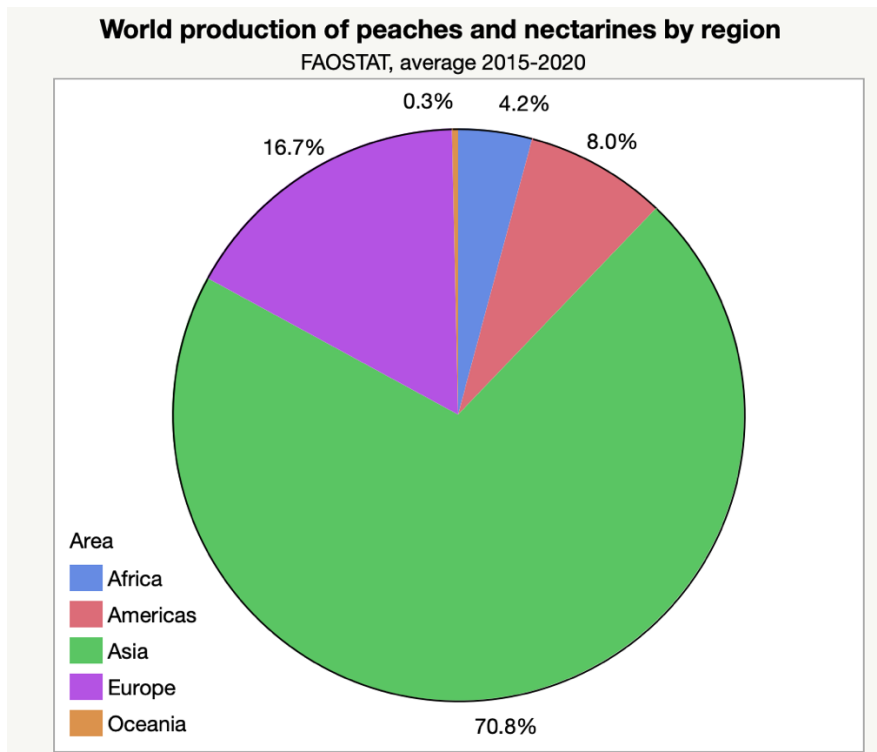
Figure 1.1. World peaches and nectarines production average from 2015 to 2020 presented as a percentage (FAOSTAT, 2021).

Main EU peach and nectarine producers
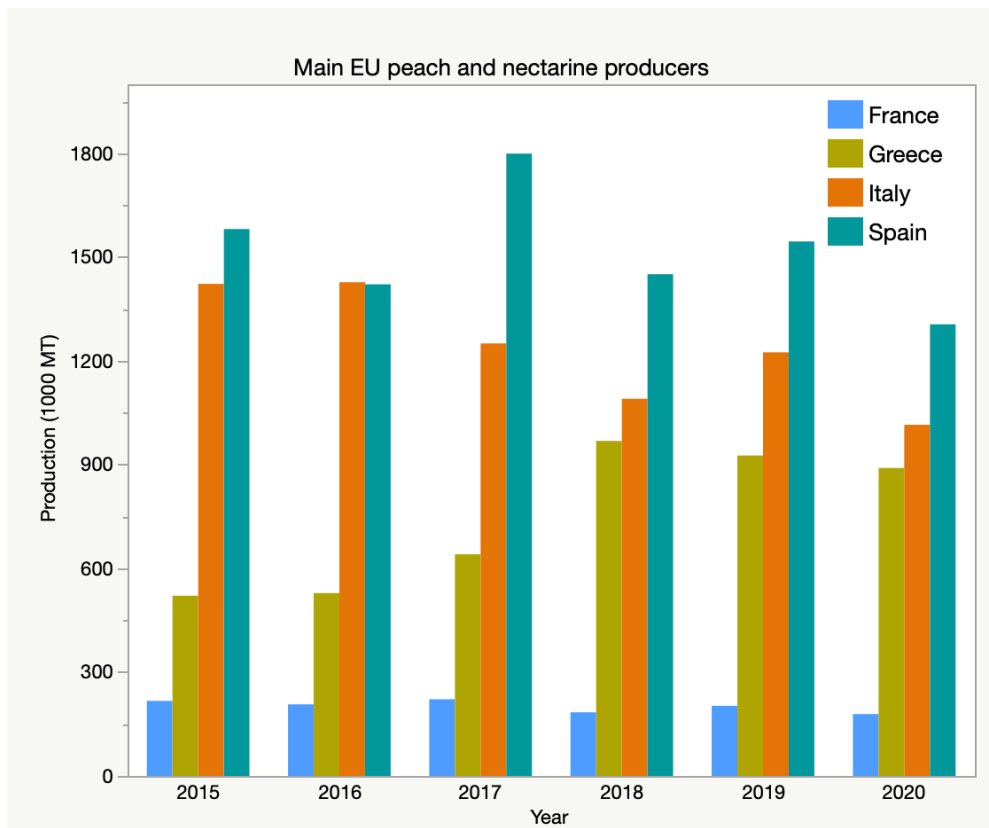
Figure 1.2. Main European peaches and nectarines production from 2015 to 2020 (FAOSTAT, 2021). Values are presented in 1000 metric tonnes (MT) per production season.

1.3. Main challenges and limitations of peach industry

Peach cultivation is still facing several biotic and abiotic challenges that have hampered peach industry. Plant diseases caused by fungal and bacterial infections are the major challenges, causing a wide variety of substantial damages at all stages of peach cultivation.

Brown rot disease caused by *Monilinia* spp. is one of the most common and damaging pre-and postharvest disease, especially in humid and warm temperatures. High humidity and warm temperature can also favor the incidence of several other diseases, for instance, anthracnose *Colletotrichum acutatum* J. H. Simmonds (Byrne et al., 2012). However, cooler temperatures favor powdery mildew caused by the obligate pathogenetic fungus *Sphaerotheca pannosa* (Wallr.) var. *persicae* (Foulongne et al., 2003), and peach leaf curl caused by *Taphrina deformans* (Berk.) Tul. (Byrne et al., 2012), both also considered serious diseases in Europe.

Regarding bacterial infections, *Xanthomonas arboricola* pv. *pruni* (Smith, 1903) is a key disease of peaches and many other *Prunus* species in several countries. The severity of this bacteria is favored mainly by wind-driven rain (Battilani et al., 1999). Several insects and mites can be key pests to peaches when adequate management is not followed, including the Mediterranean fly (*Ceratitis capitata*), especially in milder areas and in mid-season and late varieties. Aphids, bugs (i.e., brown marmorated stink bug *Halyomorpha halys* (Stål)) and western flower thrips (*Frankliniella occidentalis*) are also significant pests (Adaskaveg et al., 2008; Horton et al., 2008).

Beyond the humidity-related problems encountered throughout the latitudinal range of the peach, temperature-related challenges are seen at the extreme latitudes at which peaches are grown. Minimum winter temperatures and spring frosts are the limiting factors in high latitudes of 45° north and south or above. Flower bud death and consequently crop losses are widespread in those places due to cold temperatures (Byrne et al., 2012).

Low fruit quality and international competition and overproduction have been reported as additional challenges in the peach industry (Fideghelli et al., 1997). Furthermore, high

production expenses from annual services such as inputs (fertilizers, chemicals), labor forces, irrigation, machinery, hail protection and more can also be highlighted.

1.4. Brown rot (Monilinia spp.): taxonomy and life cycle

The fungi that cause BR belongs to the division Ascomycota, class Leotiomycetes, order Helioteliales, *Monilinia* is a genus of the Sclerotiniaceae family. The apothecium, or fruiting body, is generated from pseudosclerotia developed in mummified fruit partially or entirely buried in the earth or in detritus where the sexual spores (ascospores) are produced (Holst-Jensen et al., 1997; Martini & Mari, 2014). Depending on the host, the mycelium creates elliptical chains under warm, humid conditions. Conidia of the *Monilia*-type on tufts of hyphal branches (sporodochia). This form, which looks like beads on a thread, is called moniloid, which comes from the Latin word monile that means necklace (Byrde & Willetts, 1977).

Primary inoculum sources overwinter as mycelium in mummified fruit on the tree and in twig cankers or as pseudosclerotia in mummies on the orchard ground (Gell et al., 2009; Villarino et al., 2010). Wind and rain disseminate the conidia to sensitive host tissues, where they germinate under ideal moisture and temperature conditions.

Secondary inoculum can arise from any infected tissue in which the moisture content is sufficient for conidial sporulation. The chains of conidia provide the inoculum for other parts of the same or neighboring trees where they grow on infected tissue and act as a secondary inoculum for immature and mature fruit infection (Byrde & Willetts, 1977; Ioos & Frey, 2000). In this stage *Monilinia* acts as polycyclic, the new conidia have the potential to infect additional fruits. Several generations may occur during the growth season, depending on the climatic conditions. These conidia infect fruit and can cause brown rot in favorable climatic conditions or remain dormant in unfavorable climatic conditions. BR disease develops when these conditions become conducive for disease expression.

1.5. Main damage and symptoms of BR disease

The causal agent *Monilinia* spp. infects aerial parts of host plants, causing blighting of blooms, cankers on woody tissues, and fruit rotting, among other symptoms (Figure 1.3). Blossom blight is the earliest symptom of spring, appearing on blooms and developing when spores or conidia settle on and penetrate the flower stems of susceptible plants (Hong & Michailides, 1999; Villarino et al., 2010).

Any part of the flower, including the stigma, stamens, petals, and sepals, can get infected. On the decaying, shriveled floral portions, the mycelium develops many conidial tufts from which fresh masses of conidia are discharged. The mycelium spreads quickly through the bloom petioles, fruit spurs, and twigs, forming a depressed, reddish-brown, shield-shaped canker (Watson et al., 2002). Green fruit infections are uncommon and manifest as soft, water-soaked, dark regions; rot appears first in clustered fruit, fruit contact spots, and insect- or wind-damaged fruit, as clustered fruit is more conducive to disease growth (Martini & Mari, 2014).

When the relative humidity is high and/or the fruits are soft and ripe, conidial tufts or vegetative mycelium cover practically the whole surface of the fruit. While when the relative humidity is low and/or the fruits are not ripe, no mycelium and little or no conidial tufts form. Stem cankers occasionally develop from blighted twigs or fruit spurs by growth of mycelium in larger limbs of the tree. Gum is often exuded in the diseased area (Watson et al., 2002). The fungus begins by growing intracellularly, secreting enzymes that produce maceration and browning of the infected tissues; it quickly invades the fruit, generating conidial tufts on the already rotten region (Lee & Bostock, 2007).

Infections occurring in the field can remain quiescent until the fruit reaches ripening. Regarding quiescent infections, in which the pathogen is restricted within the host in an inactive state, host factors may signal the pathogen to remain in a state of quiescent, preventing it from transforming into an infectious form (Wang et al., 2002). Fruit susceptibility is shown to be

dramatically reduced during the period corresponding to the pit hardening stage (Mari et al., 2003); further details are discussed in chapter 3.

(A)   **(B)**
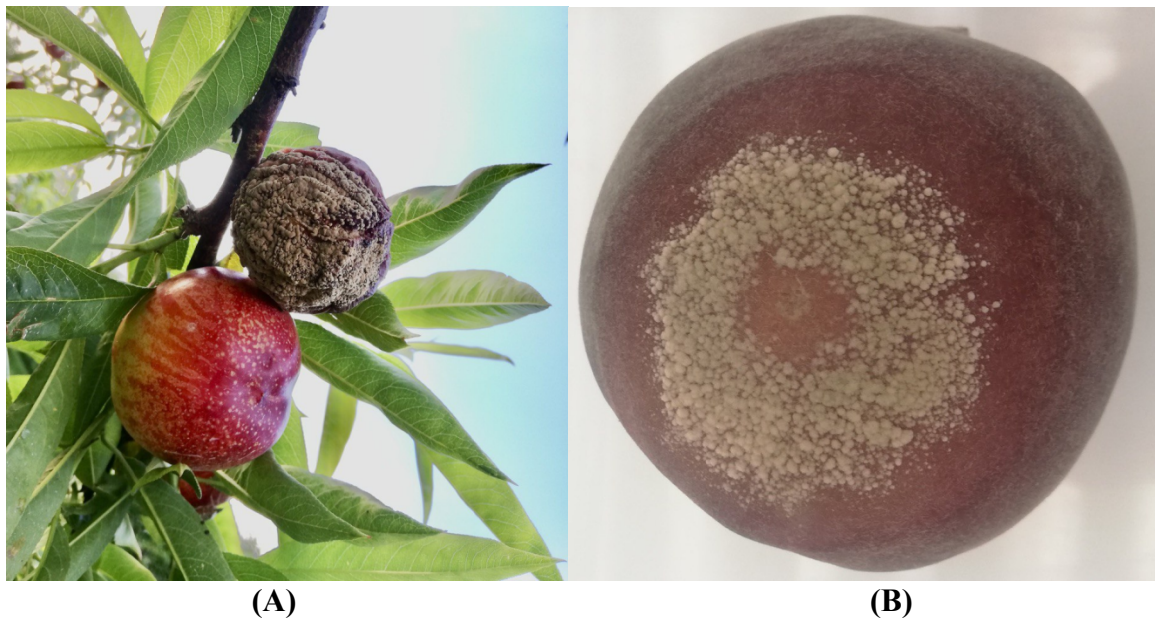
(C)

Figure 1.3. A peach fruit infected in field condition (A), and artificially inoculated in vitro (B) by *M. laxa*. New shoots of a nectarine cultivar heavily infected by *Monilinia* spp. (C) in the experimental orchard (Imola, Italy).

Conclusively, *Monilinia* may infect any plant part above soil. However, economically substantial damage of BR on peaches can be attributed to fruit losses, whereas in case of apricots, blossom infection is a key disease beside fruit infection. Although flower blight resistance varies among *Prunus* species, there appears to be no correlation between flower and

fruit resistance, necessitating selection for both floral blight and fruit response (Wagner et al.,

2005) when assessing for resistance attitude.

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CHAPTER 2. Thesis objectives

The following are the research objectives for this Ph.D. project:

1. Reviewing the factors contributing to Brown rot disease development in field and phenotyping procedures used in breeding programs and cultivar assessment for BR resistance.

2. Investigating the *in vitro* antifungal effects of important phenolic and triterpenoid compounds against *Monilinia fructicola* and *M. laxa*, that have been correlated with BR disease resistance in peaches. The follow-up objective for this part was studying the gene expression of virulence genes in *M. fructicola*.

3. Identifying QTL(s) of BR disease resistance, including other fruit traits such as maturity date, Soluble solid content, peach/nectarine in a F_2 progeny derived from "Contender" × "Elegant lady".

CHAPTER 3. Phenotyping Brown Rot Susceptibility in Stone Fruit: a Literature Review With Emphasis on Peach

Abstract:

Plant disease phenotyping methodologies can vary considerably among testers and often suffer from shortcomings in their procedures and applications. This has been an important challenge in resistance breeding to brown rot, one of the most severe pre-and postharvest stone fruit diseases caused by *Monilinia* spp. Literature about methodologies for evaluating stone fruit susceptibility to brown rot is abundant but displays significant variations across the described approaches, limiting the ability to compare results from different studies. This is even though authors largely agree on the main factors influencing brown rot development, such as *Monilinia* inocula, environmental conditions, cultivars, fruit stage and management practices. The present review first discusses ways to control or at least account for major factors affecting brown rot phenotyping studies. The second section describes in detail the different steps of fruit infection assays, comparing different protocols available in the literature with the objective of highlighting best practices and further improvement of phenotyping for brown rot susceptibility. Finally, highlighting year-to-year variability and exploring correlations of evaluation outcomes among years and assay types, suggesting that choice of phenotyping methodology must be carefully considered in breeding programs.

Keywords: brown rot; inoculum application; *Monilinia*; phenotyping; phenotypic instability; stone fruit.

Review:

Phenotyping Brown Rot Susceptibility in Stone Fruit: a Literature Review With Emphasis on Peach
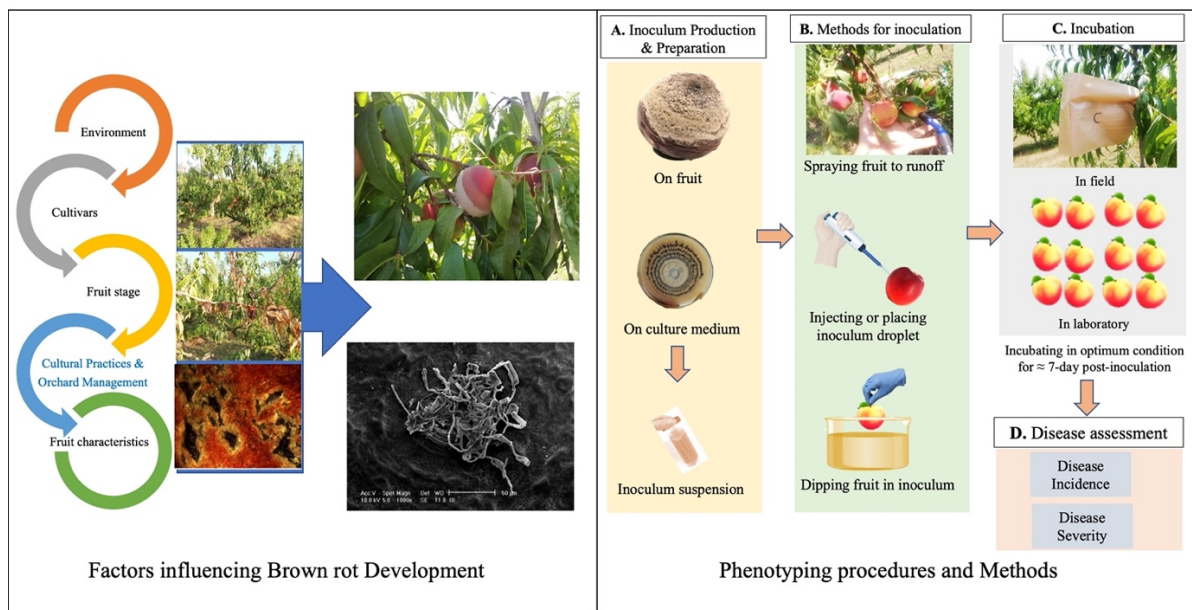
Graphical Abstract

3.1. Introduction

Brown rot (BR) caused by *Monilinia* spp. is one of the most destructive diseases in commercial stone fruit orchards worldwide. *M. fructicola* (G. Winter) Honey, *M. laxa* (Aderh & Ruhland) Honey, and *M. fructigena* (Aderh. & Ruhland) Honey are the main species causing fruit infections (Byrde & Willetts, 1977). These fungi incite losses by infecting blossoms, flowers, and fruit during the preharvest, harvest, and postharvest periods (Larena et al., 2005). Postharvest losses can be particularly severe, especially when conditions are favorable for disease development; in some cases, 80–85% of a crop may be lost (Hong & Michailides, 1998; Larena et al., 2005). When weather conditions are unfavorable, infections may remain latent until conditions become favorable for disease expression, at which point fruit rot ensues (Gell et al., 2008).

Currently, cultural practices and frequent fungicide applications are the main management measures to control BR in the field, although emerging *Monilinia* isolates resistant to fungicides have been reported (Chen et al., 2013; Egüen et al., 2015). Therefore, developing and assessing cultivars with resistance traits against BR has been the primary goal of several breeding programs.

Classic breeding approaches are time-consuming due to lengthy procedures for evaluating resistance on field-grown segregating progenies. Therefore, an important objective is to develop new tools to screen seedlings with enhanced BR resistance. Marker-assisted selection (MAS) is a valuable strategy for this purpose, as it allows the early selection of seedlings bearing favorable alleles at marker loci associated with genomic regions that control the trait of interest. In stone fruit, the mapping of quantitative trait loci (QTL) on populations derived from bi-parental crosses is presently applied (Núria Baró-Montel, Eduardo, et al., 2019; Martínez-García et al., 2013; Pacheco et al., 2014). However, genetic analyses require accurate phenotypic data for the estimation of genotype-associated variation of the trait.

BR resistance is a complex trait requiring robust, easy to apply, inexpensive and effective phenotyping methods. Many stone fruit breeders have developed protocols aiming at BR susceptibility evaluation on fruit (Núria Baró-Montel, Torres, et al., 2019; V. I. Obi et al., 2017; Pacheco et al., 2015; Pascal et al., 1994; Walter et al., 2004). Some are applied in the field, others in controlled conditions (laboratory); some are easy to use, whereas others involve laborious procedures. However, a complete understanding of the process that contributes to effective disease phenotyping is crucial for results to be reliable and repeatable. Protocols are highly dependent on adequately performing different steps. In addition, other factors influence the development of BR and also directly affect the phenotyping process. No comprehensive review is available on phenotyping methodologies for brown rot susceptibility in stone fruit to this extent.

Therefore, this review focuses on essential phenotyping protocols and procedures applied in breeding programs and cultivar evaluations for BR susceptibility in stone fruits. The objectives were to (i) summarize essential factors for BR development and phenotyping, (ii) review the protocols applied in the field and laboratory for artificial BR infection, and (iii) discuss consequences and instability in phenotyping, also in light of recent unpublished experimental results from our group.

3.2. Factors Influencing Brown Rot Development

The critical life stages of *Monilinia* spp. such as primary inoculum availability, host infection and colonization, and secondary inoculum, are the essential prerequisites for the development of BR infection. Multiple factors influence the completion of these life stages, and their knowledge is critical to develop optimized phenotyping protocols.

Principally, the brown rot life cycle includes different stages (Byrde & Willetts, 1977): blossom blight and twig canker at early spring, brown rot at late spring and summer, latent infections, and overwintered inoculum in the form of mummified fruit on trees or orchard ground.

Monilinia spp. overwinters and produces primary inoculum from two sources: mycelia in the fruit mummies, fruit peduncles, cankers on twigs and branches, leaf scars, and buds that sporulate under favorable condition; and stromata that produce ascospores in the spring (Biggs & Northover, 1985; Byrde & Willetts, 1977; Gell et al., 2009; Holtz et al., 1998; Jerome, 1958; Ogawa et al., 1995). However, mummies hanging on trees appeared to be a more viable and effective source of primary inoculum than ground mummies (Casals et al., 2015).

Secondary inoculum can emerge from any infected tissue in which the moisture content is sufficient for sporulation (Byrde & Willetts, 1977); however, non-abscised (aborted) fruit on trees and thinned fruit on the orchard floor appeared to be more critical sources (Landgraf & Zehr, 1982; M. Villarino et al., 2010).

Some authors remark the importance of quiescent infections on developing or ripening fruit which may become active when fruit mature before or after harvest (Northover & Cerkauskas, 1994). Latent infection can be particularly relevant in postharvest (Garcia-Benitez et al., 2020). Molecular techniques have been developed for detecting latent infections in stone fruit (Adaskaveg et al., 2008; Förster & Adaskaveg, 2000). Latent infection is critical for postharvest BR epidemiology, although it is less discussed at the breeding level.

3.2.1. Environment

Environment plays an essential role in disease development (Agrios, 2005). Variables such as temperature, photoperiod (light), humidity, and leaf wetness modulate canopy environment and influence fruit growth and quality (Lopresti et al., 2014), likewise BR development. For *Monilinia* spp., the most critical environmental factors seem to be temperature and humidity. Under the favorable condition, the process of *Monilinia* infection starts with the conidia germination on the fruit surface, followed by elongation of mycelia and formation of appressoria to penetrate the epidermis (M.-H. H. Lee & Bostock, 2006) or to entry through natural openings and wounds (L. De Oliveira Lino et al., 2016). Under adverse

conditions, primary infections can remain latent in blossoms and immature fruits (Cruickshank & Wade, 1992; Northover & Cerkauskas, 1994).

Temperature and humidity are primary factors to be considered in *Monilinia* spp. life cycle. The optimum temperature for mycelial development and sporulation was about 25 °C for all BR fungi (Byrde & Willetts, 1977). However, for most *Monilinia* spp., the optimum temperature for mycelial growth ranges from 15 to 20 °C, and only *M. laxa* requires 25 °C (1984). Regarding *M. fructicola* germination, the best temperature range has been reported at 15-25 °C or 21-27 °C, depending on the study (McCallan, 1930; Weaver, 1950). More recently, analyzing the influence of temperature on fruit infection, Biggs & Northover (1988) suggested that optimum temperature for cherry and peach BR infection by *M. fructicola* ranging 20-22.5 °C and 22.5-25 °C, respectively.

Bernat et al. (2017) modelled and compared the effects of temperature on brown rot, mycelia development and sporulation on peaches and nectarines for *M. fructicola* and *M. laxa*. They showed a better adaptation of *M. fructicola* and *M. laxa* to high and low temperatures, respectively. Notably, the capacity of *M. fructicola* and *M. laxa* to infect fruit seems to be maintained across an extensive temperature range, between 0-30 °C (Bernat et al., 2017). In addition, the two species significantly differ in infection and colonization speed, being *M. fructicola* more aggressive, causing larger fruit lesions and having shorter periods of both incubation and latency (M. Villarino et al., 2016). However, the risk of *Monilinia* infection is significantly reduced at low temperatures (Bernat et al., 2019).

Several reasons can explain discrepancies among studies: the relative humidity and/or temperature-by-humidity interactions; the different optimal temperatures required for fungal functions, such as germination, mycelial growth and sporulation; variations in temperature requirements putatively existing between geographic isolates of *M. fructicola*: e.g. isolates from blossoms, which develop during cool springs, grow at lower temperatures than those

developing on fruit (A. Papavasileiou et al., 2015a). However, temperatures deviating from the optimum mainly cause a delay of germination but poorly affect the final infection success (Wellman & McCallan, 1942).

Wetness or relative humidity (RH) influence the initiation and development of BR in many inter-related ways. In sweet cherry, BR incidence by *M. fructicola* doubled when wetness duration increased from 9 to 12 hours and doubled again with further increase in wetness duration (1988). Similar results were also reported on peach, where a linear increase in disease incidence was observed over the same conditions. Likewise, blossom infections by *M. laxa* were significantly influenced by both temperature and duration of post-inoculation wetness (Tamm et al., 1995). The degree and course of wetness also influenced the success of penetration of nectarine surface and disease incidence (Fourie, P. H., & Holzh, 2003). In the same way, the penetration of peach blossoms by *M. fructicola* was greatly influenced by relative humidity (Weaver, 1950). In a saturated atmosphere, access occurred through any of the floral parts, except sepals, but at a relative humidity of 80% or lower, infection was only observed through stigmas (Gell et al., 2008). A combination of those two factors determines the delay before infection and the rate of success. Under dry conditions at 15 °C, up to 40% of cherry blossoms were infected, while infections at different temperatures (5, 10 and 20 °C) were less frequent. In contrast, under 24h post-inoculation wetness, up to 70-90% of blossoms were infected at each temperature tested (Fourie, P. H., & Holzh, 2003).

Furthermore, the wind is another crucial factor, as it could modify relative humidity and conidia dispersion through air turbulence (Corbin et al., 1968), playing an essential role in the disease spread.

Finally, rain is another significant factor in BR development, assisting in dispersing and spreading inocula and providing ideal relative humidity. Further information on the

epidemiology of *Monilinia* spp. has been reviewed by Holb (2004) and Rungjindamai et al. (2014).

3.2.2. Cultivars

Despite the most relevant for breeding, qualitative sources of BR resistance have not been found in peach and other stone fruit. Some studies have identified accessions with partial resistance (often defined as highly tolerant), in which infection remains latent and/or a limited number of fruits per tree develop symptoms; however, available commercial cultivars are all relatively highly susceptible to BR. Such high susceptibility acts as a further contributor to BR development since infected fruit play as a continuous source of inoculum along the season. In peach, the Brazilian cultivar Bolinha is known to display the highest levels of BR tolerance, in terms of reduced rate of lesion development, sporulation per unit area and, particularly, disease incidence (Feliciano et al., 1987; Gradziel et al., 1998). This cultivar has been used as a BR resistance donor in conventional breeding for developing canning and low-chill peaches despite its poor fruit size and quality, high susceptibility to enzymatic browning and high rate of pre-harvest fruit drop. Besides the increased compactness of epidermal and sub-epidermal cells, the high fuzz and thick cuticle, Bolinha fruit contain a high amount of phenolic compounds compared to other BR-susceptible cultivars (Baccichet et al., 2021; Bostock et al., 1999). The case of 'Bolinha' demonstrates the challenge of breeding for BR, as traits associated with fruit resistance may conflict with commercial requirements; however, among the primary objectives of some breeding programs, resistance against BR takes precedence.

In the peach breeding programme at the University of Milan, Italy (started at the University of Bologna), an F1 population from a cross between 'Contender' × 'Elegant Lady' (Bassi et al., 1998) resulted in a higher BR tolerance level compared to the tolerance donor 'Contender' (Pacheco et al., 2014).

At UC Davis and USDA joint breeding program, improved levels of BR tolerance in some peach cultivars and advanced selections were reported. A progeny was generated by

crossing the moderately resistant cultivar Dr. Davis with an introgression line ('F8,1–42') resistant to BR, originated from an almond × peach interspecific cross (Martínez-García et al., 2013).

Furthermore, at the Clemson University peach breeding programme, some degree of resistance has been reported in materials other than 'Bolinha' and interspecific hybrids (almond × peach). An advanced selection from North Carolina State University peach breeding programme 'NC97-45' ('Contender'; descendant) (Brown et al., 2014) was reported as more tolerant to BR than parents (Fu et al., 2018), which supports the findings of Pacheco et al. (Pacheco et al., 2014) on 'Contender' as a source of tolerance to BR.

In another programme, the progeny from 'Texas' (almond) and 'Earlygold' (peach) backcross (BC1) showed a comprehensive severity and incidence of BR infection in wounded and non-wounded fruit (Núria Baró-Montel, Vall-llaura, et al., 2019). Moreover, Nicotra et al. (2006) have reported 11 advanced apricot selections and cultivars with BR resistant traits. However, studies in many cherry cultivars failed to find promising accession with fruit resistance to BR (Kappel & Sholberg, 2008; Northover & Biggs, 1995; Xu et al., 2007). In contrast to the low level of skin tolerance often found in peach, plum cultivars showed low (Pascal et al., 1994) or no BR infection (Hong et al., 1998) in inoculated intact fruits. Thus, the outcome of inoculation of intact fruit surface (skin) seems unsuitable for artificially classifying plum fruit as BR tolerant since they are still sensitive in a natural condition or when fruit are wounded.

3.2.3. Fruit Stage

Fruit susceptibility to BR varies along with the phenological growth and development stage. Several studies have investigated these variations by evaluating infection probability at different fruit stages (Mari et al., 2003; Martini et al., 2014).

In peach, fruit development is divided into four stages (S1 to S4), all highly susceptible to *Monilinia* spp. except for S2 (pit hardening) (Y. Luo & Michailides, 2001). The early fruit-

stage related susceptibility to BR on stone fruit has been previously reported (Mari et al., 2003; Leandro Oliveira Lino et al., 2020).

The first stage (S1) starts after ovule fertilization or petal fall and ends at the beginning of stone lignification. The fruit is photosynthetically active at this stage, displaying intense transpiration activity and showing the highest nutrient content (T. Thomidis et al., 2007), resulting in increased susceptibility to BR, probably due to the stomata activity, providing an entry point to the pathogen (Curtis, 1928).

The second stage (S2, pit hardening) is the most resistant to *Monilinia* spp. infection (De Cal et al., 2013; Kreidl et al., 2015; Mari et al., 2003); this stage is characterized by the accumulation of secondary metabolites, such as catechin, epicatechin and phenolic compounds, associated with the lignification of the endocarp. In artificially wounded fruit, the temporary absence of susceptibility in S2 seems to be mainly associated with the biosynthesis of specific biochemical compounds rather than a higher mechanical resistance (Mari et al., 2003). Contrary to other studies, even the pit hardening stage has been observed to be susceptible to BR infections, which remain latent until the ripening stages (Kreidl et al., 2015; Y. Luo & Michailides, 2001).

During the third stage (S3), characterized by a high rate of cell expansion and ending at fruit physiological maturity, fruit become increasingly susceptible to pathogens, including BR. At fruit maturity (S4), BR susceptibility reaches its peak starting approximately two weeks before full ripening (Biggs & Northover, 1988; Emery et al., 2000). Similar patterns were previously reported for apricot and peach (Guidarelli et al., 2014; Mari et al., 2003). The progressive decrease of resistance-compounds concentration due to fruit growth and/or structural changes affecting surface integrity would seem the most plausible hypotheses for the increased susceptibility observed at these stages (Bostock et al., 1999).

Also, in cherry, the susceptibility to *M. fructicola* fluctuates with the stage of fruit development (Northover & Biggs, 1990): young developing cherries become increasingly susceptible to infection, then they turn to be less susceptible at pit hardening and finally again become gradually more susceptible until harvest (Xu et al., 2007). Moreover, the susceptibility to *M. laxa* under field condition significantly increase with fruit maturity (Xu et al., 2007).

3.2.4. Cultural Practices and Orchard Management

Commonly applied practices in a stone fruit orchard, including crop load management, irrigation, fertilization, pruning and canopy architecture, have a major impact on *Monilinia* spp. development (Li et al., 1989). Besides fungicide application, pruning blighted twigs and removal of mummified fruit are considered the most effective control measures against BR. Cultural practices can impact the inoculum source directly via microclimate modulation such as irrigation, pruning, fertilization and indirectly via fruit thinning (Y. Luo et al., 2001).

Mercier et al. (2008) studied the combined effects of irrigation regime and pruning system. The lowest BR incidence occurred under a combination of water deprivation (about 30% of the fully irrigated treatment) and 'long' pruning (i.e., dormant plus summer interventions for the removal of epicormic shoots and young, vigorous sprouts, without trimming) in comparison with full irrigation and 'short' pruning (i.e., dormant plus summer interventions of shoot trimming). Similarly, training system and pruning (shapes with a dominant central leader) seemed to reduce brown rot incidence compared to conventional system, e.g. 'vase' systems (Bussi et al., 2015). This effect could be due to improved light penetration and reduced relative humidity in the less dense canopies that negatively affected fungal germination and sporulation.

Gilbert et al. (2007) have primarily studied the complex interplay between cultural practices, fruit growth, and BR infection risks. They showed that irrigation and fruit thinning affect fruit growth and the appearance of microcracks on the fruit surface. Frequent and high levels of irrigation on 'Zéphir' nectarine strongly increased the density of cuticular cracks

compared to water-restricted trees receiving two- to three- times less water per day. Furthermore, low crop loads dramatically increased both fruit size and the incidence of cuticular microcracks, leading to increasing BR susceptibility.

Nevertheless, management of crop load concerning fruit BR susceptibility seems difficult to be optimized. Bellingeri et al. (2018) reported opposite effects on trees subjected to different thinning treatments, with the highest BR infection observed in moderately thinned compared to intense or unthinned trees. This could be explained by a complex interaction between the probability of infection by contact (which tend to decrease along with fruit density) and cuticle cracking (which tend to increase in faster-growing fruit) (see section 3.2).

Fertilization also seems to play a role in BR susceptibility. For example, peach trees subjected to a high level of nutrients exhibited a significant increase in *M. fructicola* incidence. Other studies investigated fertilization with calcium (Elmer et al., 2007), zinc (T Thomidis et al., 2006) and boron (Thomas Thomidis et al., 2017), reporting an enhancement of fruit quality and lowering of BR susceptibility. The effect of fertilization could result in a modification of tree growth, affecting canopy microclimate or increased fruit nitrogen content (Melo et al., 2016). However, no clear correlation between seasonal changes of peach nutrient content and susceptibility to *M. laxa* was found (2007).

Monilinia spp. can mainly enter the fruit via two ways, either by actively wounding the fruit surface or through natural openings such as stomata or microcracks (Figure 3.1) (Bostock et al., 1999; L. De Oliveira Lino et al., 2016; C. Garcia-Benitez et al., 2019; C. Gibert et al., 2009; M. H. Lee & Bostock, 2007). However, *Monilinia* is also able to penetrate fruit skin directly without the need for wounds or natural openings, employing degrading enzymes and colonizing plant tissue similarly to other necrotrophic fungi (Abate et al., 2018; N. Baró-Montel, Vall-llaura, et al., 2019; C. Garcia-Benitez et al., 2017, 2019; Carlos Garcia-Benitez et al., 2016).

Besides chemical factors such as nutrients and volatiles, fruit surface characteristics such as hydrophobicity and topography are common appressorial inducers for many fungi. In nectarine fruit, the formation of *M. fructicola* appressorium at the S2 stage and their absence at the S3 stage seem to be associated with the respective high and low peel hydrophobicity (M.-H. H. Lee & Bostock, 2006).

Although *Monilinia* is a necrotrophic fungus that can infect fruit via direct penetration, fruit cracks are well-known to be the preferential entry ports (Caroline Gibert et al., 2005; Yamamoto et al., 1990). Different fruit characteristics can be accounted for reducing susceptibility to BR, which most of these defense barriers, either mechanical or biochemical, are related to the epidermis (Bostock et al., 1999; Gradziel et al., 1998; Pascal et al., 1994). Considering the active penetration of the fungi, the composition of the different epidermis layers and the mechanical traits linked to surface integrity seems to be the main characteristics to be explored in addition to active biochemical defense mechanisms.

The plant cuticle is the first protective barrier to biotic stresses, as it contains antimicrobial compounds involved in plant-pathogen interactions. However, until recently, few studies have explored the cuticle of *Prunus* fruit. Oliveira Lino et al. (2020) studied the cuticular wax composition of three nectarine cultivars and its change during fruit development in correspondence to conductance and susceptibility to *M. laxa*. Cuticular waxes greatly varied both quantitatively and qualitatively throughout fruit growth. The high conductance in the early stages was attributed to the high density of functional stomata in young fruit and the absence of the wax layer not yet formed. Besides, this absence might have also facilitated direct infection by *M. laxa* at the early stages. The variation of cuticular waxes deposition may also explain their contribution to BR resistance at pit hardening and, conversely, the susceptibility of mature fruit (showing a higher level of alkane waxes, which could favor the fungus growth).

Skin cracks are an essential factor affecting the integrity of fruit surface integrity. The link between cracking and BR incidence suggests that fruit resistance factors provided by the epidermis are, of course, no longer influential when the cuticle loses its integrity (Gilbert et al., 2007). Cuticular cracks are assumed to occur when the elastic limit of the cuticle is exceeded as a consequence of high internal pressure, especially during rapid fruit expansion (Christensen, 1973; Ohta et al., 1997). Certain cultural practices mainly promote a fast-growing phase (see 3.2**)**. Microscopic observations of fruit surface in three nectarines ('Zéphir', 'Magic' and 'C222') confirmed the formation of a dense network of microcracks in mature fruits and preferential spore germination inside the cracks (Figure 3.1). These observations suggest that BR resistance factors targeted in breeding programs should explore a combination of these two traits: low susceptibility to cracking and enhanced content of antifungal compounds.
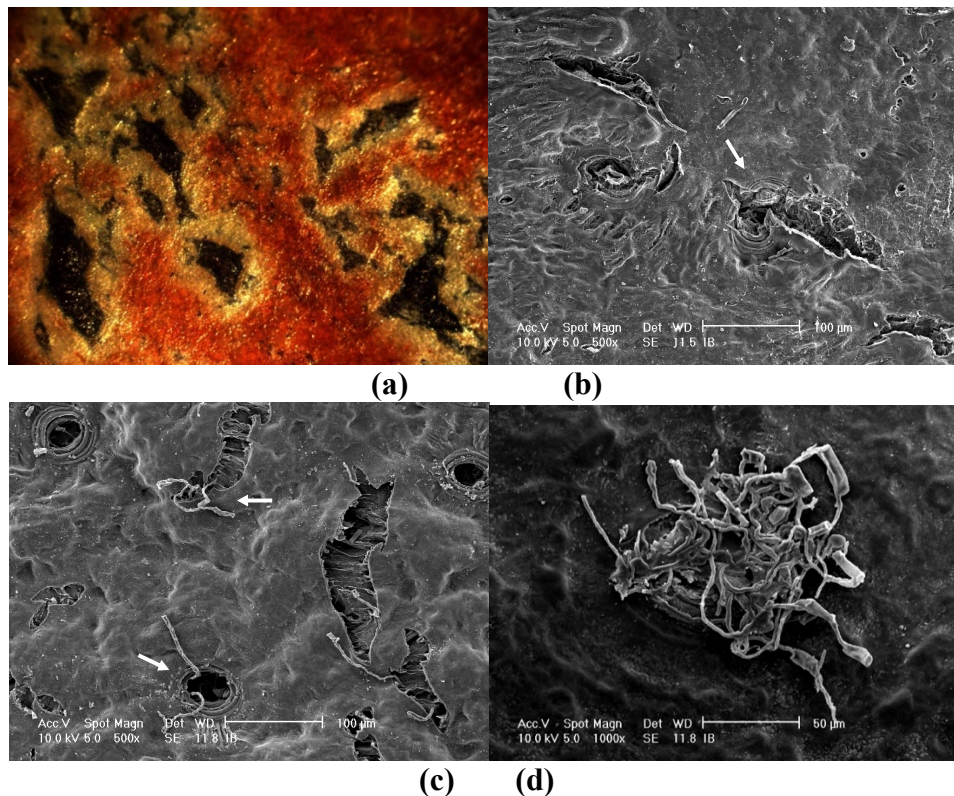
Figure 3.1. **(a)** Surface of the nectarine fruit 'Zéphir' at maturity with a dense network of microcracks under a stereomicroscope. Cracks were stained dark blue by applying toluidine blue at 0.1%. **(b)** Scanning Electron Microscopy image showing microcracks originating from a lenticel, presumably derived from a stoma (arrow) on the fruit peel of nectarine 'C222' selection. **(c)** Scanning Electron Microscopy image showing spores of *M. laxa* germinating (arrows) in the microcracks of mature nectarine fruit of cultivar Magic. **(d)** Scanning Electron Microscopy showing the development of *M. laxa* mycelia in a lenticel on the fruit peel of 'C222' selection.

3.3. Protocols for BR Susceptibility Evaluation

Some stone fruit breeders and scientists have developed protocols for BR susceptibility evaluation to be applied either in the field or controlled environments; some are easy to use, whereas others involve laborious procedures. The goal commonly sought is a robust, fast and low-cost protocol enabling the screening of a large number of progenies. This section reviews BR resistance phenotyping protocols used to evaluate artificial infection in stone fruit, focusing on cultivar evaluation and breeding programs.

Among the several prerequisites, assessed fruit should not receive fungicide treatments after flowering (Antonios Papavasileiou et al., 2020; Walter et al., 2004) since fungicide residues could bias phenotyping results. Selected fruit should also be unblemished, uniform in size and maturity (Feliciano et al., 1987), since variations in the degree of ripeness and/or the

presence of wounds or cracks could mislead conclusions about fruit susceptibility. Criteria and methods for establishing the degree of fruit maturity often vary across studies, ranging from visual assessment to the measurement of firmness, color and/or soluble solids content (SSC) (Núria Baró-Montel, Torres, et al., 2019; V. I. Obi et al., 2017). The use of the index of absorbance difference (I_{AD}) measured by a portable DA-Meter (TR Turoni, Forli, Italy) seems a reasonable and objective approach to standardize peach maturity evaluation (Núria Baró-Montel, Torres, et al., 2019; Pacheco et al., 2015; Zhang et al., 2017; Ziosi et al., 2008). In addition, a stereomicroscope was used to examine fruit surface with the aim of discarding injured fruit before inoculation (1998). However, this procedure is difficult to implement as a routine check.

3.3.1. Fruit Preparations Before Inoculation

In laboratory assessments, fruit are carefully handpicked and usually subjected to preparations before inoculation (Gradziel et al., 2003). Primarily, damaged and field infected fruit are excluded (Gradziel et al., 2003) without considering possible latent infections coming from the field that has not yet been activated. Dissipating field heat or precooling of fruit is the first care to slow down biological activities. To this end, different fruit temperatures and durations treatments, for example, storage at 0, 0.5 and 4 °C for few days up to few weeks, have been tested (Núria Baró-Montel, Torres, et al., 2019; Hong et al., 1998; Martínez-García et al., 2013) until the day of assessment. However, prolonged storage is not recommended since low temperatures may interfere with critical physiological properties and modify fruit susceptibility. Storing fruit for short periods gives more flexibility to organize inoculation. For example, Gradziel et al. (1998) kept fruit at 22 °C for 12h to homogenize the batches harvested on different days and simulated the practice of fruit storage in postharvest and commercialization period.

Postharvest disinfection of fresh fruit is considered an essential step before handling (Feliziani et al., 2016). Similarly, this practice has been employed in screening stone fruit for

BR susceptibility before inoculation with *Monilinia* spp. to eliminate field contaminations or competing organisms that may interfere during artificial infection. Fruits were surface-sterilized by bleach at 10% or 8%, with different concentrations of sodium hypochlorite (NaOCl) (Bernat et al., 2017; Kappel & Sholberg, 2008; Martínez-García et al., 2013; Nicotra et al., 2006; Antonios Papavasileiou et al., 2020), calcium hypochlorite (Fourie & Holz, 1985), or less concentrated chlorines solutions ranging from 0.5 to 2% (Gell et al., 2008; Kreidl et al., 2015; Nicotra et al., 2006; V. I. Obi et al., 2017; Tian & Bertolini, 1999; M. Villarino et al., 2016; Xu et al., 2007). Also, ethyl alcohol has been used as a surface sterilizer, mainly at 70% concentration, before or after disinfecting with chlorine compounds (Gell et al., 2008; Kappel & Sholberg, 2008; M. Villarino et al., 2016). However, no consensus method for disinfecting fruit before inoculation emerged from these protocols, as different concentrations and combinations of hypochlorite, ethyl alcohol and timing have been used. However, in all methods, the process ends up by carefully rinsing fruit in water to remove the disinfectants, followed by air drying. Overall, the treatments above might be considered as somehow disruptive of the fruit surface and, putatively, a modification of its susceptibility to infection. This was the reason behind the use of only water for fruit cleaning (Bassi et al., 1998; Northover & Biggs, 1995).

Baró-Montel et al. (2019) have thoroughly investigated the effect of different type and concentration of disinfectants on wounded and non-wounded fruit before inoculation. They reported a lower disease severity in disinfected wounded fruit. However, in non-wounded fruit, a significant increase in disease severity was reported when the most aggressive (10% NaClO) disinfectant treatment was applied. Finally, they also observed a rise in BR incidence after dipping the fruit in tap water without a disinfectant, suggesting that water could promote pathogen growth and facilitate the infection process.

The use of a water bath (recommended as a technique to reduce postharvest infections) deserves further attention. Spadoni et al. (2015) have shown a stimulating effect on the germ tube of *M. fructicola* conidia on the fruit surface immediately after heat treatment at 60 °C for 60 sec. Volatile organic compounds emitted from heat-treated peaches have been putatively implicated in the stimulation of conidia germination and the increased BR incidence when inoculation occurred immediately after bathing.

Even though these surface compounds were not affected, the water bath might influence other compounds such as proteins and water-soluble metabolites involved in the fruit-fungus interaction trade-off pathway. A similar increase of BR incidence has been reported for peach and nectarine (Bernat et al., 2019) and nectarine 'Red Jim' fruit (Garcia-benitez et al., 2020) when subjected to water dumping followed by incubating at 20 °C 65-100%RH.

Even though fruit disinfection is an important operation in postharvest to avoid secondary infections, our results recommend utmost precautions before subjecting fruit to the water bath since this procedure seemed to increase the susceptibility of nectarine fruit to BR and may activate latent infections in postharvest handling.

3.3.2. Strain Conservation and Inoculum Production

Monilinia spp. culture could be maintained for long-term storage on different media such as potato dextrose agar (PDA) at 5 °C (De Cal et al., 1990) or 4 °C (V. I. Obi et al., 2017), and 2% Malt extract agar at 2 °C in darkness (Tamm & Fluckiger, 1993). There are other methods for storing fungi; for example, in our lab, we maintained *Monilinia* spp. spores in an aliquot of 20% glycerol with potassium dihydrogen phosphate buffer at -20 °C or -80 °C.

Before running any experiments, good quality inoculum should be prepared. Therefore, *Monilinia* spp. cultures are activated on nutrient media at optimum temperatures (25 °C). Inoculum preparation from a single-spore isolate allows using the same isolate throughout the experiment. However, some authors used the isolated *Monilinia* spp. directly from seasonal

infected stone fruit: in this case, series of subcultures are needed to purify the inoculum from contaminants.

Moreover, the assessment of pathogenicity and virulence among *Monilinia* species revealed a significant variability even between isolates of each species (Janisiewicz et al., 2013; Kreidl et al., 2015). Thus, it is recommended to check the stability of pathogenicity before running experiments. According to Koch's postulates, such a practice can be performed by infecting intact fruit (e.g., peach) (V. I. Obi et al., 2017). In our lab, working on *M. laxa* and *M. fructicola*, we observed reduced growth competence of *Monilinia* spp. on V8 juice agar (V8A) after several subcultures. Therefore, we periodically regenerated new cultures from aliquots stored at -20 °C or isolating from actively infected fresh fruit (Figure 3.2). This process was repeated every three months to maintain maximum growth speed.

Screening large progenies for BR susceptibility requires a tremendous amount of inoculum to be prepared weekly to achieve an identical concentration of viable conidia throughout the experiment. The media composition may impact the rapidity of growth and sporulation, the number of spores produced, and viability.
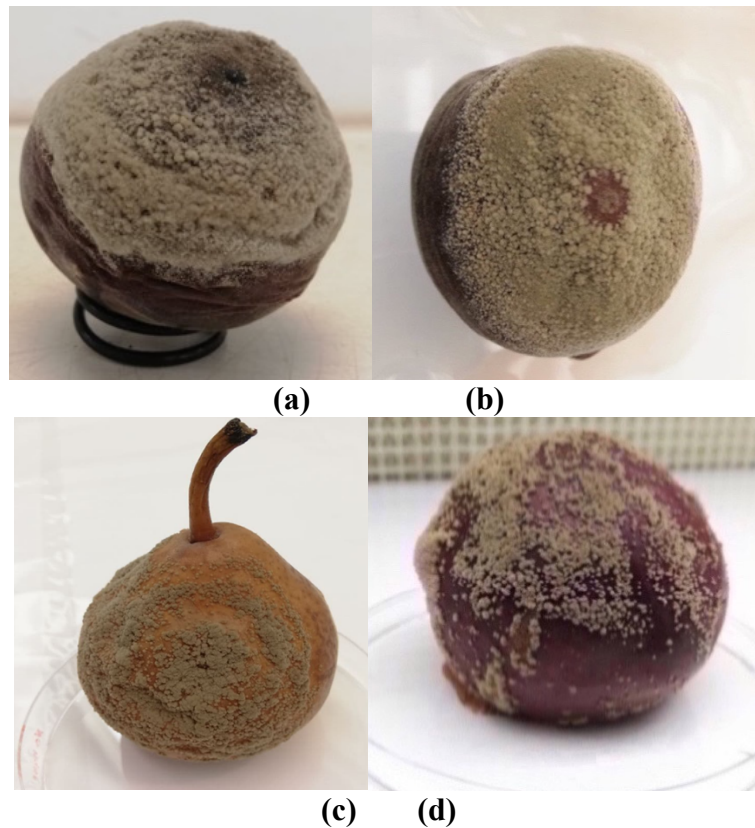
Figure 3.2. *M. fructicola* inoculum production and activation on peach **(a)** and *M. laxa* inoculum production and activation on peach, pear and plum **(b, c** and **d**, respectively**)** at 7-day post-inoculation. The inoculated fruit, with 10 µl at 10^5 conidia ml^{-1} suspension concentration for each species, were incubated in a culture chamber at 24/18 °C and 16/8 h light/dark photoperiods, in clear plastic boxes with maximized relative humidity.

A PDA and V8A are the most common media used for inoculum production for *Monilinia*; other less frequent media include peach or tomato juice agar and glucose-asparagine-yeast extracts (Table 3.1). Producing the inoculum directly on fruit is a valid and viable option (Figure 3.2), with the precaution of previous disinfection with alcohol. The use of canned fruit is also reported (Walter et al., 2004). Phillips (1984) reported that spores produced on PDA were less aggressive and smaller in size than those cultivated on peach and nectarine fruit. Hence, a culture media as V8A may be preferred for high quality and amount of sporulation.

For inoculum production, culture plates (i.e., V8A or PDA) are incubated between 20-25 °C under different photoperiods. Light is regarded as an essential promoter for conidia

production. Authors have tried to produce inoculum under different photoperiods: 12 h light/dark or 16 h light/8 h dark; also, continuous light or dark were tested (Table 3.1), even though *M. fructicola* appeared to require shorter photoperiods than *M. laxa* to effectively sporulate (Bernat et al., 2017), a 16 h light/8 h dark photoperiod is based on our experience advisable to promote sporulation for both.

The time required to promote sporulation of *Monilinia* Petri dish cultures is another phase that differently approached in literature. Depending on the type of medium and incubation condition, authors have used 5 to 14 days old cultures for inocula (Bernat et al., 2017; Mari et al., 2003; V. I. Obi et al., 2017; Tamm & Fluckiger, 1993). Though this period is critical, it should not exceed 14 days, especially for sporulation quality.

Table 3.1. Phenotyping protocols for evaluating brown rot disease susceptibility in stone fruit.

Fruit species	*Monilinia* spp.	Maturity determination	Wounded or Unwounded (intact)	Production of Inoculum	Mode of inoculation	Inoculum concentration (conidia/ml)	Incubation condition	Assessment time	Disease assessment	Reference
Peach	*M. fructicola*	Fruit color determinations by spectrophotometer	Unwounded, wounded	V8A	Drop 10 µl	2.5×10^4	Humidified plastic containers at room temperature	3 days	Disease incidence, disease severity (lesion diameter)	(Martínez-García et al., 2013)
Peach	*M. fructicola*	Mature (firm ripe) and mature green	Unwounded, wounded	PDA	Drop 10 µl and a 5-mm mycelial disk	2×10^5	23 -25 °C/ 90% RH in dark	(24, 48 and 73, 96 hr.), rote diameter (48, 72, and 96 h) and sporulation 7 days	Disease incidence, disease severity (rot diameter), sporulation amount	(Feliciano et al., 1987)
Peach	*M. fructicola*	Commercial maturity	Unwounded	PDA	Drop 10 µl	2×10^4	22–25°C / 95% RH, in dark	3 days	Disease incidence	(Gradziel et al., 1998)
Peach, Nectarine, Plum	*M. fructicola*	Commercial maturity	Unwounded, wounded	PDA + acidified lactic acid	Drop 20 µl	$1 \times 10^6, 10^5, 10^4, 10^3, 10^2$	20 °C/ 95% RH in plastic cardboard boxes	5 to 7 days	Disease incident and severity (lesion diameter)	(Hong et al., 1998)
Peach, Nectarine	*M. fructicola*	Maturity classes based on (I_{AD})	Unwounded, wounded	PDA supplemented with tomato pulp	Drop	2.5×10^4	20 °C and 85% RH storage boxes	3 and 5 days	Brown rot incidence (%), lesion diameter	(Núria Baró-Montel, Torres, et al., 2019)
Peach, Nectarine	*M. fructicola, M. laxa*	Commercial maturity	Wounded	PDA	Drop 15 µl	1×10^4	0, 4, 10, 15, 20, 25, 30, 33 °C with ±1 °C / 85% RH, dark or 12-h light photoperiod	12 hours for *M. fructicola* and 5-7 days for *M. laxa*	Lesion diameter, presence, or absence of sporodochia.	(Bernat et al., 2017).
Peach, Apricot, Sweet cherry, Plum	*M. fructicola, M. laxa*	Commercial maturity	Wounded	V8A	Drop 30 µl	1×10^5	22 °C/ high RH, in containers	6 days	Disease severity (rot diameter)	(A. Papavasileiou et al., 2015b)
Peach, Nectarine	*M. fructicola, M. laxa, M. fructigena*	NA	Wounded	PDA	Drop 25 µl	1×10^4	22 ± 2 °C/ light and in humidity chambers lined with a moist paper	7 days	% Brown rot incidence, lesion diameter, sporulation, spore germination, mycelium length	(M. Villarino et al., 2016)
Peach, Nectarine, Apricot, Plum	*M. fructicola, M. laxa*	Commercial maturity, immature fruit	Unwounded, wounded	V8A, PDA	filter paper discs soaked in suspension, drop 10 µl	1×10^4	22–25 °C/ (90–100 %) in plastic boxes lined with a damp paper towel and the lids closed	7 days	Pathogenicity and disease incidence	(Kreidl et al., 2015)
Peach, Sweet cherry	*M. Fructicola*	Different maturity date	Unwounded	PDA	Drop 30 µl	$1 \times 10^5, 10^6$	15 to 30 °C with 2.5 °C intervals, then at 20 °C / >95% RH, in plastic boxes	6 days	Disease severity (scaling 0 to 3) and percentage of fruit infection	(Biggs & Northover, 1988)

Fruit species	*Monilinia* spp.	Maturity determination	Wounded or Unwounded (intact)	Production of Inoculum	Mode of inoculation	Inoculum concentration (conidia/ml)	Incubation condition	Assessment time	Disease assessment	Reference
Peach	*M. laxa*	Maturity at 0,6 I_{AD}	Unwounded	NA	Spray	1×10^5	Fruit left on the tree bagged in plastic or paper bags	7 days	Disease incidence% in the field	(Pacheco et al., 2015)
Peach	*M. laxa*	NA	Unwounded	NA	Spray	1×10^5	at 25±2°C/ 95–100% RH	7 days	Brown rot infection number, per cent of rotted skin (lesion)	(Bassi et al., 1998)
Peach, Nectarine	*M. laxa*	NA	Unwounded	NA	Sprayed to runoff	$1 \times 10^4, 10^6$	23°C/ in trays lined with moist paper and plastic film. 16-h photoperiod.	7 days	Incidence (%) of fruit rot	(Gell et al., 2008)
Peach, Nectarine	*M. laxa*	Optimum maturity	Unwounded, wounded	Peach fruit	Drop	25×10^3	23 °C / 40–60% RH, in darkness	5 days	Measuring brown rot incidence (%), lesion diameter (mm) and colonization extent (mm)	(V. I. Obi et al., 2017)
Peach Apricot, plum	*M. laxa*	Commercial maturity	Unwounded, wounded	Fruit	Drop 20 µl	1×10^6	23 °C / high RH	10 days unwounded; 5 days wounded	Disease incidence, disease severity (lesion diameter)	(Pascal et al., 1994)
Peach, Apricot	*M. laxa*	Commercial maturity	Unwounded, wounded	V8A	Dipping fruit for (1 min) inoculum	1×10^5	20 C and 95% RH	7 days	Brown rot incidence %	(Mari et al., 2003)
Peach, Plum	*M. laxa*	Mature fruit from the market	Wounded	PDA, canned peaches	Dipping for 30 sec in inoculum suspension or a drop	$1 \times 10, 10^2, 10^3, 10^4, 10^5$ spore/cm³	21 °C, wrapped in plastic bags	5 days, or 4 to 6 days	Disease incidence %	(Fourie & Holz, 1985)
Apricot	*M. fructicola M. laxa*	Mature apricots	Unwounded, wounded	Tinned apricot halves	Drop 30 µl	1.5×10^4	15–22°C	48, 66, 72, 96 and 120 h	Lesion area, spore counts, storage rot, cuticle thickness	(Walter et al., 2004)
Apricot	*M. laxa*	Mature visually	Unwounded	PDA	Drop (drip)	1×10^5	22 °C covered with polythene bags	7 days	Percentage infection and scaling to resistant: 0-10%; moderately susceptible: 11%- 30%; susceptible: 31%-50%; highly susceptible: > 50%.	(Nicotra et al., 2006)
Sweet and sour cherry	*M. fructicola*	NA	Unwounded	PDA	Drop 30 µl	$1 \times 10^6, 10^5, 10^4, 10^3$	20 °C/ 95%RH	6 days	Percentage fruit infection, lesion development	(Northover & Biggs, 1990)
Sweet cherry	*M. fructicola*	Commercial maturity	Unwounded	NA	Spraying	1×10^4	13 °C 95-97% RH in the growth chamber	8, 11 days	Disease incidence	(Kappel & Sholberg, 2008)
Sweet cherry	*M. laxa, M. fructigena*	5 to 6 weeks after blooming	Unwounded, wounded	PDA, Apple fruit	Spray	1×10^5	20 °C under light	7 days	Incidence of infection in field and polyethene tunnel	(Xu et al., 2007)

Fruit species	*Monilinia* spp.	Maturity determination	Wounded or Unwounded (intact)	Production of Inoculum	Mode of inoculation	Inoculum concentration (conidia/ml)	Incubation condition	Assessment time	Disease assessment	Reference
Prune	*M. fructicola*	Different stages	Wounded	Acidified PDA	Injecting $\approx$0.1 ml inoculum	5×10^3	Left on the tree	27 days or more	Disease incidence (%), and natural infection in the field	(Yong Luo et al., 2005)

Abbreviations: V8A: V8 juice agar, PDA: potato dextrose agar, °C: degree Celsius, RH: relative humidity, NA: not available, IAD: index of absorbance difference, %: percentage, mm: millimeter, h: hour, $\approx$: approximately.

3.3.3. Inoculum Preparation

Inoculum suspension is prepared by flooding the culture plates or washing-off fruit with distilled water and wetting agent such as Tween 20 or 80 at 0.01% (Núria Baró-Montel, Torres, et al., 2019) or 0.05% (Mari et al., 2003) to scrape the conidia. Vigorous shaking or centrifugation of the suspension is needed to break conidia chains, followed by filtering through different means to reduce the mycelium parts in the suspension as much as possible. Strainers with pore size ranging from 25 to 40 µm, or layers of cheesecloth or lens tissue, among others, could be used. Finally, conidia concentration in the suspension is evaluated by counting aliquots by a hemocytometer or other counting chambers; the suspension is then adjusted to the desired concentration.

In literature, inoculum concentration ranged from 10^2 to 10^6 conidia ml^{-1} depending on fruit ripening stage or integrity (intact or wounded). In the case of ripe fruit, concentrations from 10^3 to 10^5 conidia ml^{-1} should not be exceeded to highlight resistance, as applying a high inoculum pressure would lead to generalized infections.

Immature fruit require higher concentrations (around 10^6 conidia ml^{-1}) to obtain significant infections; the suggested level is probably the maximum that could occur in field conditions when fruit are ripe (Northover & Biggs, 1990). Hong et al. (1998) demonstrated enlargement of lesion diameter with increased inoculum concentration in wounded peaches; simultaneously, a concentration of 10^5 conidia ml^{-1} was required for unwounded fruit to get lesions around 10 mm diameter at 3-day post-inoculation. At lower concentrations (namely 10^2, 10^3 and 10^4 conidia ml^{-1}), fruit infections were delayed with significantly smaller lesion diameters. Overall, a concentration of 10^5 conidia ml^{-1} appears to be an effective inoculum concentration, particularly for inoculating intact fruit.

3.3.4. Field and Laboratory Protocols

In general, protocols can be divided into two categories: protocols applied in the field (or *in-situ*) and laboratory.

Field protocols are intended to quickly screen a high number of trees through the artificial inoculation of tree-attached fruit. Very few protocols are available for field evaluation. Luo et al. (2005) inoculated tree-attached plum fruit by injecting 100 µl of *M. fructicola* conidia suspension at different growth stages and subsequently monitoring BR development. In a semi-field condition, Xu et al. (2007) developed a protocol to evaluate the effect of fruit age and wetness duration on the BR infection of tree-attached cherry fruit under polythene tunnel. A polythene bag was used to maintain adequate humidity; the inner side of the bag and the branch (including leaves and fruit) were wetted before inoculation by spraying distilled water. Then about 8 ml inoculum was sprayed onto the fruit on each branch until runoff, and then the polythene bag was placed over the branch and sealed with tape for different wetting periods before removing the bags.

More recently, in a field condition, Pacheco et al. (2015) developed a protocol to screen large peach progenies in-situ to set up a more time- and cost-effective method to screen BR susceptibility in breeding programs.

Laboratory protocols provide a more accurate evaluation of the resistance displayed, although time-consuming as several steps are involved: fruit harvest, followed by preparation (as described in section 33.3.1); arranging fruit in trays; inoculation, either on intact skin or after wounding in different ways. Inoculations by droplet or spray usually are practiced at different inoculum concentrations and incubation periods (see section 3.3). Finally, observing fruit infection can be performed daily, and several indicators can be recorded (see section 33.3.8). Both field and laboratory protocols have advantages and disadvantages (Table 3.2) and are contingent on the final objective and the quantity of material to be screened.

3.3.5. Wounded or Unwounded Fruit
Overall, injuring the fruit in the process of inoculation is a method to investigate the tolerance of the flesh while infecting non-wounded fruit inspects the skin resistance. Since fruit skin is the first barrier to fungal invasion (Núria Baró-Montel, Torres, et al., 2019), the

tolerance of the flesh is expected to be low; therefore, most of the studies focus on non-wounded fruit.

Several authors comparatively studied wounded and unwounded artificial inoculations (Table 3.1). Generally, stone fruit are successfully infected by both wounded and non-wounded methods, except for plums that appeared to infected only by wounding (Fourie & Holz, 1985; Hong et al., 1998; Pascal et al., 1994).

Most of the studies show no correlation between skin and flesh resistance. As expected, unwounded fruit display less susceptibility, suggesting that most of the resistance lies in the skin (Núria Baró-Montel, Torres, et al., 2019; Bostock et al., 1999; Feliciano et al., 1987; Gradziel & Wang, 1993; M. H. Lee & Bostock, 2007; Martínez-García et al., 2013; Pascal et al., 1994). Conversely, Mari et al. (2003) observed a correlation between susceptibility of wounded and unwounded fruit in peach and apricot: they explained the results in light of a typical biochemical response of both skin and flesh. Finally, as evident in almost all literature, wounding deprives the fruit of its main barrier against pathogens (Hong et al., 1998; Xu et al., 2007), resulting in higher infection and severity levels compared to intact fruit.

Table 3.2. Advantages and disadvantages of field and laboratory-based protocols to evaluate fruit resistance level.

Evaluating Environments	Advantages	Disadvantages	References
Field	Relatively faster in manipulation. Plenty of accessions can be evaluated in a short time.	High variability, which may lead to low repeatability of the result. Environmental factors may impair the level of the recorded susceptibility.	(Vitus Ikechukwu Obi et al., 2018; Pacheco et al., 2015)
Laboratory or controlled condition	Enables fruit preparation before inoculation, such as disinfection, wounding. Facilitates the post-inoculation evaluation of traits such as fruit weight, acidity, Brix. Provides repeatable environmental conditions. Fruit manipulations relatively easier. Inoculum load could be precisely placed on fruit sides (cheeks). Allows recording of many parameters.	Not exactly representing the natural (field) condition. It is more laborious.	[7,55,106]

3.3.6. Artificial inoculum application

Several methods have been used in artificial fruit inoculation, e.g., spraying, dropping, injecting, dipping. However, a comprehensive comparison among different methods is still lacking. Techniques are chosen based on their applicability and reliability in coherence with the whole protocol. For instance, spraying until inoculum runoff is mainly used in the field investigation since other methods are difficult to apply on a tree-hanging fruit. Above all, this approach probably imitates the best way in which inoculum naturally arrives at multi-points on fruit in the field, via splashing (Pacheco et al., 2015; Maria Villarino et al., 2011; Xu et al., 2007).

In the laboratory, droplet fruit inoculation, dipping fruit in suspension and fruit spraying are the main methods used for non-wounding fruit inoculation. On the other hand, some other methods are mainly used for applying inoculum to the wounded fruit, such as placing or directly injecting an inoculum droplet and attaching active mycelia plug to the wound (Table 3.1). The wounds can be made by inserting a disinfected needle or a sharp blade into the fruit peel. However, for both wounded and non-wounded, the position and amount of the inoculum

are important and should be well maintained. The fruit cheeks are frequently chosen to deposit the drop inoculum, regardless of being wounded or non-wounded. At maturity, cheeks are considered the least susceptible fruit part to microcracking compared to suture, pedicel cavity and stylar region, as reported for nectarine (Gilbert et al., 2007) and cherry fruit (Peschel & Knoche, 2005; Schumann et al., 2019). Some authors have explicitly considered the position of depositing the inoculum droplet on the sun-exposed cheek (Núria Baró-Montel, Torres, et al., 2019; Pacheco et al., 2014). The suspension amount per droplet may range from 10 to 30 µl regardless of inoculum concentration. Inoculation by paper discs soaked in a suspension of conidia and then laid on the fruit is a less common method (Kreidl et al., 2015). Furthermore, non-ionic polysorbate such as Tween 20 or 80 at low concentrations from 0.005% to 0.05% is often added to the suspension (Gradziel et al., 2003; V. I. Obi et al., 2017; Pacheco et al., 2015; Walter et al., 2004), as surfactant (wetting agent) in conidial suspension. Its effects and functions have been widely studied on the inoculum preparation and viability of fungal biocontrol agents (Mishra et al., 2013; Mwamburi et al., 2015; Oliveira et al., 2015). However, the influence of those surfactants on *Monilinia* has not been particularly addressed.

3.3.7. Incubation

Incubation is the time that *Monilinia* spp. requires to colonize inoculated fruit and display visible symptoms. However, this period may vary depending on the method of inoculation. For example, the time required to show the infection is shorter on wounded than non-wounded fruit; for peach, only two days are needed, while for plum, it takes four days (Hong et al., 1998). Baró-Montel et al. (2019) have measured lesion diameter at 3-7 days post-inoculation and observed a significant increase in lesion diameter in wounded fruit at 4 to 6 days post-inoculation. In contrast, for non-wounded fruit, the measurements were delayed up to seven days. Overall, most authors have considered seven days as an appropriate incubation period (Table 3.1).

Similarly, inoculated fruit can be incubated under the same conditions described in section 33.3.2. Regardless of the stone fruit species, a diverse range of temperatures and humidity was used. However, predominantly inoculated fruit are incubated at ranges of 20-25 °C and 85-100% RH in a growth chamber or arranged in plastic boxes to secure the high relative humidity. On few occasions, fruit were incubated at lower humidity of 40-60% RH, which might not be optimal (Table 3.1). Furthermore, inoculated fruit are incubated at different photoperiods, such as continuous light or dark, and 12/12 h or 16/8 h light/dark photoperiods (Table 3.1). Since *Monilinia* spp. can successfully infect stone fruit at different light conditions, setting a photoperiod seems more reasonable. For example, 58 W white light in a 12/12 h light/dark cycle increased disease severity and sporulation more than continuous darkness in inoculated nectarines with *M. laxa*, while different photoperiods did not affect BR incidence (Rodríguez-Pires et al., 2021).

3.3.8. Infection Assessment

Infection assessment is the final step of the phenotyping methodology when the state of infection is assessed and recorded. Two main variables are predominantly used: disease incidence and disease severity.

The disease incidence calculated as the number of infected fruits out of total inoculated. When the assessment is carried out in the field, this is the only available variable since no time follow-up is possible. Notably, disease incidence is the only variable recorded in cherry since measuring the progress of lesion diameter is difficult, given the small size of the fruit (Table 3.1).

The disease severity is measured as the mean of lesion diameter of the rotten area, originated from inoculum point on the fruit surface. This is easier to record when only a single drop is deposited on the fruit, which is an indicator of the rapidity of the disease advancement (Table 3.1). Hong et al. (1998) have not accounted for lesions that did not originate from the inoculation points since they were considered a natural infection. Furthermore, Biggs &

Northover (1988) have transformed the disease severity of unwounded peach and sweet cherry fruit inoculated with *M. fructicola* to a scale of 0 to 3, where 0 = no visible infection; 1 = necrosis not wider than the inoculum drop; 2 = necrosis wider than the width of the inoculum drop, but without sporodochia; and 3 = sporodochia present on the necrotic lesion.

Notably, the BR development may be delayed in non-wounded compared to wounded fruit treatments (Walter et al., 2004). Also, a delay of lesion appearance and severity reduction is reported when inoculum concentrations were lowered from 4×10^4 to 5×10^2 conidia ml^{-1} (Tian & Bertolini, 1999). Consequently, for both cases, *Monilinia* spp. required more time to penetrate and develop on fruit.

3.4. Inconsistency of Infection Results

In plant biology, phenotypic instability is sometimes considered a form of plasticity in response to variations in environmental factors such as nutrients, water availability and temperature (Bradshaw, 1965). Likewise, several hosts and related environmental factors may cause phenotyping inconsistency, in particular across years or methodologies. Pacheco et al. (2014) noticed an inversion of the behavior of the two accessions 'Contender' and 'Elegant Lady' for BR diameter between 2009 and 2010. In contrast, Martínez-García et al. (2013) reported general consistency in ranking within a peach progeny over the three seasons tested. However, variation in resistance or susceptibility between two years was also reported.

3.5. Conclusions

Phenotyping is a crucial step in breeding stone fruit for brown rot resistance. *Monilinia* spp. are necrotrophic fungi requiring several factors to infect stone fruit successfully. In the early stages of fruit development, success primarily depends on pathogen inocula and environmental conditions. Subsequently, other crop-related factors such as cultivar, fruit-related traits and development stage, and field management practices play a significant role in BR development. As the main barrier to infection, fruit skin characteristics seem to be critical, also considering that microcracks and natural openings are the main entrance points of the

pathogen. Despite many efforts initiated in breeding programs, more obviously for peaches and nectarines, any truly BR-resistant stone fruit cultivars are commercially available. While further attempts and contributions by stone fruit breeders are expected, the first step to success relies on the optimization of the phenotyping protocols. This literature review highlights the variability in applied procedures and non-consensus methodologies. All steps of the phenotyping protocol are crucial to ensure good infection performance, from fruit sampling to inoculum preparation and application. Fruit preparation before inoculation requires utmost attention: for example, when the natural pathogen pressure in the orchards is not too high, the best advice is not to disinfect fruit before inoculation. Besides, injuring the fruit seems to be a dead-end since an infection that has reached the flesh no longer stops. Moreover, the choice between spray in orchard and inoculum droplet in the lab lies in the objectives of the test and other variables to record and phenotyping capacity (workforce). Finally, it seems that inoculum droplet and spray tests do not give the same information regarding fruit susceptibility. Overall, even taking all possible precautions discussed, inconsistency could be expected, and multi-year assays are highly recommended to gather valuable results.

3.6. References

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CHAPTER 4. Stone fruit phenolic and triterpenoid compounds more significantly modulate gene expression of *Monilinia* spp. than its characteristics during in vitro growth

Abstract:

Phenolic and triterpenoid compounds are essential components in the stone fruit skin and flesh. They are thought to possess general antimicrobial activity. However, regarding brown rot disease, investigations were confined to only a limited number of phenolics, especially chlorogenic acid. The activity of triterpenoids against *Monilinia* spp., as an essential part of the peach cuticular wax, has not been studied before. Here we investigated the anti-fungal effect of some phenolics, triterpenoids, and fruit surface compound (FSC) extracts of peach fruit at two developmental stages on *Monilinia fructicola* and *Monilinia laxa* characteristics during *in vitro* growth. A new procedure for assaying anti-fungal activity of triterpenoids, which are notoriously difficult to assess *in vitro* because of their hydrophobicity, has been developed. Measurements of colony diameter, sporulation, and germination of second-generation conidia were recorded. Furthermore, the expression of twelve genes of *M. fructicola* associated with germination and/or appressorium formation and virulence-related genes was studied relative to the presence of the compounds. The study revealed that certain phenolics and triterpenoids showed modest anti-fungal activity while dramatically modulating *M. fructicola* gene expression. *MfRGAE1* gene was overexpressed by chlorogenic and ferulic acids and *MfCUT1* by betulinic acid, at 4- and 7-days post-inoculation. These findings effectively contribute to the knowledge of fungi-plant interactions via biochemical compounds.

Keywords:

Anti-fungal activity; gene expression; *Monilinia fructicola*; *Monilinia laxa*; Phenolics; Triterpenoids; Virulence-related genes

4.1. Introduction

Brown rot (BR), caused mainly by *Monilinia fructicola* (Wint.) Honey and *Monilinia laxa* (Aderh. & Ruhl.) Honey, is one of the most damaging fungal diseases of stone fruit worldwide. Because resistant cultivars are not commercially available, fungicide remains the primary tool for reducing the incidence of BR on stone fruit. Ascospores and conidia are the primary sources of inoculum for *Monilinia* spp. during seasonal crop infection cycles (Byrde and Willetts, 1977; Landgraf and Zehr, 1982). Dispersed conidia clinging to the fruit surface eventually germinate under suitable temperature and humidity conditions, and mycelia break through the fruit cuticle. Despite the fact that *Monilinia* spp. enter through wounds and microcracks on the fruit skin (Gibert et al., 2009; Mustafa et al., 2021; Oliveira Lino et al., 2016), they can also employ degrading enzymes (exoenzymes) during fruit surface penetration, invasion, and colonization, like many other necrotrophic pathogens (Byrde and Willetts, 1977).

Cuticle is a hydrophobic coating that covers the surface of all terrestrial plants' aerial parts (leaves, flowers, fruit, and non-woody stems) (Müller and Riederer, 2005). It has two main components, a structural matrix called cutin and cuticular waxes, distinguished by their solubility in organic solvents. The former cannot be dissolved due to its polymer structure, while the latter is dissolved in solvent extraction (Pollard et al., 2008; Walton, 1997). Waxes are embedded in the cutin and form a continuous layer on the surfaces, making it possible to discriminate between intracuticular and epicuticular wax layers (Jetter et al., 2000). On the one hand, the cuticle is an inert mechanical support and barrier against biotic and abiotic stresses; it is a dynamic, metabolically active tissue (Walton, 1997; Ziv et al., 2018). Even though the cuticle provides a source of nourishment for microbes and animals, it also includes toxic peroxidases, phenolics, and activated oxygen compounds (Lattanzio et al., 2006; Oliveira Lino et al., 2016).

Secondary metabolites embedded in fruit skin and flesh may oppose or reduce fungal infection to some extent (Pusztahelyi et al., 2015). The variability of total phenolics between

different peach cultivars and the decline pattern along fruit development was proposed to explain the differential behavior of cultivars towards BR resistance and the fruit resistance evolution between stages (Andreotti et al., 2008; Kubota et al., 2000; Lee et al., 1990; Obi et al., 2020). Fruit from specific peach accessions, such as the South American cv. Bolinha shows a high level of resistance to *M. fructicola* and up to three times the level of chlorogenic acid (CGA) of susceptible accessions at equal stages of maturation (Bostock et al., 1999). CGA, neochlorogenic, and caffeic acids decline considerably from stage II to maturity (Bostock et al., 1999; Lee and Bostock, 2007), while fruit susceptibility to BR increases, supporting the hypothesis that they may play an important role in fruit resistance. CGA, neochlorogenic acid, caffeic acid (CA), pyroquilon, and epicatechin were among the primary phenolic acids studied *in vitro* for antifungal efficacy on the spore germination and mycelial growth in *Monilinia* spp. (Bostock et al., 1999; Lee et al., 1990; Lee and Bostock, 2007; Villarino et al., 2011). Unfortunately, CGA and CA at concentrations up to 5.0 mM did not inhibit spore germination or mycelial growth in culture, while epicatechin appeared to reduce spore germination of *M. fructicola* (Bostock et al., 1999; Lee and Bostock, 2007). In another study, CGA and pyroquilon did not affect *M. laxa* growth while reducing the level of melanin-like pigment (Villarino et al., 2011). However, CGA and CA markedly inhibited the production of cell wall degrading enzymes such as polygalacturonase and cutinase (Bostock et al., 1999; Lee and Bostock, 2007) and suppressed the production of melanin-like pigments in *M. fructicola*. Villarino et al. (2011) suggested that it could interfere with the early skin penetration process. In addition, Lee & Bostock (Lee and Bostock, 2007) have investigated the penetration of *M. fructicola* at different peach fruit developmental stages. They observed that *M. fructicola* produced fewer appressoria on immature fruit (stage I) and mature fruit (stage III) than on fruit at stage II. They suggested that the production of numerous appressoria on the stage II could play a role in quiescence, perhaps by serving as resting structures until fruit reach maturity. Besides these extensive

works, few studies investigated the effects of other phenolic compounds. Recently, Hernández et al. (2021) reported the conidial germination and mycelium growth inhibitory effect of other phenolic acids, namely ferulic and p-coumaric, and flavonoids (naringin, hesperidin and neohesperidin) on *in vitro* cultures of *M. fructicola*.

Triterpenes are another important class of secondary metabolites included in cuticular waxes. They potentially act as signaling molecules and protect against pathogens and pests (Thimmappa et al., 2014). Cuticular waxes include a high concentration of triterpenoids, some of which are considered phytoanticipins, these anti-fungal metabolites are preformed inhibitors constitutively present in healthy plants (Ribera and Zuñiga, 2012). In a study by Oliveira Lino et al. (2020), several triterpenoids were associated with peach BR disease resistance in immature fruit of Zephir nectarine. When the fruits had the least infection probability in stage II, ursolic acid (UA) and oleanolic acid (OA) were the most prevalent compounds. These two triterpenoids are known for their broad antimicrobial properties (Jesus et al., 2015), and these compounds and their derivatives have been characterized for their anti-fungal activity (Shai et al., 2008). However, the literature relative to the effects on *Monilinia* spp. of triterpenoids is scarce. Indeed, when evaluating compounds with high hydrophilicity, the success of the interaction between the tested compounds and fungi is not always guaranteed. As a result, based on our knowledge, no study on the inhibitory effects of triterpenoids has been conducted in *Monilinia*. The use of an airbrush to apply wax components on agar media or coverslips, as described by Belding et al. (2000), brings up interesting perspectives to assess these kind of compounds.

Besides the exploration of colony traits, such as growth speed, sporulation, and germination rates, and traits related to the infection process, such as formation of appressorium and melanization, it is also interesting to focus studies on the enzymatic and proteomic arsenal of the fungi, especially these involved in virulence and fitness functions. In addition, gene

expression perturbations may also be a good indicator of the impact of a compound on pathogen biology. In this way, works reported the impact of some compounds on enzymes production by *Monilinia* spp. and on the expression of genes potentially linked to the pathogenicity of *Monilinia* spp. (Baró-Montel et al., 2019; Bostock et al., 1999; De Cal et al., 2013; Lee et al., 2010; Rodríguez-Pires et al., 2020; Wang et al., 2002). Various essential degrading enzymes are synchronized during fungal infection to overcome the physical and chemical barriers buried in the skin. The role of these enzymes is well established in the closely related necrotrophic pathogen *Botrytis cinerea* (Li et al., 2020), and they have been used as homologs to study their roles in *Monilinia*. In particular, cell wall degrading enzymes (CWDEs), namely glycosyl-hydrolases, oxidoreductases, lyases, and esterases, have been reported to be related to virulence (Choquer et al., 2007; Kubicek et al., 2014; Lee et al., 2010; Quoc and Bao Chau, 2016; ten Have et al., 1998). All potential pathogens encounter cutin during direct host penetration of above-ground plant parts (Walton, 1994). Therefore, cutinase is an enzyme that pathogenic fungi may use to facilitate penetration of the cuticle (Bostock et al., 1999). Cutinases, pectin lyases, α-glucosidases, polygalacturonases, proteases, and xylanases are among the few CWDEs examined in *Monilinia* spp. in terms of pathogenicity (Byrde and Willetts, 1977; Chou et al., 2015; De Cal et al., 2013; Garcia-Benitez et al., 2019; Rodríguez-Pires et al., 2020; Wang et al., 2002).

The conclusion of this brief review of the literature is that little is known about the inhibitory effects of triterpenoids on the growth and fitness of *Monilinia* spp., and on the impact of phenolics and triterpenoids on the modulation of CWDE genes. Therefore, in this study, we pursued two complementary objectives: (1) investigating the inhibitory effects of phenolic compounds (chlorogenic and ferulic acids), terpenoids (oleanolic, ursolic, betulinic acids), and fruit surface compound (FSC) extracts on the growth and fitness of *M. fructicola* and *M. laxa;*

(2) surveying the effect of exposure of *M. fructicola* to these compounds on the regulation of 12 genes linked to fitness and virulence.

4.2. Material and methods

4.2.1. Fungal Isolates and Inoculum preparation

Monospore isolates of *Monilinia fructicola* and *M. laxa* were obtained from an infected apricot branch and mummified apricot, respectively, from an experimental orchard belonging to INRAE, located near Valence (Drôme, France) in March 2011. Species-specific primers were used to confirm both *Monilinia* spp. as reported by Hughes et al. (2000). Both fungi were grown and maintained on V8 agar medium at 24 °C under a 16/8-hour light-dark cycle for short periods. While conidia suspensions were stored in monopotassic phosphate buffer with 20% glycerol at -20 °C for prolonged preservation. Conidia suspensions were made from active cultures that were 7–10 days old whenever needed for running any experiments. Petri cultures were flooded with 3 ml of sterile distilled water and gently scraped with a spatula. The total conidia and mycelia were collected and shaken for 1 min in a tube containing glass beads to dismantle conidial chains. Subsequently, the suspension was passed through a falcon strainer with pore size of 40 μm to sift mycelia parts in the suspension. Malassez counting chamber was used to determine conidia enumeration and adjusted to the desired concentration.

4.2.2. Reagents

Chlorogenic acid (CGA), oleanolic acid (OA) and ferulic acid (FA) were purchased from Sigma-Aldrich (Saint-Quentin-Favallier, France). Other compounds, ursolic acid (UA) and betulinic acid (BA) were purchased from Extrasynthèse (Genay, France).

Preparations of fruit surface compounds (FSC) extract

Two immature fruit stages of Zephir nectarine were selected for extracting fruit surface compounds (FSCs): stage I and stage II (pit hardening), which correlate to high and low susceptibility to brown rot (Mari et al., 2003), respectively. The mass extraction method was carried out according to Oliveira Lino et al. (2020). Around a hundred fruits were harvested at stage I, and about fifty at stage II. A representative part of the lots was measured for the three

diameters to determine the average surface area of fruit and calculate the total surface area of the fruit lots. All the fruit were washed in reverse osmosis treated water, air-dried, and then immersed in chloroform (VWR, Normapur) for 30 seconds under frequent agitation. Extracts were then filtered through a paper filter to remove any debris, and the volume were measured as well. Liquid chromatography was used to analyze the amounts of triterpenoids and phenolic acids.

Application of compounds and FSC extracts onto the medium

CGA. CGA stock solutions were prepared by dissolving it in pure ethanol (EtOH) and then diluted in ultra-pure water to reach concentrations of 5, 10 and 20 mM in 10% EtOH. The stocks were first filtered on a 0.22 μm pore size filter (Falcon®). The solutions were amended to an autoclaved V8 juice Agar medium to obtain to 0.5, 1.0, and 2.0 mM concentrations at 1% EtOH, and the pH was adjusted to 5.2 to minimize CGA degradation. Altogether, 20 ml of media and the amended compounds were distributed onto plastic Petri dishes. Petri cultures with 1% EtOH was employed as a control.

Triterpenoid and FSC extracts. Three triterpenoid compounds OA, UA, and BA at 2.3 mM concentration and one phenolic compound FA (used to test the efficacy of the protocol) at 13 mM were dissolved in chloroform. A 3 ml of each compound was spread on the surface of a glass Petri plate (9 cm diameter) containing V8 juice agar using a glass pipette. When applied on the plates, the proportion of individual compounds were around 50 μg/cm² for AO, UA, and BA. This amount was determined to represent the actual concentration found on fruit surface (Table 4.1), while FA concentration was 122 μg/cm².

By the same approach, 3 ml of FSC extracts (stage I and stage II) were spread on the surface of glass Petri plates (9 cm diameter) containing V8 juice agar using a glass pipette. This amount was calculated to distribute a concentration per unit area of oleanolic and ursolic acids comparable to that found on the fruit surface, at each stage.

Initial extracts were concentrated on a vacuum rotary evaporator, and then a calculation was made to determine the concentration required to deposit the extracts on the surface of Petri dishes to form a layer of compound mixture which resemble the spread on fruit skin (Table 4.1). The extracts of three years of collection were used for the tests. Thus, the amount of oleanolic acid deposited were around 5 µg/cm² at stage I and 36 µg/cm² at stage II, and the amount of ursolic acid was around 19 µg/cm² at stage I and 99 µg/cm² at stage II (Table 4.1). In both the triterpenoid and FSC extract tests, 3 ml of pure chloroform was spread on plates containing the same quantity of medium and was used as a control.

Table 4.1. Oleanolic and ursolic contents in the fruit extracts at the two stages and on the Petri dishes.

| Year (season) | Fruit stage | Number of fruits | Fruit content | | Content in the extracts applicated to the Petri plates | |
			oleanolic acid (µg/cm²)	ursolic acid (µg/cm²)	oleanolic acid (µg/cm²)	ursolic acid (µg/cm²)
2013	stage I	292	3.7	13.2	4.0	13.0
2017	stage I	159	4.8	20.7	6.0	27.0
2018	stage I	60	9.0	33.6	5.0	18.0
Average	stage I	170.3	5.8	22.5	5.0	19.3
2013	stage II	50	33.5	101.0	34.0	103.0
2017	stage II	50	34.1	99.0	39.0	113.0
2018	stage II	40	37.7	84.6	36.0	80.0
Average	stage II	46.7	35.1	94.9	36.3	98.7

Plates loaded with solutions of triterpenoids, FSC extracts, and their controls were kept uncovered for about 30 minutes under a sterile chemical fume hood to allow the chloroform to evaporate completely, and they were regularly agitated by hand to leave a layer of compounds on the medium surface.

4.2.3. Inoculation and incubation of culture plates

Plates of all cultures (CGA, triterpenoids and FSC extracts) were inoculated the same way by, either *M. fructicola* or *M. laxa*, placing 10 µl conidia suspension at 10^5 conidia/ml^{-1} concentration to the center of the medium. After the droplet was dried in the center, inoculated Petri dishes were sealed with Parafilm® and incubated in a culture chamber at 24 °C under a

16/8 h light-dark cycle. Each test consisted of 3–4 Petri culture replicates per treatment and control, and each trial was repeated at least twice independently.

4.2.4. Assessment of fungal response to compounds and FSC extracts
Growth, Sporulation and Germination of second-generation conidia

Colony diameters of 3–4 replicate plates were measured in two perpendicular lines crossing from the sowing center. Values were compared to their corresponding control (untreated culture). Measurements were started at three days post-inoculation (dpi) daily and continued up to 10 dpi. Only measures from the 3 and 7 dpi were included in the final analysis.

Each culture plate was flooded with 3 ml of sterile distilled water and gently scraped the entire plate. Total conidia and mycelia were gathered and added to a tube containing glass beads. The mixture was agitated for 1 minute to dismantle conidia chains from mycelia. Then the suspension was separated and passed through a strainer (Falcon®) with pore size of 40 μm to separate conidia from mycelia. Sporulation was measured after 11dpi. Conidial concentration was determined using a Malassez counting chamber.

Sporulation from the previous step was diluted several times based on the concentration determined and adjusted to a manageable concentration to count. The conidial germination was determined by placing 4 droplets with the size of 50 μl of diluted suspension per replication on Petri dishes containing a thin layer of PDA. Plates were incubated for 20±3 h in a closed plastic box with maximized humidity at room temperature. Under a light microscope with 100x magnification, germinated and ungerminated conidia of more than 100 conidia per plate were recorded. The percentage of germinated conidia for three replicates was compared to the control.

4.2.5. Assessment of modulation of *Monilinia fructicola* gene expression by compounds and FSC extracts
Cultural conditions and Mycelia harvest

For evaluating the impact of studied compounds and FSC extracts on the regulation of virulence-related genes, three Petri plates of each treatment and control were harvested at each

incubation period. For CGA, the entire culture (approximately 3.0 g) was harvested at three incubation periods of 4, 7, 11 dpi, while other compounds and FSC extracts were collected at 4 and 7 dpi and instantly stored in -80 °C until use.

Selection of Candidate Genes and Primer design

A total of 12 genes were selected from the literature according to their putative functions described either on *Monilinia* spp. or *B. cinerea*. Selected genes from *B. cinerea and M. laxa* were analyzed *in silico* using BLAST Batch Entrez tools (https://www.ncbi.nlm.nih.gov/sites/batchentrez) from the NCBI database to produce an expressed sequence tag (EST) ortholog in *M. fructicola*. Primers were designed using software Primer 3.0 (https://primer3.ut.ee). The primer sequences and amplicon lengths are listed in Table 4.2.

Table 4.2 Details of the candidate genes with their putative function, primer sequence and amplicon size, considered in this study for *Monilinia fructicola*.

No.	Gene name	Putative function	5' to 3'sequence [oligonucleotides]	Amplicon (bp)	Described in	Reference	In *M. fructicola*
Germination/appressorium formation related genes							
1	*MfBMP1*	Germination induction and pathogenicity	F: AAAGACGGAGTTGCTGCTTC R: CGGATCCGAATCCTTTCGAG	116	*B. cinerea*	(Zheng et al., 2000) (Doehlemann et al., 2006)	This study
2	*MfPLS1*	Appressorium mediated penetration into intact host plant leaves (virulence).	F: GTCTCGGCGAATCTCTTGTT R: CATGTAGCCGTGCACTTTGA	143	*B. cinerea*	(Gourgues et al., 2004)	This study
Cell wall degrading enzyme (CWDE) genes							
3	*MfCUT1*	Production of cutinase which breaks down the cutin on the fruit surface	F: GGATCCCCAGTTCAAGGTG R: GTCTCCGGCGTGACAGAT	60	*M. fructicola*	(Wang et al., 2002)	
4	*MfPNL3*	Pectin lyase 3.	F: GCGTCTCCAACGTCATCATC R: TGAGCCTTCCAGCAGCATTA	176	*M. laxa*	(Rodríguez-Pires et al., 2020)	This study
5	*MfRGAE1*	Pectin dependent; The assistance of rhamnogalacturonan acetyl esterase.	F: CGGAGAGACCGTCTACACAT R: GGGAGGTGTAGGACCAAGTG	135	*M. laxa*	(Rodríguez-Pires et al., 2020)	This study
6	*MfPG1*	Endopolygalacturonase, overexpression of *Mfpg1* reduces virulence in *M. fructicola*.	F: TGTCAACGTTACCGGTGGT R: TTGACACCAGATGGGAGACC	60	*M. fructicola*	(De Cal et al., 2013)	
7	*MfPG3*	Endopolygalacturonase: Not clear function, expressed with high pH (when M. fructicola during fruit infection)	F: ATGGTAGAGGTATCACGATCACTG R: CGGCACCATTTCCTTCAAT	60	*M. fructicola*	(De Cal et al., 2013)	
8	*MfPG6*	Endopolygalacturonase: Not clear function, expressed with high pH (when M. fructicola during fruit infection) acidify	F: GGCCCGTTCATCTTTTCAC R: CCCTGCTGTGTTGTTGAGAG	77	*M. fructicola*	(Chou et al., 2015)	

9	*Mfcda1*	Putative chitin deacetylase gene (cda1) and conidial germination.	F: AAGGCCCTTGCTTCAACAAC R: AAACTGCAGTGGAAGATCGC	168	*B. cinerea*	(Leroch et al., 2013)	This study
10	*MfLAE1*	[ROS] Involved in secondary metabolism and virulence	F: GGTGAAGTTCTTGGGCTTGA R: TCCCAGGACTCCACTTCTAGT	118	*B. cinerea*	(Schumacher et al., 2015)	This study
Reactive oxygen system (ROS)-related genes							
11	*MfNoxR*	ROS, actin cytoskeleton regulation, decrease in sporulation	F: AGGCGCTGAAGGAATTTGAC R: TCGAAACGCCTTGTTGGAAA	168	*B. cinerea*	(Siegmund et al., 2013)	This study
12	*MfOfd1*	**Stress responses** and virulence, increased ROS.	F: GGATGGGGCGATGATGGTAATG R: GGCTCTCCTTTGGGCTCTGGT	149	*M. fructicola*	(M. M. Zhang et al., 2020)	

4.2.6. Isolation of total RNA and cDNA synthesis

Total RNA was isolated from frozen fungal samples using the ready kit NucleoSpin®RNA Plant and fungi (Macherey-Nagel) according to the manufacturer's instructions. RNA was quantified using a spectrophotometric at 260 and 280 nm absorbance (Thermo Scientific NanoDrop™) and tested for integrity by agarose gel electrophoresis. According to the manufacturer's instructions, one microgram of total RNA was used as the template for cDNA synthesis using oligo (dT) primer (AffinityScript QPCR cDNA Synthesis Kit; Agilent Technologies) and specific reverse primer of genes. The completed first-strand cDNA synthesis reactions were diluted (1:10), and diluted cDNA was used as the template for qPCR analysis.

4.2.7. Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR was performed on a Stratagene MX Pro 3005 amplification system. For amplification, 7 µl of SYBR Green PCR master mix (Brilliant III with High ROX, Agilent Technologies), 0.5 µl of each primer (10 µM), 2 µl of diluted cDNA (1:10), and 5 µl RNase free water were used in a 15 µl reaction mixture. The thermocycling program consisted of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min. Each reaction was carried out in triplicates. All qPCR reactions were normalized by the threshold cycle value (Ct) compared to three internal reference genes *α-Tubulin*, *RPL13* and *TEF2* in *M. fructicola* following the $2^{-\Delta\Delta CT}$ method for relative quantification as according to Livak & Schmittgen (2001). The reference genes were expressed homogenously and showed no significant changes between modalities. Statistical analyses were performed for the normalized gene expressions

by adapting scripts from the R package (RqPCRBase) developed by Hilliou & Tran (2013). Thereby, for each gene relative to tested compounds, a fold change relative to control was computed. Positive and negative values correspond respectively to the genes' over and under expression due to adding the compound in the culture compared to the control (no compound).

Statistical analysis

Results of colony diameter underwent a two-way analysis of variance in which the days post-inoculation was considered a second parameter. A one-way ANOVA was used with a t. test using an R-based tool and ggplot2 to generate graphs to evaluate the mean differences for sporulation and germination. Treatments were compared to controls, and a P-value of 0.05 was used to indicate significance. All studies were carried out at least twice independently, with each treatment receiving at least three replicates.

4.3. Results

4.3.1. Anti-fungal Effect of Phenolic and terpenoid compounds and FSC on *Monilinia laxa* and *M. fructicola*

Chlorogenic acid

The amendment of CGA in media proved to have no inhibitory effect on growth of both *Monilinia* spp., whatever the concentrations. On the contrary a significant speed increase of colony growth was observed for *M. laxa* at both 3- and 7-days post-inoculation (dpi) compared to their respective control (untreated culture). As for *M. fructicola*, colony growth was less enhanced at 3 dpi (only 1.0 mM was significant), whereas the increase of growth was also obvious at 7 dpi. Contrarywise, the sporulation of both species was reduced significantly with less effect on *M. fructicola* compared to *M. laxa* at 11 dpi after full colony growth. Furthermore, germination of second-generation conidia of *M. laxa* gathered in the assays with 0.5 and 1.0 mM CGA showed significant reduction, however no statistical reduction of germination was observed for *M. fructicola* compared to control (Figure 4.1).
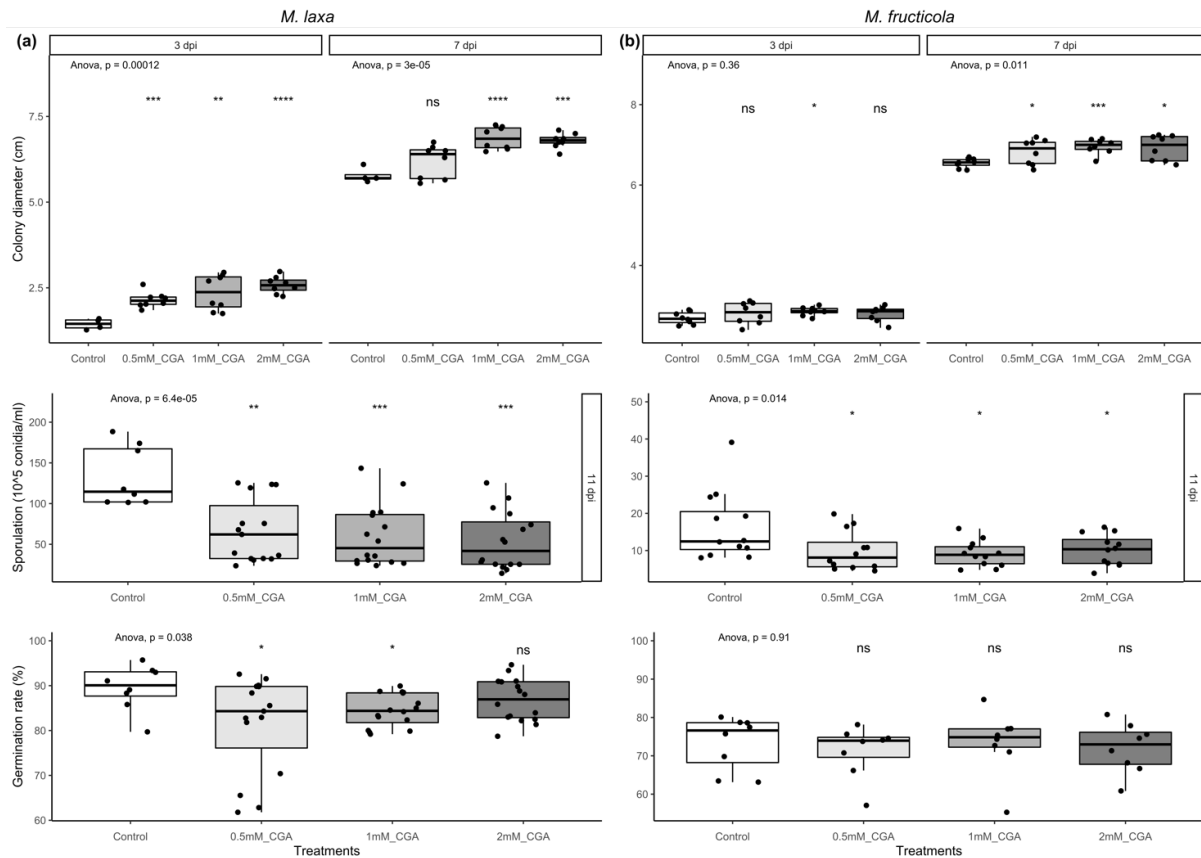
Figure 4.1 Anti-fungal effect of chlorogenic acid (CGA) on *Monilinia laxa* (**a**) and *M. fructicola* (**b**), by *in vitro* assessments, at three concentrations amended to V8 juice agar. Colony growth diameter (cm) was measured at 3 and 7 dpi, sporulation (10^5 conidia/ml) at 11 dpi, and the germination of second-generation conidia was assessed from spores harvested after 11 dpi as well. A final concentration of 1% EtOH in the medium was employed as a control. The differences are assessed using a t-test with a 0.05 p-value.

Triterpenoid compounds and Ferulic acid

The suitability of the protocol to test hydrophobic compounds was confirmed by the obtention of significant effects of ferulic acid on the three characteristics of fungi growth and fitness. Indeed, with both *Monilinia* spp., a significant inhibitory effect was observed on the fungal growth (especially at 7dpi) and sporulation, while the effect on germination of second generation of conidia was limited to *M. laxa* (Figure 4.2). The anti-fungal effect of oleanolic acid against *M. laxa* and ursolic and betulinic acids against *M. fructicola* was then observed. Applying a thin layer on the Petri dishes with 50 µg/cm² of each compound did not induce any inhibitory effect on growth and fitness of the two *Monilinia* species.
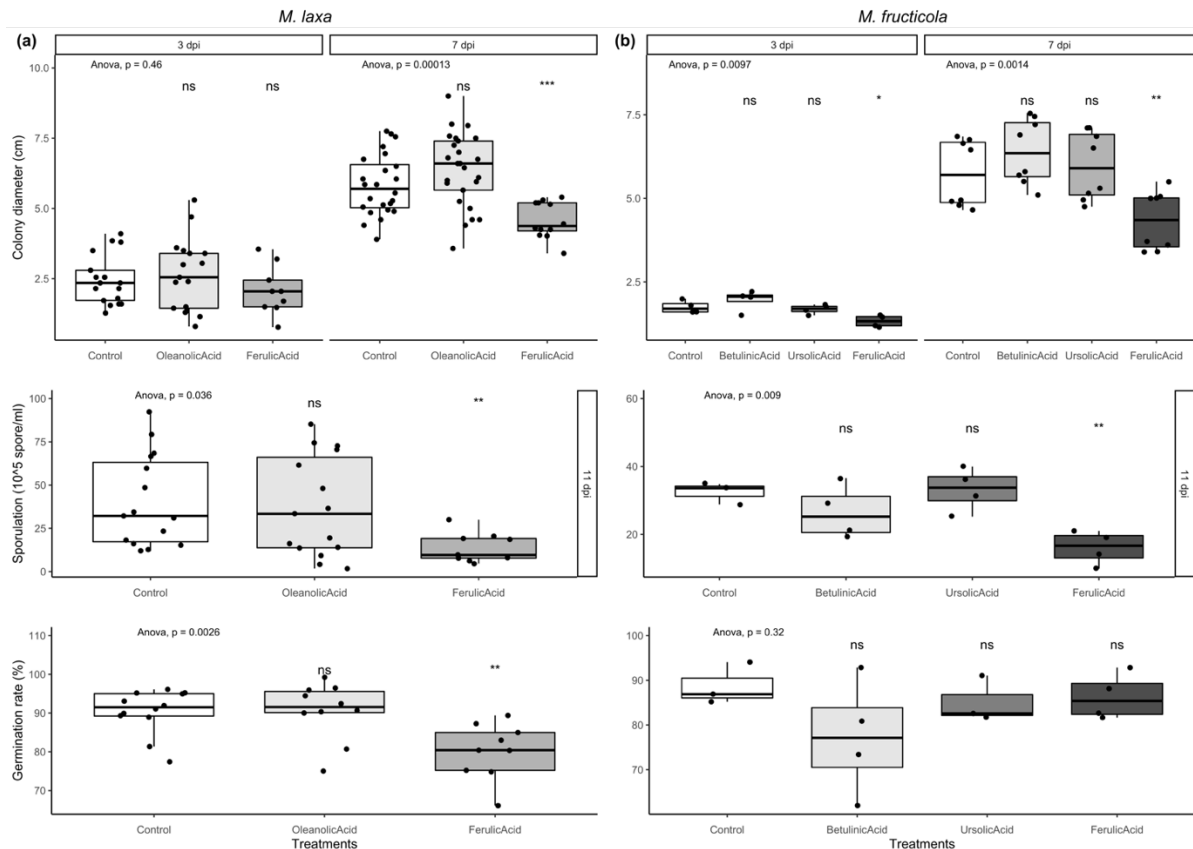
Figure 4.2. Anti-fungal effect of individual compounds on *Monilinia laxa* (**a; right**) and *M. fructicola* (**b; left**), by *in vitro* assessments. The concentration of compounds applied was 2.3 mM (or 50 µg/cm² on medium surface) for each, while ferulic acid was 13.4 mM (or 122 µg/cm²), dissolved in chloroform. Three ml of each compound was spread on the surface of V8 juice agar medium. Colony growth diameter (cm) was measured at 3 and 7 dpi, and sporulation (10^5 conidia/ml) after 11 dpi, and the germination of second-generation conidia was assessed from spores harvested after 11 dpi as well. Control was only 3 ml chloroform (pure) spread on the medium. The differences are assessed using a t-test with a 0.05 p-value.

Effect of Fruit Surface compound (FSC) extracts

The two FSC extracts of Zephir nectarine stages I and II were assessed on *M. laxa* and *M. fructicola* cultures. No inhibitory effects were observed; instead, in the case of *M. laxa,* the colony growth diameter was increased at 7 dpi compared to control, and a significant increase was observed at 3 dpi for *M. fructicola*. At full colony growth, after 11 dpi, the sporulation and germination of second generation were not affected compared to their respective control for both FSC extracts and with both *Monilinia* spp. (Figure 4.3). The concentrations in OA and UA present in the layers made with stage II FSC extracts (around 36 and 99 µg/cm² respectively) were very similar to the concentrations applied testing separately OA and UA bought from industry (50 µg/cm²), except they were applied together and mixed with the other

minor constituents of the waxes. Same results were obtained (no effect) apart from the promotion of colony growth of *M. laxa*, which can be attributed to the minor compounds in FSC extracts.
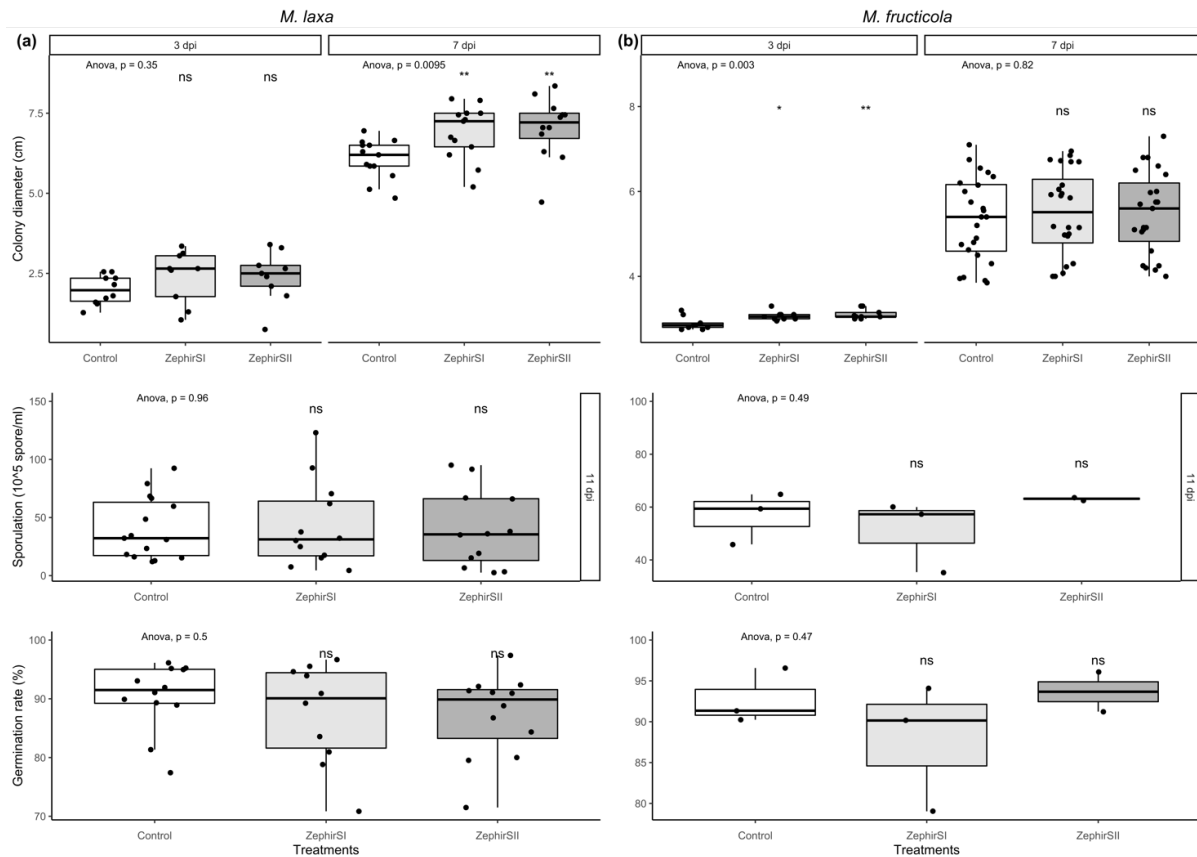
Figure 4.3. Anti-fungal effect of two fruit surface compound extracts at stage I and II of fruit development of cultivar "Zéphir" on *Monilinia laxa* (a; right) and *M. fructicola* (b; left), by in vitro assessments. Three ml of each compound was spread on the surface of V8 juice agar medium. Colony growth diameter (cm) was measured at 3 and 7 dpi and sporulation (10^5 conidia/ml) after 11 dpi, and the germination of second-generation conidia was assessed from spores harvested after 11 dpi as well. Control was only 3 ml chloroform (pure) spread on the medium. The differences are assessed using a *t-test* with a 0.05 *p-value*.

4.3.2. Effect of phenolic and terpenoid compounds and FSC extracts on Gene expression

Quantitative real-time PCR (qRT-PCR) was used to quantify the gene expression of 12 genes in the presence of compounds or extracts, compared to their corresponding control. For the CGA experiment, total mycelium was harvested after three incubation periods (4, 7, and 11 dpi), while for the other compounds and FSC extracts, total mycelium was harvested after two incubation times only (4 and 7 dpi).

Chlorogenic acid (CGA)

Among the 12 genes tested for gene expression in response to the presence of 2.0 mM CGA amended, *MfRGAE1*, *MfPG1* and *cda1* showed significant overexpression at 4 dpi, and *MfRGAE1* was also overexpressed at 7 dpi. In addition, *MfLAE1*, *MfOfd1* and *MfPG3* were slightly overexpressed at 4 dpi, though significantly. On the contrary, *MfCUT1* was the only gene down-regulated throughout the period compared to control. Besides, *MfNoxR*, *MfOfd1*, *MfPG1* and *MfPLS1* displayed significant under-expression at 11 dpi only. Some genes such as *MfPG1*, *MfOfd1* and moderately *MfPLS1*, shifted from up-regulation at 4 and 7 dpi to significant down-regulation at 11 dpi compared to control (Figure 4.4).

Figure 4.4. Chlorogenic acid (CGA) effect on the expression of 12 germination/appressorium formation and virulence-related genes in *M. fructicola*. qRT-PCR was monitored during growth on 2.0 mM CGA concentration at different incubation periods (4, 7, and 11 dpi). Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$) compared to control (untreated culture), i.e., negative values correspond to an under-expression of the genes due to adding CGA in the culture. The black and white bars correspond to significant and non-significant (ns) values, respectively.

Ursolic and Betulinic acids

In general, ursolic and betulinic acids displayed similar profiles of modulation of gene expression over the 12 genes tested (Figure 4.5). However, ursolic acid had a more negligible effect than betulinic acid. Indeed, many genes were significantly affected by adding 2.3 mM ursolic acid to the medium but only moderately and this effect remained at 4 and 7 dpi. *Mfcda1*,

MfCUT1, *MfOfd1*, *MfPG1*, *MfPNL3* were significantly overexpressed at 4 and 7 dpi and *MfRGAE1* at 4 dpi only. On the contrary, *MfNoxR* was the only gene significantly down-regulated by ursolic acid relative to control. Surprisingly, betulinic acid distinguishingly up-regulated *MfCUT1* mainly at 7 dpi with a fold-change compared to control reaching more than 15. On the other hand, *MfPG1* and *MfPG3* were significantly down-regulated at 7 dpi. In addition, *MfPG1*, again (as with CGA), shifted from significant up-regulation at 4 dpi to significant down-regulation at 7 dpi.
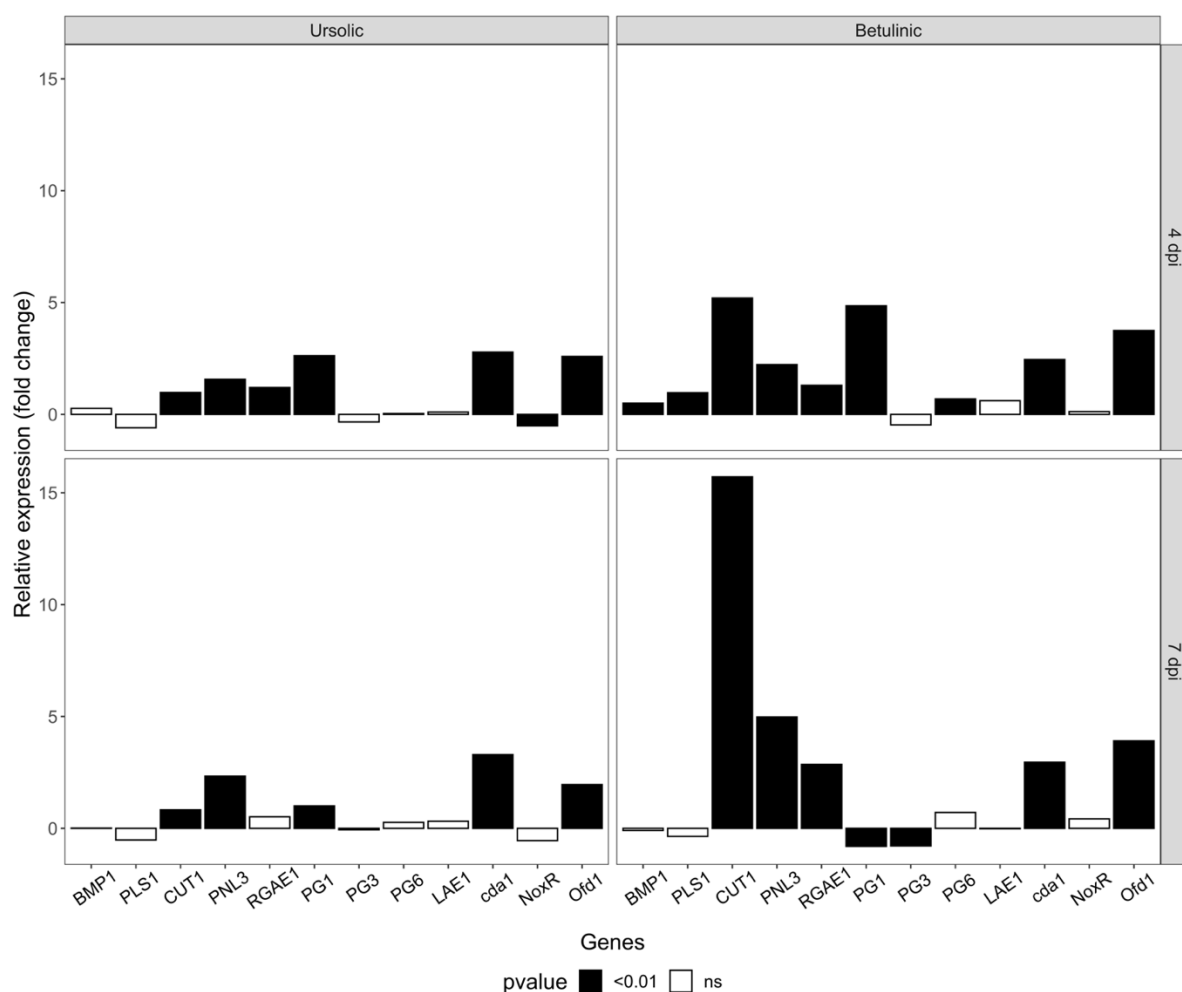
Figure 4.5. Ursolic and betulinic acids effect on the expression of 12 germination/appressorium formation and virulence-related genes in *M. fructicola*. qRT-PCR was monitored during growth on 2.3 mM (or 50 µg/cm² on medium surface) concentration of each compound at different incubation periods (4 and 7 dpi). Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$) compared to control (untreated culture). The black and white bars correspond to significant and non-significant (ns) values, respectively.

Ferulic and Oleanolic acids

Neither of the two acids, ferulic nor oleanolic, caused the under-expression of any studied genes (Figure 4.6). On the contrary, they triggered a strong overexpression of some specific genes. *MfRGAE1* was largely up-regulated by ferulic acid at 4 and 7 dpi (~ 20 and 10-fold change, respectively). Regarding oleanolic acid, *MfPG6* showed significant high up-regulation at both 4 and 7 dpi compared to control (respectively, 18- and 20-fold change). *MfRGAE1* and *MfCUT1* were remarkably up-regulated by oleanolic acid respectively at 4 and 7 dpi (25- and 10-fold change, respectively).
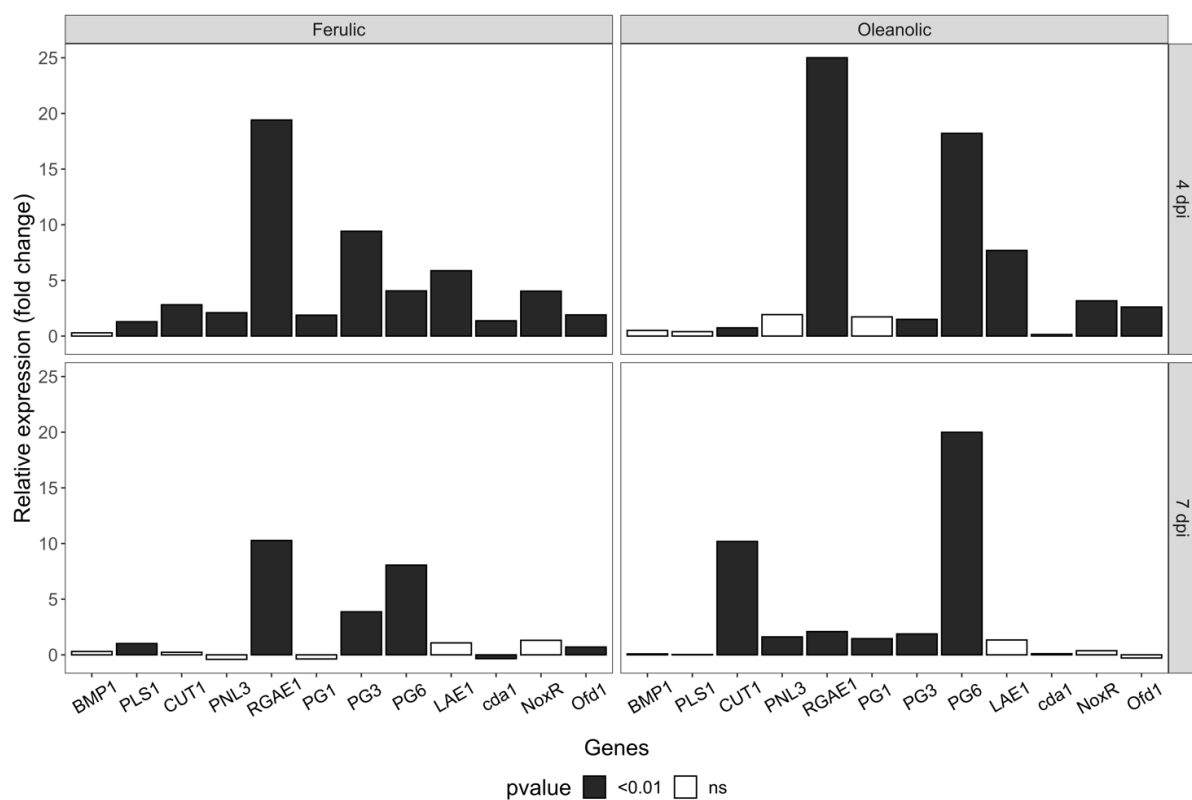
Figure 4.6. Ferulic and oleanolic acids effect on the expression of 12 germination/appressorium formation and virulence-related genes in *M. fructicola*. qRT-PCR was monitored during growth on 2.3 mM (or 50 µg/cm² on medium surface) for oleanolic acid while for ferulic acid was 13.4 mM (or 122 µg/cm²) at different incubation periods (4 and 7 dpi). Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$) compared to control (untreated culture). The black and white bars correspond to significant and non-significant (ns) values, respectively.

Fruit surface compound (FSC) extracts

Regardless of the genes, both FSC extracts had a minimal effect at 4 dpi, with low fold changes even if significant (Figure 4.7). On the contrary, at 7 dpi most of the genes had an expression significantly modulated, with different effects depending on the FSC extracts. It is

worth noting that the two genes related to germination/appressorium formation, *MfBMP1* and *MfPLS1*, were more over-expressed in the case of stage I FSC extracts (ZeSI) compared to stage II FSC extracts (ZeSII). In addition, *MfOfd1* showed a significant overexpression due to ZeSI at 7 dpi with nearly 14-fold change. *MfPG3* and *MfPG6* were both up-regulated by the two FC extracts, but *MfPG3* was much more impacted by ZeSII (15-fold change compared to 7-fold change). Also, ZeSII largely impacted *MfLAE1* at 7dpi inducing overexpression of 9-fold change.
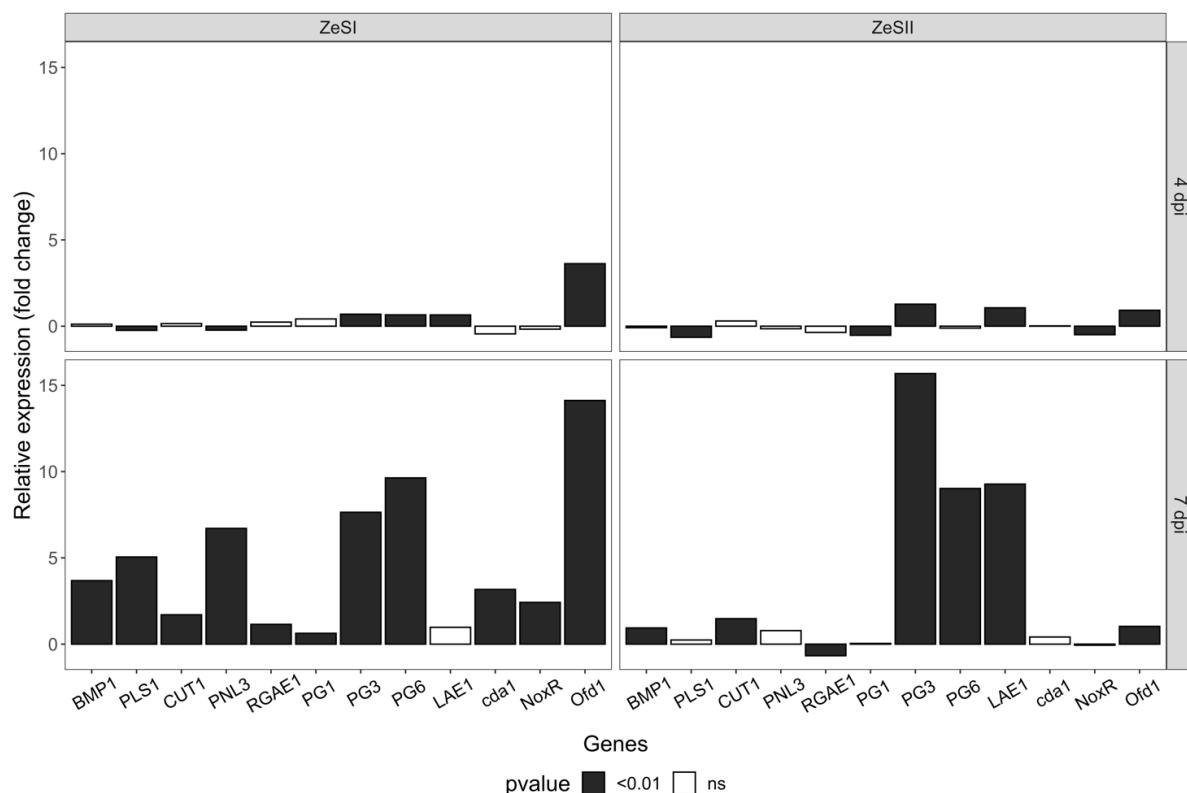
Figure 4.7**.** The effect of Fruit surface compound (FSC) extracts of fruit developmental stage I (ZeSI) and II (ZeSII) of Zeph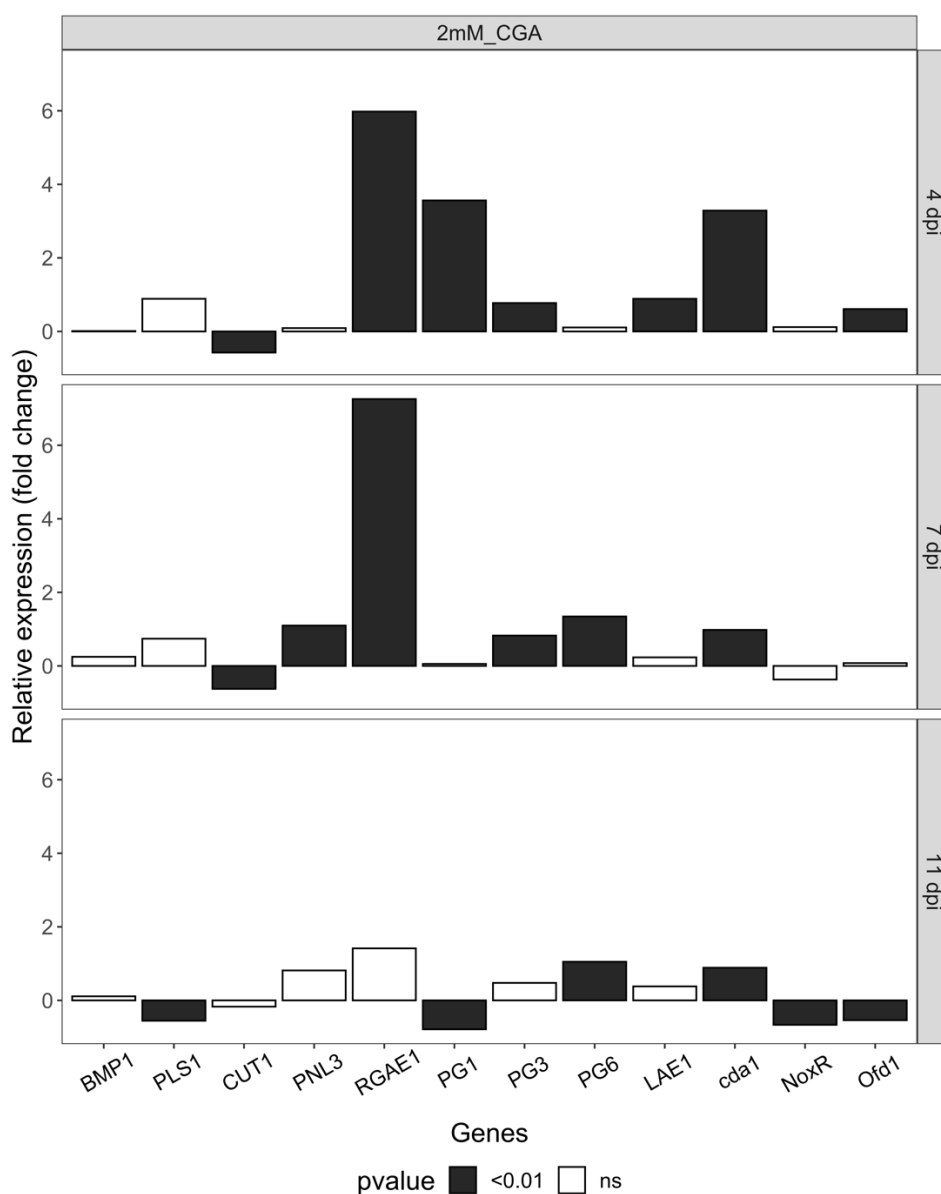ir nectarine on the expression of 12 germination/appressorium formation and virulence-related genes in *M. fructicola*. qRT-PCR was monitored during growth on each extract at different incubation periods (4 and 7 dpi). Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$) compared to control (untreated culture). The black and white bars correspond to significant and non-significant (ns) values, respectively.

4.4. Discussion

Monilinia fructicola and *M. laxa* are the two most important causal agents of brown rot (BR) in stone fruits. Because phenolic and terpenoid compounds are found in almost all stone fruits (Lara et al., 2020), and some of them have been correlated to BR resistance in different cultivars and at some fruit developmental stages (Oliveira Lino et al., 2020), their anti-fungal effect was investigated either individually or collectively.

In this study, the anti-fungal activity of two phenolics (chlorogenic and ferulic acids), three triterpenoids (oleanolic, ursolic, betulinic acids), and two fruit surface compound (FSC) extracts from two Zephir nectarine fruit developmental stages have been assessed.

Antifungal effect of the compounds and extracts
Chlorogenic acid (CGA)

At three concentrations (0.5, 1.0, and 2.0 mM), chlorogenic acid (CGA) did not inhibit colony growth diameter and germination of second-generation conidia generated from treated plates. On the contrary, it increased the colony growth slightly. However, the sporulation was reduced for both fungi compared to untreated control. These findings align with earlier research that featured that even higher concentration of 5.0 mM CGA did not limit colony growth and spore germination (Bostock et al., 1999). In another study, Lee and Bostock (Lee and Bostock, 2007) found that at a dose of 0.5 mM, conidia germination was not inhibited on flower petals or fruit, but it did reduce appressorium formation from germinated conidia. Furthermore, they reported that applying CGA to peach petals and cherry fruits significantly reduced lesion development caused by *M. fructicola*. The anti-fungal action of CGA against *Monilinia* spp. is ambiguous in literature, as it is mainly dependent on both the observed fungal features and the test settings (methodologies) of *in vitro* culture and/or on plant tissue.

Ferulic acid

The validation of anti-fungal properties of triterpenes via *in vitro* tests requires setting up specific protocols since they are difficult to dissolve and amend in the medium due to their waxy and very hydrophobic nature. So, it is challenging to study the triterpenoids in liquid

form or mixed with other nutritious media. As ferulic acid (FA) is broadly recognized as an effective anti-fungal against many pathogenic fungi, it was taken as control to test the protocol to be further used for the triterpenoids. Since we obtained the expected results for FA antifungal effects and the deposit was homogeneous without voids and clumps, we concluded that the protocol developed in this study was adequate to study the inhibitory effect of other complex compounds to be dissolved such as triterpenoids. For both *M. fructicola* and *M. laxa*, FA appeared to significantly inhibit colony development, sporulation, and germination of second-generation conidia compared to control. Our findings are in accordance with a recent study that brought out an anti-fungal activity of FA against *M. fructicola* (Hernández et al., 2021). However, their results should be taken with caution. Indeed, using a measure of medium turbidity as an indicator of fungal development seems risky since phenolic compounds such as FA and CGA interfere with the color of the mycelium.

Although the study by Fernández et al. (2011) reports the presence of FA in peach fruit, Oliveira Lino and colleagues (2020) did not detect FA in its free form; instead, it was found in the form of feruloyl quinic acid in leaves and feruloyl-2,3- dihydroxy-urs-12-en-28-oic acid in peach fruit. In conclusion, FA is a promising anti-fungal compound to fight against BR, but it does not seem possible to increase its content in peach to create resistant varieties against *Monilinia* spp. since it is hardly detected in peach fruit.

Triterpenoid compounds

Triterpenoids are major components of fruit cuticular waxes, among them ursolic acid (UA) and oleanolic acid (OA) being the most prevalent in peach (Ludeña-Huaman and Ramos-lnquiltupa, 2019) and nectarine (Oliveira Lino et al., 2020). The quantity of UA and OA may vary depending on the fruit stage and cultivar. For instance, in Zephir nectarine at stage II, the quantity of UA and OA reached very high levels of 95 and 35 $\mu g.cm^{-2}$, respectively. These amounts then decreased at later stages when fruit approached maturity (Oliveira Lino et al., 2020).

Particularly UA is considered as an active anti-fungal compound (Mahlo et al., 2013; Shai et al., 2008; Shaik et al., 2016; Shu et al., 2019), especially against *Alternaria alternate*, since it induces disturbance of membrane permeability and integrity, and intracellular ROS accumulation, resulting in the lysis of the pathogen (Shu et al., 2019). Regarding OA, fewer anti-fungal activity has been reported, for example, against *B. cinerea* and *S. sclerotiorum* (Zhao et al., 2013). In addition, betulinic acid (BA) also has antimicrobial and/or anti-fungal properties (Shai et al., 2008). BA is not broadly detected in peach fruit waxes except in small amounts in fruit peel of *P. davidiana* and some *P. persica x P. davidiana* hybrids (Oliveira Lino, 2016). In other plants, it has been found in limited amounts in grape berry and olive cuticular waxes; however, it is mainly found in the bark of the white birch *Betula alba* L (Yogeeswari and Sriram, 2010) and the cuticular wax of *E. globulus* fruit (Pereira et al., 2005). Through a kinetic study, Oliveira Lino et al. (2020) revealed that oleanolic and ursolic acids contents were strongly correlated with BR disease resistance in nectarine ("Zephir"). To our knowledge, oleanolic, ursolic, and betulinic acids had not been studied before concerning brown rot and this is the first assay against *Monilinia* spp. Our results were quite disappointing since no inhibitory effects could be highlighted against *M. laxa* and *M. fructicola* mycelial growth, sporulation, and germination of second-generation conidia at 2.0 mM concentration, for any of the triterpenoids tested. This could be due to fungi unable digest and utilize waxy triterpenoids , as previously reported in other fungi with apple cuticular waxes (Belding et al., 2000).

Fruit surface compound extracts

Generally, plant cuticle plays a vital role in plant-pathogen interactions, but the processes are poorly understood. A greater understanding of the mechanisms and the development of effective strategies to exploit plant cuticle for plant defense are needed (Ziv et al., 2018).

Many compounds can be found in peach and nectarine exocarp extracts, such as phenolics, flavonoids, hydroxycinnamates and flavonols, which appeared to be more present in the peel than in the pulp (Lara et al., 2015; Yeats and Rose, 2013). In particular, peach cuticle was found to be composed of 53% cutan, 27% waxes, 23% cutin, and 1% hydroxycinnamic acid derivatives (mainly ferulic and p-coumaric acids) (Fernández et al., 2011). The marked evolution of the composition of cuticular waxes in parallel with the evolution of resistance to *Monilinia* spp. during the stages I and II of peach fruit growth (Oliveira Lino et al., 2020), invited to validate a cause-and-effect link *in vitro*. The FSC extracts used in this study were primarily comprised of triterpenoid derivatives along with other minor compounds (Oliveira Lino et al., 2020), and the results showed no inhibitory effect of stages I and II of FSC extracts on *M. laxa* and *M. fructicola* growth and fitness, but a slight colony increase, especially for stage II.

As source of anti-fungal compounds, pomegranate skin extracts were shown to limit growth and conidia germination of *M. laxa* and *M. fructigena* (El Khetabi et al., 2020). Identical results were observed with orange peel extracts and combinations of polyphenolics on *M. fructicola* (Hernández et al., 2021). Such studies make it possible to assess synergies between compounds that may be fundamental to yield significant effects against the pathogen and reproduce results obtained directly on fruit. Unfortunately, testing stage II FSC extracts did not showed the results that the *in vivo* experiments suggested. Probably the resistance of the fruit at stage II not only come from FSC extracts and may involve other mechanisms that require fruit integrity.

Gene expression

Our follow-up objective was to investigate the effect of some compounds on the modulation of gene expression of *M. fructicola* in a medium (4 and 7 dpi) to long-term (11 dpi) exposure. A set of 12 genes were studied whose putative functions are regarded necessary to pathogenicity in *Monilinia* spp. and *B. cinerea*.

Appressorium formation and/or Penetration-related genes

MfBMP1, *MfPLS1* homologs to *BMP1* and *PLS1,* respectively, have been reported to play a critical role in the host penetration and appressorium formation in *B. cinerea* (Doehlemann et al., 2006; Gourgues et al., 2004; Zheng et al., 2000). The *MfPLS1* gene, which encodes a tetraspanin-like protein, has been identified in many plant pathogens associated directly with host plant penetration (Clergeot et al., 2001; Gourgues et al., 2004; Siegmund et al., 2013; Veneault-Fourrey et al., 2005). Another study reported a close association between *MfPLS1* and NADPH oxidase complexes (NoxR) in *B. cinerea* and their role in regulating penetration peg emergence (Siegmund et al., 2013). Regarding the *BMP1* gene, the mutant of *B. cinerea* was unable to infect (penetrate) intact or wounded host tissue, although its growth on the medium was not affected (Gourgues et al., 2004; Zheng et al., 2000). These results testify to the difficulty of studying these genes whose expression is regulated by the surface of the fruit by the pathogen. This may explain the low expression observed in our experiments, even in the control cultures (data not shown). Indeed, appressorium formation and/or penetration-related genes should be up-regulated when the fungus recognizes the fruit surface. Plate cultures and single compounds added may not constitute actual signals to trigger the expression of these genes. On the contrary, stage I FSC extracts only provoked an up-regulation of these two genes. We can thus infer that stage I FSC extracts contained promoting compounds inducing the penetration/appressorium formation that were no longer present in stage II FSC extracts. These conditions of low gene expression do not make it possible to explore the effect of the compounds. Extra studies are required to fully unravel the effect of the compounds studied. Probably developing tests on fruits could overcome the lack of recognition by the pathogen and resolve the lock linked to the low expression of genes under control conditions.

Cell wall degrading enzymes (CWDEs)

The cell wall of plants comprises many different layers with various compositions, i.e., pectin, cutin, cellulase, protecting the plant from biotic and abiotic stresses. Plant pathogenic

fungi produce extra-cellular enzymes that can degrade plants' cell wall components. Fungi can digest plant cell wall polymers to obtain an important nutrient source and enables cell penetration and spread through plant tissue. Many studies have been focused on determining the role and importance of extra-cellular cell wall-degrading enzymes (CWDE) related to the virulence of plant pathogenic fungi (Brunner et al., 2013; Kubicek et al., 2014; Li et al., 2020; Quoc and Bao Chau, 2016).

Cutin has been shown to up-regulate genes involved in penetration of host tissues, whether from leaves or fruit. *Monilinia* needs to induce cutinase to break down the fruit's cutin (Bostock et al., 1999; Chiu et al., 2013; Wang et al., 2002). Indeed, cutinase is both involved in the penetration of the host cuticle at the early infection stage and in the degradation of a large amount of cuticle during lesion expansion (Yu et al., 2017). When *MfCUT1* is overexpressed, the pathogenicity of *M. fructicola* rises (Lee et al., 2010). Early studies on CGA and caffeic acid reported their ability to either down-regulate the expression of the *MfCUT1*, an *M. fructicola* gene encoding a cutinase enzyme (Wang et al., 2002), or inhibit the cutinase activity (Bostock et al., 1999). Therefore, *MfCUT1* was given special attention in our study. At the three incubation periods (4, 7, 11 dpi), we detected an attenuation of *MfCUT1* with 2.0 mM CGA concentration which may slow down the fungal penetration. On the contrary, we observed overexpression of *MfCUT1* with betulinic and oleanolic acids and may speculate that these compounds were recognized as cuticle compounds, thus their presence promoted pathogenicity.

Pectinase-related Genes

Pectin is one of the main components of the middle lamella, acting as a dynamic matrix embedding the cellulose-hemicellulose primary cell wall and providing strength and support to fruit (Caffall and Mohnen, 2009; ten Have et al., 2002). Pectin also influences cell-wall properties and texture in fruit (Brummell et al., 2004). In this context, enzymatic degradation accomplished by pectinases is of particular interest due to their ability to weaken the cell wall,

causing tissue maceration, the characteristic symptom of soft rot diseases (Walton, 1994). Indeed, further evidence of the importance of pectin-degrading enzymes was provided by Blanco-Elate et al. (2014), who showed that endo- and exo-PGs and RGases were the most abundant carbohydrate-active enzymes (CAZymes) among these expressed during infection of lettuce leaves, tomato and grape berries by *B. cinerea*.

MfRGAE1 codes for a protein member of the rhamnogalacturonan acetylesterase (RGAE) protein family. Among the pectin-dependent related genes, *MfRGAE1* has been previously studied and reported to be pectin dependent in *M. laxa* (Rodríguez-Pires et al., 2020). *MfRGAE1* was considerably up-regulated in the presence of 2.0 mM chlorogenic, ferulic, and oleanolic acids in our investigation, even though no pectin was supplemented to the medium. Consistent with the literature, the overexpression of *MfRGAE1* was higher at shorter incubation time (4 dpi) than at longer. Contrary to these three compounds, ursolic and betulinic acids had limited effect on this gene expression. Therefore, it would seem these different acids are not plant cuticle-related indicators and do not play the same role in the activation of cutinase synthesis. Surprisingly, in the presence of FSC extracts, *MfRGAE1* expression was only slightly modulated. Moreover, the regulation of *MfRGAE1* was opposite between the two FSC extracts since it was significantly overregulated with stage I FSC extracts while down-regulated with stage II FSC extracts. Minor compounds differentially present in these extracts probably play a role in recognizing the cuticle by the pathogen and the activation of cutinase genes.

Three genes from the endoPG family were studied, namely *MfPG1*, *MfPG3*, and *MfPG6*. It was reported that the overexpression of *MfPG1* in *M. fructicola* reduced the lesion infection (Chou et al., 2015). In our study, *MfPG1* was significantly overregulated at 4 dpi in the presence of 2.0 mM CGA. We could speculate that one reason why CGA correlates well with disease resistance could be its promoting effect on *MfPG1* expression. The function of the

other two genes, *MfPG3* and *MfPG6,* is less known apart from the fact that they have been associated with high pHs in the medium (De Cal et al., 2013). They were significantly up-regulated with FSC extracts. *MfPG6* was also significantly overexpressed with oleanolic acid.

MfLAE1 gene was significantly up-regulated by the stage II FSC extract corresponding to the period when the fruit is resistant to *Monilinia* spp. This up-regulation might be due to the higher concentration of OA in FSC extract at stage II than at stage I since comparable overexpression was observed in the presence of OA alone. In *B. cinerea*, the deletion of *LAE1* gene resulted in mutants with reduced pathogenicity, light-independent conidiation and sclerotia formation loss (Schumacher et al., 2015; M. Z. Zhang et al., 2020). In another fungus, i.e., *Trichoderma reeseimm*, was shown that the putative protein methyltransferase *LAE1* controls cellulase gene expression (Seiboth et al., 2012).

Finally, *MfOfd1* gene is a redox-related gene linked with stress response and virulence of *M. fructicola* (M. M. Zhang et al., 2020). In their study, the knockdown of the *MfOfd1* gene did not affect mycelial growth but resulted in a decline in conidiation and modification of the osmotic stress signal transduction pathway and tolerance to oxidative stress. In our study, this gene was significantly highly overexpressed at 7 dpi in the presence of FSC extracts from stage I, corresponding to the susceptible fruit stage.

4.5. CONCLUSION

Among compounds studied, only ferulic acid showed effective inhibitory effects on *M. fructicola* and *M. laxa* growth, while chlorogenic acid effect was limited to sporulation reduction. However, the gene expression study strongly suggested that chlorogenic acid and other compounds could play a significant role in the fungi-plant interactions during the infection process. The tested compounds modulated some genes essential for *Monilinia* spp. infection in a two-way manner, with some acting as infection inhibitors and others acting as promotors. The genes modulated are involved in different fungal functions linked to growth or infection. Since a particular compound may slow down/inhibit only one step of fungal infection, the concept of generalized infection inhibitor needs to be reassessed. It could be split into distinct specialized features such as germination, penetration, growth, sporulation and more based on the infection steps. Two consequences flow from this. First, aside from enlarging the list of genes to study in connection with candidate compounds, other features such as appressorium or peg formation, fungal melanization, and sclerotia production are other important fungal characteristics that may be modulated by the compounds to investigate in the future. Second, the solution to reduce fruit infection by *Monilinia* spp. may lie in breeding varieties capable of synthesizing a cocktail of compounds, each acting on a specific function of the fungus.

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CHAPTER 5. Genome-wide QTL analysis for Brown rot (*Monilinia laxa*) resistance in F$_2$ progenies from "Contender" × "Elegant lady"

Abstract:

Brown rot (BR), caused by *Monilinia* spp., causes significant pre-and post-harvest losses in stone fruit production, particularly peach. This chapter investigates the genetic background responsible for disease resistance in peach by detecting Quantitative Trait Loci (QTL) and attempts to identify molecular markers for assisted selection (MAS) in peach. This study investigated phenotyping fruits on tree for disease susceptibility and other fruit quality parameters were considered. Three F$_2$ progenies, derived from three selfied F1 selections obtained from "Contender" (C, resistant) × "Elegant Lady" (EL, susceptible), were studied for two seasons (2019 and 2021). The whole progeny was genotyped by Single-Primer Enriched Technology (SPET) and a recently developed 18K SNP array. The genome-wide QTL analysis showed intriguing areas relevant to disease resistance, mainly the QTLs on chromosomes 2 and 4, which may be candidates for future MAS applications. Several other QTLs were detected for other fruit quality traits, including maturity date, soluble solid content and fruit weight.

Keywords:

Brown rot; Disease resistance; fruit quality; *Monilinia laxa*; Marker-assisted selection (MAS); QTL analysis.

5.1. Introduction

Brown rot (BR), caused by *Monilinia* spp., is a devastating fungal disease that affects peaches and nectarines worldwide. Any of the three *Monilinia* species, *M. laxa*, *M. fructicola*, and *M. fructigena*, can cause it (Byrde & Willetts, 1977). Brown rot causes direct yield losses by infecting flowers (flower and twig blight) and fruit rot at preharvest and post-harvest. Post-harvest losses are usually more severe than preharvest losses, and they often occur during storage and transportation, sometimes even harming fruit during processing (Byrde & Willetts, 1977; Hong et al., 1998). However, decaying fruits generally account for most peach losses, even though damage can also be severe in some cases on shoots and blossoms (Zehr, 1982). The mainstay of the disease's control strategy is the application of fungicides, which BR is the primary reason for fungicide usage. In peach orchards, the amount of applied fungicides can be very high when weather conditions are favorable to infection (Yoshimura et al., 2004).

In addition, today's concern about health problems caused by chemical residues has increased chemical-free products forward (Byrne, 2005). It has been reported that the pathogen has gained resistance to dicarboxamides, benzimidazoles (BZIs), and sterol demethylation inhibitor (DMI) fungicides (Luo & Schnabel, 2008) and that it may adapt to more fungicides in the future. This causes scientists and producers to become increasingly concerned, prompting them to look for potential solutions to these issues. On the other hand, fertilizer, and frequent fungicides application, are thought to taint fruit, causing customers to complain about flavorless fruits. BR caused by *M. laxa* has recently become a bottleneck for stone fruit production in Europe due to increased demand for high-quality fruit and a progressive decline in the quantity of accessible fungicides due to legislation (Rungjindamai et al., 2014). Consequently, cultivars that are less sensitive to brown rot are suggested to decrease fungicide applications. Despite the highlighted concerns, critical factors directly contributing to BR disease development are thoroughly discussed in Chapter 2, including main favorable

environmental conditions, cultivar susceptibility, cultural practices, and different fruit characteristics.

Bolinha has been recognized as a resistant cultivar to BR and used as a resistant donor in some breeding programs (Feliciano et al., 1987; Fresnedo-Ramírez et al., 2017; Fu et al., 2018, 2021). However, these resistant cultivars have several defects, such as tiny, unmarketable fruit, thick skin with a prominent fuzz, high phenolic, and high enzymatic browning. Phenolic acids, including chlorogenic acid, caffeic acid and triterpenoids have been correlated to disease resistance during fruit development stages in peach; for more details, see chapter 3.

Only a few research on QTLs for BR disease resistance have been published, and in most cases, bi-parental segregating progenies were used for the analysis. BR resistance in peaches was previously reported to be quantitative (Baró-Montel et al., 2019; Fu et al., 2021; Martínez-García et al., 2013; Pacheco et al., 2014), implying that linked genetic information is spread over several genes (polygenic). Time-consuming and cost of field-grown segregating trees impede BR-resistant breeding projects. Therefore, a crucial objective is to develop new strategies for early seedlings selection with higher BR resistance. For these objectives, marker-assisted selection (MAS) could assist the traditional plant breeding approach, making the selection process more efficient, effective, reliable, and cost-effective (Collard et al., 2005). QTL mapping on populations from biparental crosses has been used to uncover such molecular markers in *Prunus* (Pacheco et al., 2014).

Martínez-García et al. (2013) performed a preliminary QTL analysis on Pop-DF, a peach population obtained from crossings between a susceptible cultivar 'Dr. Davis' and the resistant 'F8,1–42', a peach introgression line derived from an almond × peach interspecific hybrid. The study, which used *M. fructicola* for inoculation, revealed three QTLs, two of them in linkage group (LG)1 and one in the LG4 of *Prunus* genome, which QTL regions comprised two potential candidate genes coding for PAMP-triggered immunity, and effector-triggered

immunity (ETI) proteins. In another study, QTL analysis was performed on the F1 progeny from the cross between commercial cultivars Contender (moderate field resistance) × Elegant lady (highly susceptible) associated with skin resistance mapped on linkage group LG2, and flesh resistance on LG3 by a *Monilinia fructigena* isolate (Pacheco et al., 2014). The evaluation of interspecific BC1 population generated from the almond and peach varieties 'Texas' × 'Earlygold' revealed 12 QTLs associated with BR resistance. Except for LG1 and LG3, the location of these putative QTLs conferring BR resistance was found in all LGs. However, according to the authors, no consistent QTLs were detected during the two phenotyping seasons, although two QTLs mapped in G4 were near stability (Baró-Montel et al., 2019). Unlike previous studies, recently Fu et al.(2021) applied a genome-wide association study on 26 cultivars and progeny from 9 crossings using 'Bolinha' as a source of tolerance in their new study. Except for chromosome 3, the study found 14 single nucleotide polymorphisms (SNPs) significantly correlated with BR infection responses to *M. fructicola*, with 10 SNPs related to peach peel and 4 SNPs to flesh throughout the whole genome.

The knowledge about this complex trait (BR disease resistance) is increasing. However, some critical factors may lead to variations in QTL results (Chapter 2)(Pacheco et al., 2014):

- Phenotyping processes or protocols, including environmental issues for the disease and plant, are critical to be considered.

- The genetic backgrounds of the analyzed populations.

- The statistical approaches and models used to discover the QTLs.

Specific genetic backgrounds seem responsible for susceptibility of skin and flesh, implying that the genes that cause susceptibility in the skin are different from those that cause susceptibility in the flesh (Pacheco et al., 2014). Conversely, flesh resistance or, more realistically, a delay in disease colonization is an unattainable target since wounded fruits get

infected and colonized by *Monilinia* regardless of the cultivar and incubation condition (see chapter **2** for more details).

This section of the project aimed to identify molecular markers associated with BR resistance to increase and underpin a better understanding of the genetic basis for this complex trait. Therefore, a linkage map based on SNP markers was constructed for the "C×EL" F_2 population, and QTL analyses were performed with phenotypic data from two years of artificial inoculation experiments and naturally occurring BR disease as well. Other traits were also recorded and used for correlation analysis.

5.2. Material and methods
5.2.1. Plant materials
The population used in this study encompassed 273 F_2 siblings derived from the selfing of three F1 selections, namely BO92038140, BO92038071, and BO92038046 (Bassi et al., 1998), in turn originated from the cross of Contender and Elegant Lady peaches(Figure 5.1). Contender is a freestone, yellow fruit of around 180 g with a melting, high-quality melting flesh, round shape, resistant to browning, and moderately tolerant to brown rot in the field (partial resistance); it ripened the first week and the second week of August, in 2019 and 2021, respectively. Fruits were ripened by the second and third week of August in 2019 and 2021, respectively (Figure 5.2). Elegant lady is an attractive freestone, yellow peach of about 170 g with a melting, high-quality flesh, round shape, very susceptible to brown rot in the field. Trees were grafted on 'GF677' rootstock and planted in 2016 at 1 × 4 m with two to three replicates per seedling at the MAS.PES peach germplasm collection located in the experimental farm 'M. Neri' in Imola (Emilia-Romagna region, northern Italy). Trees were managed according to standard cultural practices. Fruits were thinned within 40–60 days after blooming and setting a crop load proportional to tree vigor. No fungicides were applied.
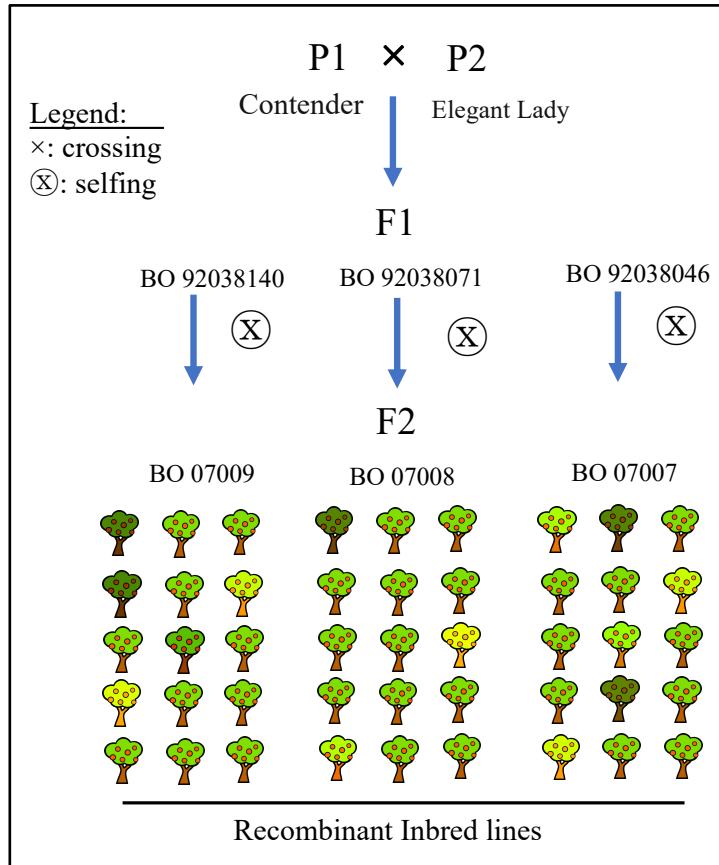
Figure 5.1: Pedigree of the F$_2$ population structure derived from Contender (seed parent) × Elegant lady (pollen parent).

Figure 5.2: Fruit of the two parents Contender (left) and Elegant Lady (right) at full maturity.

5.2.2. Phenotyping procedures

Inoculum preparation

For artificial inoculation, *M. laxa* was isolated from an infected peach fruit in 2019. After purification, the culture was maintained on PDA or V8 juice agar. Species-specific primers were used to confirm the *M. laxa*, according to Hughes et al. (2000).

For the inoculum, disinfected peach fruits were inoculated by mycelial plugs and incubated at 23 ±2 °C with 80% RH for 7 days. Conidia suspension was prepared before each application by washing off artificially infected fruits with sterile aqueous 0.05 % Tween 20. Afterward, the suspension was added to a tube containing glass beads and agitated for 1 min to dismantle conidial chains. Subsequently, the suspension was passed through a falcon strainer with a pore size of 40 μm to remove mycelia parts in the suspension. Malassez counting chamber was used to determine conidia enumeration and adjusted to 10^5 conidia/ml^{-1} at final concentration.

Physiological maturity determination of fruit

Trees were considered ready to inoculate when nearly 60% of the total fruits reached physiological maturity by the *Index of Absorbance Difference* (I_{AD}) (Ziosi et al., 2008), measured non-invasively in the field with the portable ΔA-meter instrument (Synteleia S.R.L., Italy). Ten fruits per cultivar were investigated every week, and a value of lower than 0.7 I_{AD} was set as a threshold for an individual's readiness to be inoculated. Fruits with deferred maturity were dropped.

Artificial inoculum application

Based on fruit availability, nearly ten intact and healthy fruit per accession were inoculated on tree with 10^5 conidia/ml^{-1} by a hand sprayer until runoff. Five fruits per individual were mocked with distilled water with few drops of 0.05 % Tween 20. All Inoculated fruits were wrapped with paper bags (20 x 50 cm size) to optimize the condition for *Monilinia* development (Figure 5.3).

Infection in the field

After seven days of incubation, bags were checked for infection presence. Fruits infected were recorded for both artificial inoculation and control. The degree of susceptibility of a cultivar not inoculated in the natural state was then determined by recording the number of fruits hanging on the plant (both infected and uninfected).

Peach fruit quality parameters

On the day of BR inspection in the field, ten representative unbagged fruits were randomly picked from each progeny. Fruits were assessed for weight (FW) and soluble solid content (SSC). Fruit weight was measured by a precision scale and the I_{AD} was recorded once more while measuring the fruit quality traits. A hand-held refractometer (Atago, Milan, Italy) was used to quantify the total amount of sugars in fruit cheeks as solid-soluble content (SSC%).
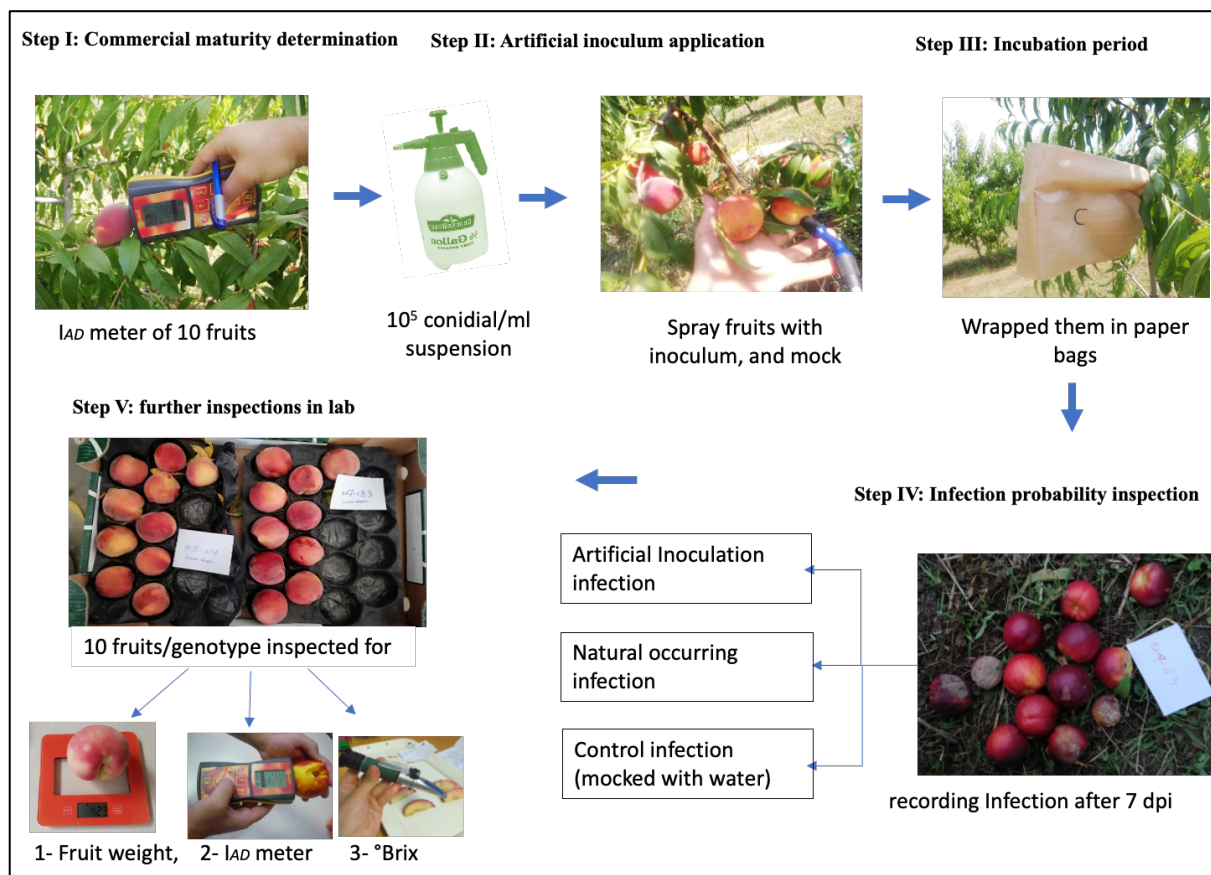

Figure 5.3. The steps for phenotyping field BR incidence and fruit-quality parameters in the laboratory.

Phenotypic Data Analysis

Descriptive statistic of data was implemented in R environment (version 4.1.1). Correlation between traits was analyzed by Spearman's correlation coefficient (ρ) and corrplot

matrixes were generated with Package "corrplot" version 0.91 (Wei et al., 2017). Histograms were produced in R base and tested using the Shapiro–Wilk test ($p < 0.05$). The principal component analysis (PCA) was produced using the package "factoextra" (Kassambara, 2020). Because most traits had low correlations between 2019 and 2021, QTL analysis was done for each year separately or combinedly (data average for both years).

5.2.3. DNA extraction and quality inspection

Genomic DNA was isolated from young leaves collected from the parents and the entire progeny following Doyle & Doyle (1987), with minor modifications. Briefly, leaves (100 mg) were lyophilized and ground into a powder in a 2 ml Eppendorf containing glass beads then shacked for 40 seconds. Dried ground samples were supplemented by 800 μl of extraction buffer (mix of 49 ml 3% CTAB and 1 ml of pure 2-mercaptoethanol) and then incubated in a water bath at 65 °C for 30 minutes. Tubes were inverted by hand every 10 min to mix the contents during the incubation time Then, 800 ul of Chloroform/Isoamylalkohol (24:1) mixture were added and thoroughly mixed. After cooling at room temperature, samples were centrifuged at 14,000 rpm for 15 minutes at 4 °C. The supernatant was transferred into new tubes containing 500 μl of 100% Isopropanol. Samples were incubated at –20 °C for 20 minutes to precipitate the DNA. The tubes were then centrifuged as described previously for 40 minutes, and the resulting DNA pellets were washed twice with 70% ethanol and dissolved in 120 μl and stored in autoclaved ultra-distilled water. DNA concentration and purity were measured spectrophotometrically with a Genesys 180 spectrometer (Thermo Scientific, USA) using Hellma TrayCell cuvettes (Hellma GmbH, Germany). DNA samples were stored at –20 °C until genotyping.

5.2.4. Genotyping and GWAS analysis

The studied C×EL progeny and parents were genotyped by Single Primer Enrichment Technology (SPET). Polymorphisms were selected in order to include the recently developed 18K SNP array peach, the IPSC peach 9K SNP array (Verde et al., 2012) and a 9K SNPs add-

on generated from high-coverage whole-genome resequencing data (Gasic et al., 2019). Additional probes were added to reach a total number of 25K polymorphic sites, selected by peach genome data from the PeachVarDB database (Cirilli et al., 2019). A subset of individuals (32) was genotyped with 18K SNP chip array only. In order to homogenize the two genotyping systems, only the SPET SNPs in the 18K SNP array were used. For SNP marker positions, the Peach Genome Assembly V2.0 was taken as a reference (Verde et al., 2017). After removing markers with a missing rate of 10% or a minor allele frequency (MAF) of more than 5% from the genotyping data, a total of 13,628 high-quality SNPs were left for GWAS analysis.

Single-marker QTL analysis was performed in R using the package "GAPIT3" (Lipka et al., 2012). Four algorithms, "GLM", "MLM", "CMLM", "FarmCPU", and "Blink", were compared for each trait; which best algorithm was Fixed and random model Circulating Probability Unification (FarmCPU) (Liu et al., 2016) for our data set and maintained for GWAS analysis and the final outputs. Significance thresholds were calculated by random permutation test (PT) with 1000 replicates considering the genome-wide LOD scores corresponding to $p = 0.05$.

5.3. Results
5.3.1. Phenotypic results
Individuals from the C×EL F$_2$ progeny were phenotyped in two seasons: 219 individuals in 2019 and 194 in 2021, with 154 being repeated in two seasons. However, due to a distractive frost in late spring, no fruit were available in the summer of 2020 for phenotyping.

Distribution of partial resistance phenotype and maturity date
To determine the degree of resistance of the progenies, three parameters of fruit infection in the field were considered: artificially inoculated infection, natural field infection (NFI) and control (with water). Figure 5.4 shows the distribution of brown rot (BR) disease incidence and maturity date throughout two years of phenotyping.

Generally, disease resistance parameters behaved identically in both years, and they were highly skewed towards (0 % infection) and were not normally distributed according to the Shapiro–Wilk test (Table 5.1).
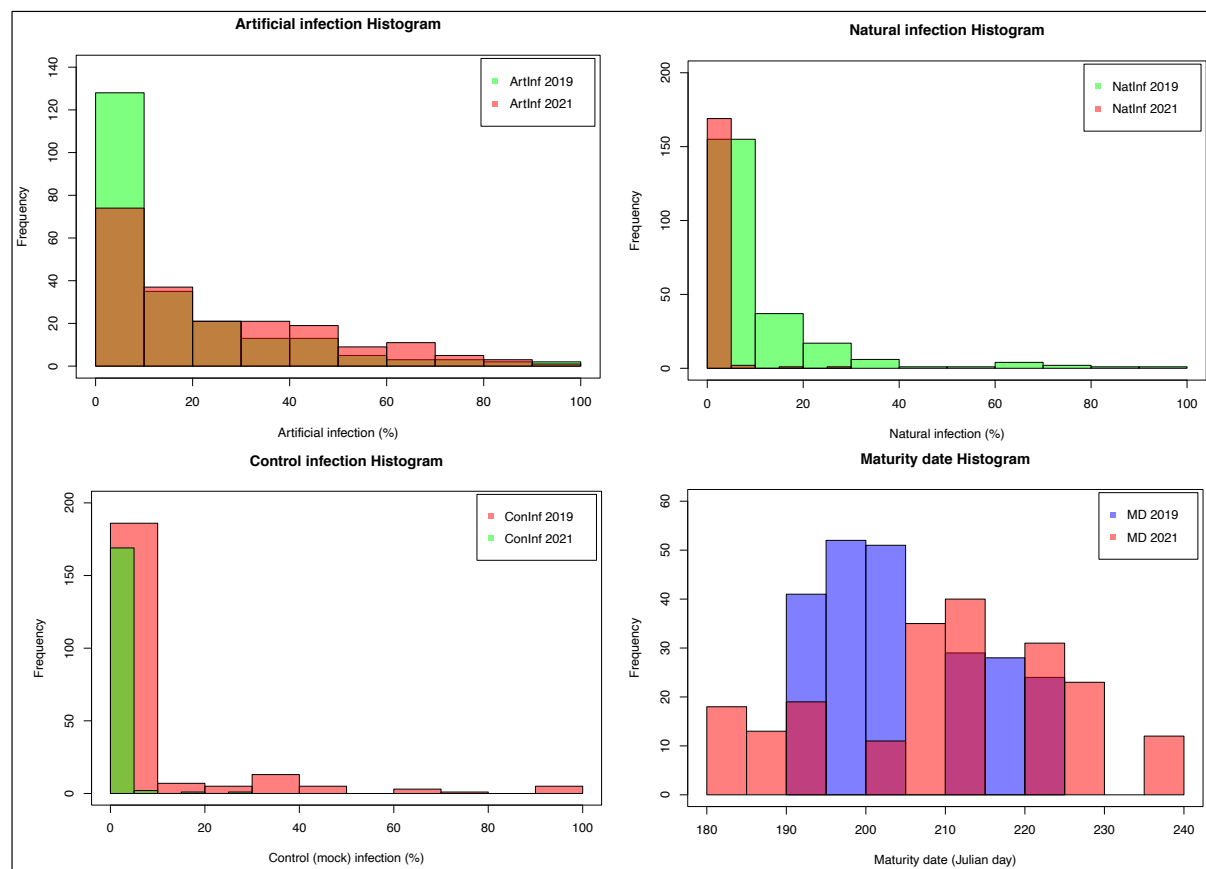

Figure 5.4. Progeny distribution for f brown rot disease incidence parameters and maturity date for the season in 2019 and 2021. Normality and density curves represent the data distribution across seasons of phenotyping.

Distribution of quality traits

Distributions of quality traits recorded for two years of phenotyping are presented in Figure 5.5. Soluble solid content and fruit weight showed a normal distribution in both seasons, according to Shapiro–Wilk test (Table 5.1). SSC ranged between 8 to 23 °Brix, depending on the seedling, while FW was extended from 60 to 165 grams.
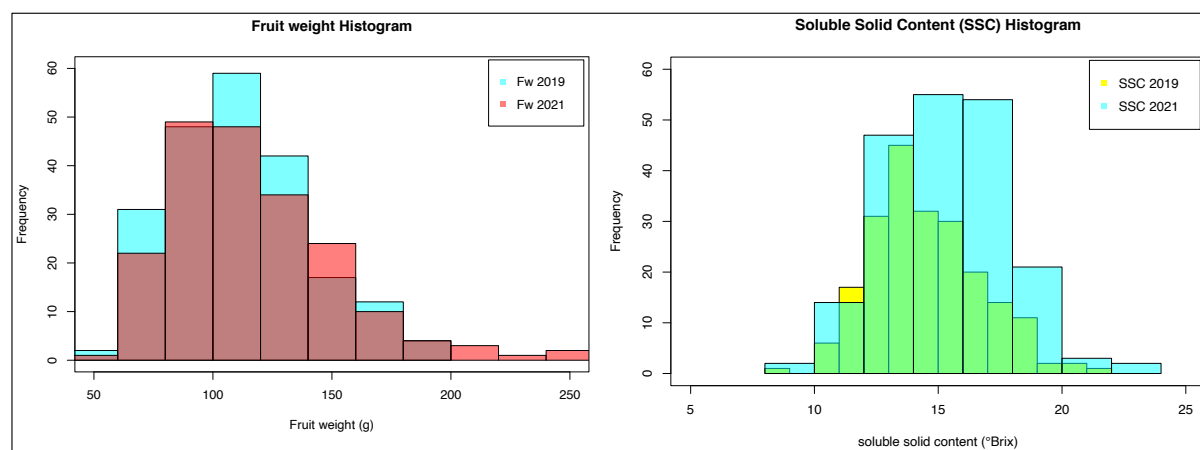
Figure 5.5. Progeny distribution for fruit weight and soluble solid content traits for the years 2019 and 2021.

Table 5.1. Shapiro-Wilk Normality Test of Wilk and p-values for the recorded traits. Lower p-values at <0.05 indicate non-normality.

Names	Wilk-values	P-values
Partial resistance phenotype, Maturity date and Fruit quality traits		
Artificial inoculated infection: Artinf.19	W = 0.75405	p-value = 1.131e-14
Artificial inoculated infection: Artinf.21	W = 0.90096	p-value = 1.265e-08
Control (mocked fruit) infection: ConInf.19	W = 0.4002	p-value < 2.2e-16
Control (mocked fruit) infection: ConInf.21	W = 0.42983	p-value < 2.2e-16
Naturally field infection: Natinf.19	W = 0.6464	p-value < 2.2e-16
Naturally field infection: Natinf.21	W = 0.14553	p-value < 2.2e-16
Maturity date: MD.19	W = 0.90723	p-value = 2.931e-08
Maturity date: MD.21	W = 0.94722	p-value = 1.712e-05
Fruit weight: Fw.19	W = 0.97268	p-value = 0.005365
Fruit weight: Fw.21	W = 0.92799	p-value = 7.762e-07
Soluble solids content: SSC.19	W = 0.98821	p-value = 0.2713
Soluble solids content: SSC.21	W = 0.99297	p-value = 0.6802

5.3.2. Correlation of the BR resistance and fruit quality

For the individuals phenotyped throughout the two seasons (2019 and 2021), the non-parametric correlation coefficient between disease resistance and other fruit quality parameters

was calculated. In 2019, a strong positive correlation ($\rho= 0.65$, *P-value* of 0.001) between artificially inoculated infection and natural field infection was shown, as well as between artificially inoculated infection and control infection. The correlation between field infection and control infection was also significant. Moreover, the correlation between artificially inoculated infection and control infection with *Index of Absorbance Difference* (I_{AD}) was negative $\rho = -0.33$ and $- 0.29$ with a *P-value* of 0.01, respectively (Figure 5.6).

In 2021, no relationships among the three disease resistance measurements were found. Indeed, very few fruits were infected naturally and in control compared to 2019. Despite the low infection level in 2021, a negative correlation was observed between the artificially inoculated infection and the natural field infections (NFI) with maturity date. Furthermore, there was a positive association between the infected control fruit and fruit weight.

Comparing the two seasons, correlation among the three disease resistance measurements was very weak or insignificant (Figure 5.6). Regarding fruit quality traits, maturity date and soluble solid content (SSC) were positively correlated in both seasons. The maturity date between 2021 was 2019 was correlated statistically with $\rho = 0.62$ and *P-value* $=0.001$. In 2021, there was a negative correlation between fruit weight against I_{AD} and SSC at $\rho = -0.55$ and -0.55, *P-value* $=0.001$, respectively (Figure 5.6).
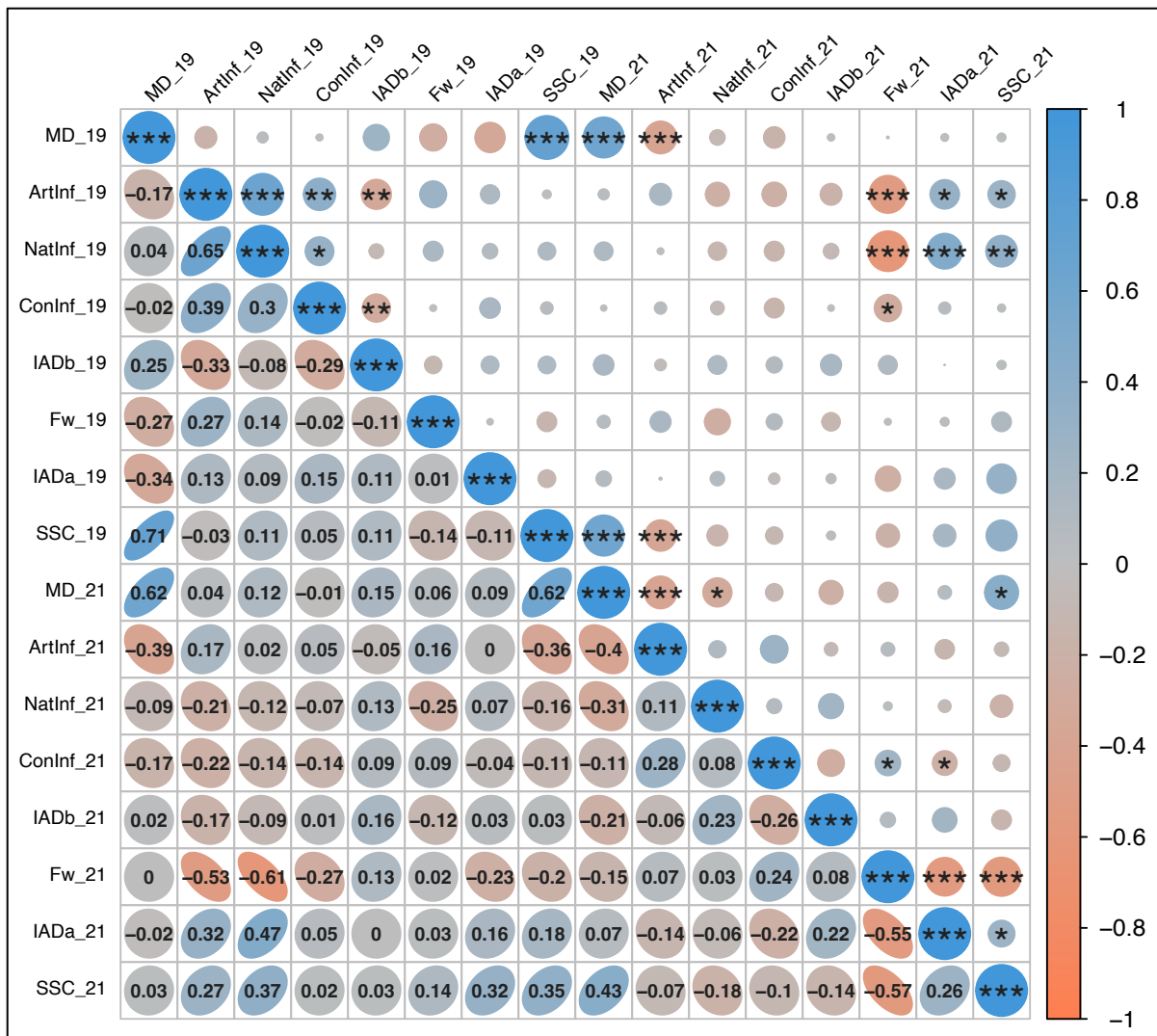
Figure 5.6. Heatmap correlation matrix for non-parametric Spearman's correlation coefficient among infection phenotypes and fruit quality traits recorded during harvesting season 2019 and 2021. Significance: 0.001***, 0.01**, 0.05*. The following abbreviations were assigned; MD, maturity date, Artinf, Artificial infection; NatInf, natural field infections; ConInf, Infection in control, IADb and IADa, *Index of Absorbance Difference* (I_{AD}) before inoculum application and at inspection date of the infection, respectively; SSC, soluble solid content (°Brix), and FW, fruit weight

5.3.3. Principle component analysis

The principal component analysis (PCA) was performed on the sub-dataset of combined (averaged) data from the two seasons (2019 and 2021) with 273 individuals for the disease resistance measurements and fruit quality traits. The first two principal components have captured the variance of 29.5% and 20.8%, respectively. Maturity date and SSC contributed the most, and other disease resistance phenotypes appeared with a lower contribution in the Dim1. Fruit weight, on the other hand, had a much smaller impact, as seen in Dim2 (Figure 5.7).
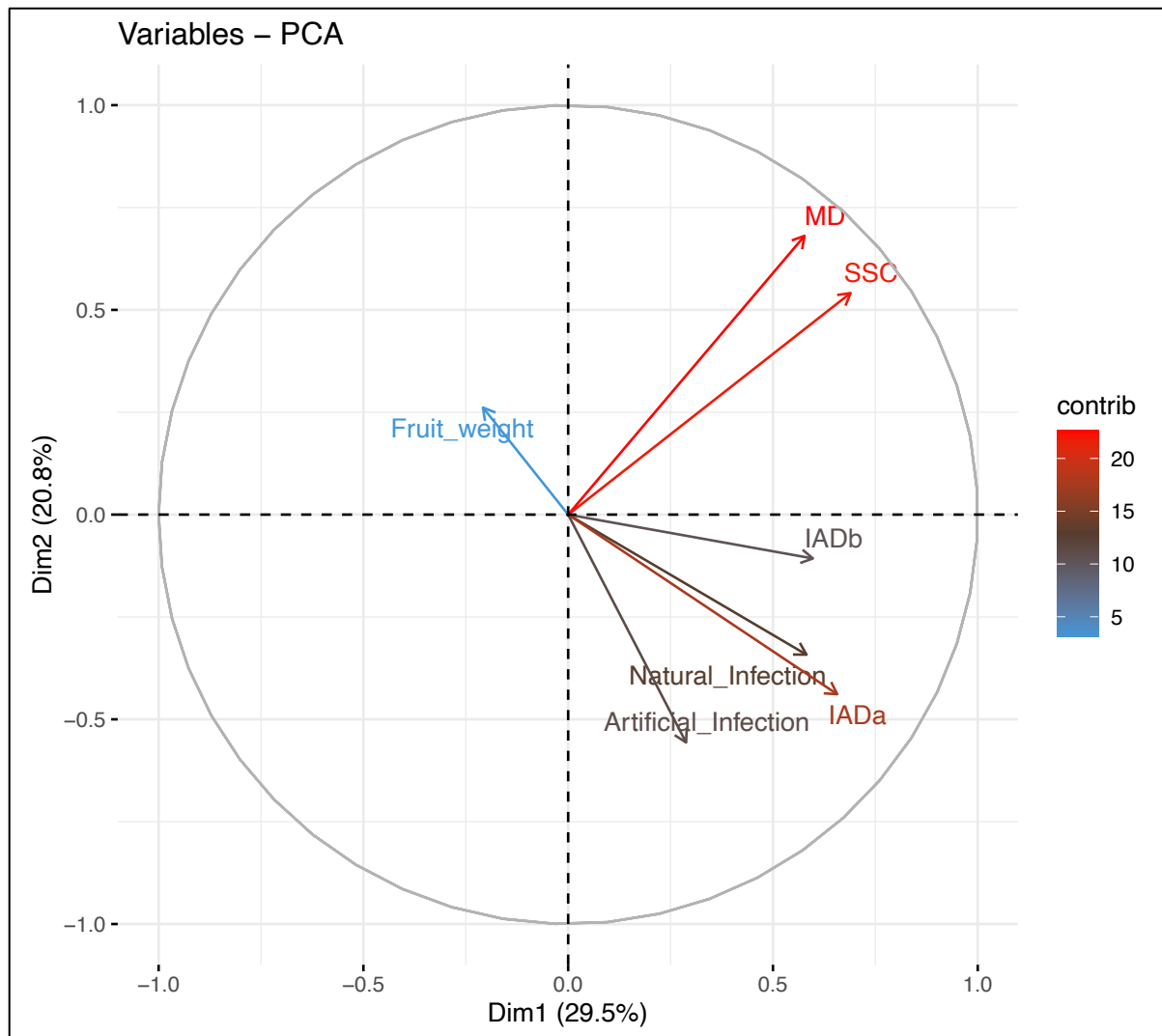
Figure 5.7. Principal component analysis represents the relation between disease-resistant phenotypes and fruit quality traits. MD, maturity date, Artinf, Artificial infection; Natinf, Natural infection; ConInf, Infection in control, IADb and IADa, measurement of Index of Absorbance Difference (IAD) before inoculum application and at the inspection date of infection, respectively; SSC, soluble solid content (°Brix), and FW, fruit weight.

5.3.4. Genome-wide QTL Analysis
Disease resistance QTLs

Between the two years, different disease resistance loci were detected. Altogether QTLs were detected on all chromosomes (Chr) except Chr 6. In 2019, seven QTLs were detected for artificial infection (Figure 5.8), while in 2021, only one major QTL locus was detected on Chr 2 with SNP181763. Only the naturally field infection (NFI) observation in 2019 was considered for QTL analysis since poor infection was detected in 2021, which revealed five QTLs on 2, 3, 5, and 8 chromosomes. The QTL found on Chr 8 (SNP12342) was constant with the artificial infection and NFI measurements in 2019 (Figure 5.8).

Figure 5.8. Manhattan and QQ plots of -log *p* values estimated from BR fruit infection observations for both artificial and natural field infection in 2019 and 2021 using FarmCPU algorithm.

Table 5.2. Quantitative trait loci (QTL) were detected for brown rot disease resistance and some fruit quality traits.

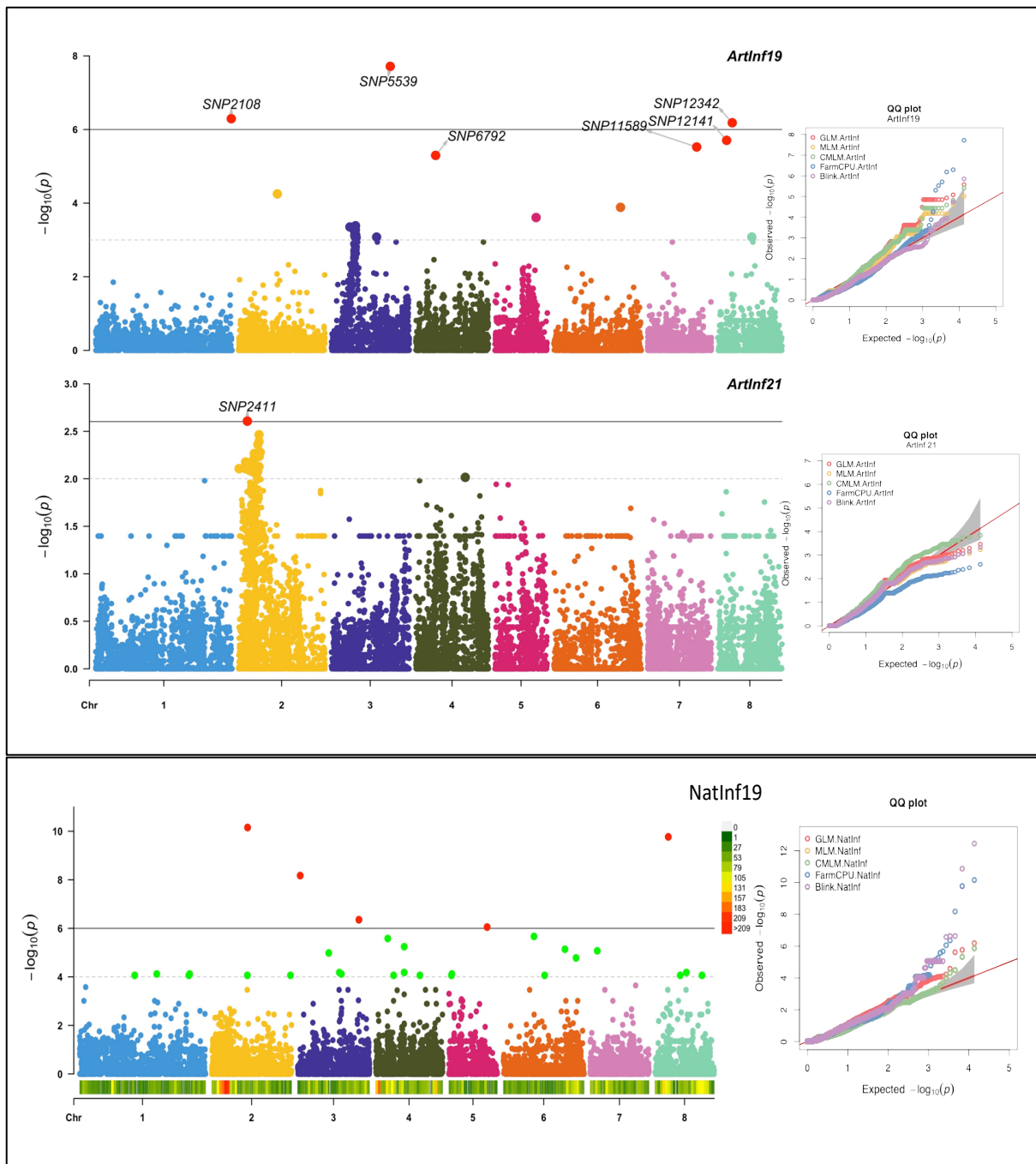| Trait	Year	SNP	Chr	position	*P*value
Artificial infection	2019	SNP5539	3	20688433	1.93E-08
		SNP2108	1	47593157	5.09E-07
		SNP12342	8	5096159	6.55E-07
		SNP12141	8	3135398	1.97E-06
		SNP11589	7	17197891	2.99E-06
		SNP6792	**4**	**7025432**	**5.05E-06**
		SNP3603	2	13669298	5.62E-05
	2021	**SNP2411**	**2**	**3136339**	**2.47E-03**
Naturally occurred infection	2019	SNP3603	2	13669298	7.01E-11
		SNP12342	8	5096159	1.70E-10
		SNP4491	3	1041498	6.71E-09
		SNP5722	3	23305031	4.42E-07
		SNP8810	5	14599271	8.89E-07
Maturity date	2019	**SNP7110**	**4**	**11108143**	**3.15E-28**
		SNP9097	6	3534150	1.87E-07
	2021	**SNP7110**	**4**	**11108143**	**1.32E-38**
		SNP11523	7	15730605	4.47E-13
Soluble solid content	2019	**SNP7110**	**4**	**11108143**	**4.01E-13**
		SNP8846	**5**	**15796862**	**5.72E-13**
		SNP1299	1	29637780	1.07E-06
	2021	**SNP7110**	**4**	**11108143**	**1.96E-11**
		SNP8884	**5**	**16786885**	**2.16E-07**
Fruit weight	2019	SNP1010	1	22970797	2.77E-05
		SNP8849	5	15861398	5.54E-05
		SNP3173	2	7155329	8.03E-04
	2021	SNP9059	6	2980381	1.47E-04
		SNP3764	2	16477856	4.90E-04
		SNP11436	7	14160041	5.13E-04
Skin hairiness (nectarine/peach)		**SNP8884**	**5**	**16786885**	**2.9E-20**

* SNP, single nucleotide polymorphism; Chr, chromosome.

Fruit quality QTLs:

A major QTL for maturity date (SNP7110, 4) was detected on Chr 4 for both years, while other four minor QTLs were detected on Chr 6 (Table 5.2). A major locus for SSC was detected (SNP420094), which colocalized with the major loci for maturity date on Chr 4. Other three loci were also detected for SSC on Chr 1, 4, and 5 (Figure 5.9). Four QTLs (SNP520429, SNP387034, SNP640028, and SNP767386) were detected in 2019 for fruit weight, while only one was detected in 2021 on Chr 6 (SNP538101) (Table 5.2, Figure 5.9). Altogether, 10 QTLs on all chromosomes were shown, except Chr 1 and 3.
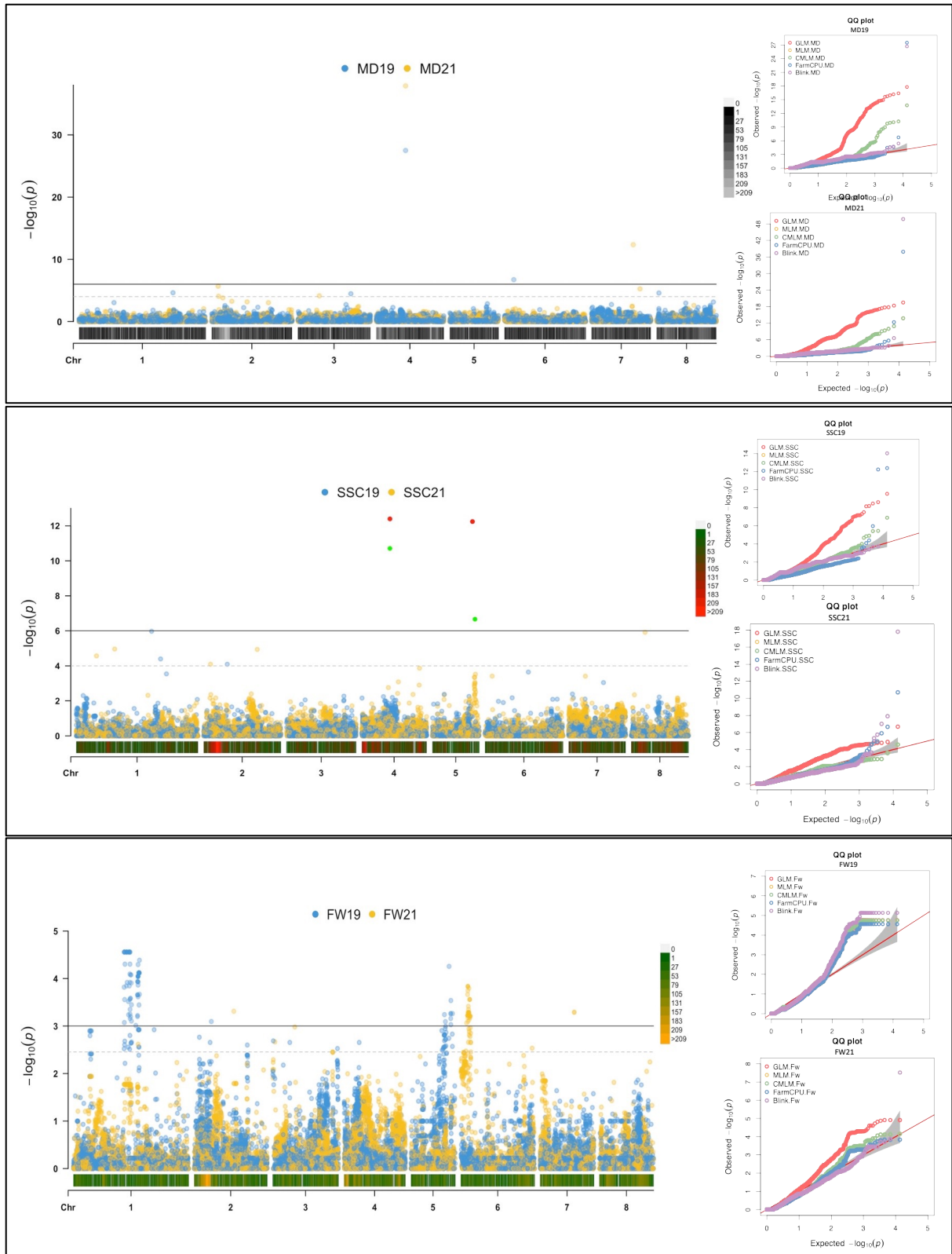
Figure 5.9. Manhattan and QQ plots representing fruit quality trait QTLs: Maturity date (MD), Soluble solid content (SSC), and fruit weight (FW) on the 8 chromosomes of *P. persica* genome. The phenotypic data from 2019 and 2021 using the FarmCPU algorithm.

Skin hairiness (nectarine/peach).

In the assessed seedlings, only one progeny (BO07009) out of the three was segregated to the fuzzless skin (nectarine). There were 16 nectarine siblings among the 76 siblings in the progeny (Elegant Lady is known to be heterozygous for the trait). The QTL analysis revealed one Mendelian QTL on Chr 5, on the position 16786885 (Figure 5.10).
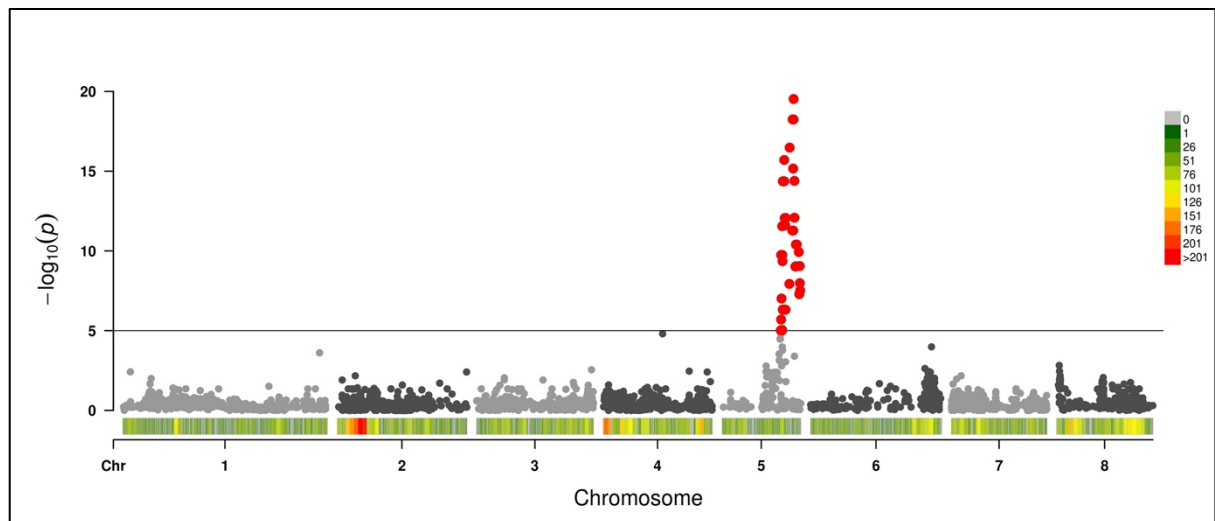
Figure 5.10 Manhattan plot showing the Mendelian QTL for fuzzless skin (nectarine) located on chromosome 5.

5.4. Discussion

This study aimed to explore the genetic makeup (background) behind the resistance/susceptible phenotype of peach fruit to brown rot (BR) disease. Our study was performed on three F_2 progenies from a "Contender" x "Elegant lady" cross during two seasons. In 2019 there was a positive correlation between artificial and field infection on both naturally occurring and control fruits. This implies that the *Monilinia* inoculum was present in the orchard. However, this was not the case in 2021. Susceptibility variation across years has been attributed to environmental factors influencing fruit barrier efficiency (Gibert et al., 2009; Pacheco et al., 2014). Nevertheless, another important factor was involved in our case, as the natural inoculum was inadequate in 2021, which can be explained as the very poor fruit set in 2020 due to severe frost damage.

On the other hand, the infection in control treatment in 2021 was negligible, confirming the low natural *Monilinia* inoculum hypothesis. Another important factor is the maturity date since a negative correlation with BR susceptibility was observed in 2021. This was opposite of the observations reported by Pacheco et al. (2014). This discrepancy might be due to seasonal differences in environmental conditions (humidity, temperature, and wind), particularly rainfall amount, frequency and distribution. A negative correlation between the BR susceptibility and the degree of ripening was observed. Based on our data, an I_{AD} lower than 0.7 was a reasonable threshold to be considered before fruit inoculation. A higher can negatively affect the results, while lower I_{AD} represents over-ripening and the fruit can easily be damaged during handlining or it could be more prone to other pathogens. Therefore, we can propose a range of 0.7 to 0.2 as an optimum range for evaluating fruit resistance for BR.

There was a significant positive correlation between MD and SSC; however, this correlation differed between the two years. SSC seems primarily affected by the fruit development period and maturity date as broadly indicated by the reduced SSC of early-ripening fruits compared to the mid-to-late ripening ones (Cirilli et al., 2016). Our findings

show no correlation between fruit BR infection and SSC in which peach fruits are infected regardless of SSC level. Fruit weight was not correlated to BR susceptibility in both seasons; however, it was negatively correlated with SSC and I_{AD} at the date of disease inspection.

Genome-wide QTL analysis for BR resistance

Some studies on BR resistance factors have been published using bi-parental F1 progenies so far (Baró-Montel et al., 2019; Fu et al., 2018; Martínez-García et al., 2013; Pacheco et al., 2014) or, more recently, genome-wide association studies (GWAS) (Fu et al., 2021). In most cases, reported QTLs for BR resistance were unstable or with minor effect. For example, the BR resistance assessment of an interspecific BC1 population generated from the almond and peach varieties 'Texas' × 'Earlygold' revealed a total of 12 QTLs associated with BR resistance. Except for LG1 and LG3, the location of the putative QTLs conferring BR resistance was found in all LGs. However, according to the authors, no consistent QTLs were detected during the two phenotyping seasons, although two QTLs mapped in G4 were near stability (Baró-Montel et al., 2019). Recently Fu et al.(2021) applied a genome-wide association study on 26 cultivars and progeny from 9 crossings using 'Bolinha' as a source of tolerance in their new study. Except for Chr 3, the study found 14 single nucleotide polymorphisms (SNPs) significantly correlated with BR infection responses to *M. fructicola*, with 10 SNPs related to peach peel and 4 SNPs to flesh throughout the whole genome.

In our study, the QTL found in the lower region of Chr 2 can be particularly interesting since a QTL in a nearby region was previously reported in F1 (Pacheco et al., 2014), though it was not detected in both seasons. This QTL appears to have been passed down to both F_1 and F_2 progenies. The QTL mapped on the F1 population was associated with markers M1a and UDP96-013, being these two markers heterozygous in Contender (donor parent) and homozygous in Elegant lady (susceptible) (Pacheco et al., 2014). In the nearby, two resistance gene analogs (RGAs) have been attached to LG2 in the "*Prunus* resistance map" reported by Lalli et al. (2005). One of these RGAs is found in the T× E-2 group's lower area, which is co-

localized with a powdery mildew resistance QTL (Foulongne et al., 2003). Another study on the QTLs for bacterial spot of leaf and fruit tissues of peach indicated the presence of several putative R genes in the detected QTL regions (Yang et al., 2013).

These RGAs genes, on the other hand, encode nucleotide-binding site leucine-rich repeat (NBS-LRR)-type proteins, which have been linked to the regulation of biotrophic pathogen resistance responses. This results in a hypersensitive response (HR) to a host-induced cell death at the site of infection that prevents pathogen expansion via nutrient limitation (Jones & Dangl, 2006; Mengiste, 2012). Further evidence for the importance of this region on the Chr 2 has been more recently provided. Which powdery mildew resistance genes (*Vr3*) have been fine mapped, and they narrowed the region down to 270-kb consisting of a cluster of 27 genes (Marimon et al., 2020). The second interesting QTL in our study was located in the middle of Chr 4 is worth investigating since this QTL has been reported in a similar position on LG4 by Martínez-García et al. (2013).

When no artificial inoculation evolved, primary or traditional cultivar evaluation for BR susceptibility in *Prunus* species was relied on field evaluation. The effectiveness of this method is determined chiefly on the availability of inoculum from the previous season and appropriate conditions for BR, which is influenced by seasonal weather conditions. In our study, natural field infection was considered in addition to artificial infection. In 2019, the QTL analysis discovered five QTLs, two of which were also discovered using artificial infection. In 2021, however, the data was exempted from QTL analysis since no or little field infection was identified.

Fruit quality trait QTLs

Maturity date (MD). MD is a critical aspect in marketing fresh fruit, particularly those with a short shelf life like peach (Pirona et al., 2013). MD is reported to be pleiotropic (Eduardo et al., 2011). Our study detected a major QTL for MD on the Chr 4 (SNP7110, P-value = 8.5E-34), which was stable across the years. Also, four other minor QTLs have appeared on Chr 6.

The MD QTL on the Chr 4 is well established and reported in many previous studies (Eduardo et al., 2011; Elsadr et al., 2019; Pacheco et al., 2014; Pirona et al., 2013; Rawandoozi et al., 2021).

Soluble solid content (SSC). Several QTLs with minor effects, typically clustered together, are responsible for peach fruit's overall and individual sugar content. Due to substantial environmental impacts, many of these QTLs are unstable, with low LOD scores and tiny percentages of explained phenotypic variability (Cirilli et al., 2016). In our study, four QTLs were detected on Chr 1, 4, and 5. However, QTLs in the middle of Chr 4 and towards the end of Chr 5 were appeared to be important hotspots for SSC. The QTL on Chr 4 seemed flanked with maturity date and is considered a major and stable QTL (Cirilli et al., 2016). Maturity date has an important effect on the SSC; therefore, they were significantly correlated in our study. Late maturity cultivars have a higher SSC because they have longer days to accumulate sugars. Our results align with the previous finding as similar results have been reported on Chr 4 and 5 (Quilot et al., 2004) and Chr 4 (Eduardo et al., 2011).

Fruit weight (FW). Because large fruit are better valorized in the fresh market, peach breeding programs have prioritized finding QTLs linked with fruit weight and/or size. Previous studies reported that the FW of peach is controlled by multiple QTLs allocated across the genome (Dirlewanger et al., 1999; Eduardo et al., 2011; Cassia da Silva Linge et al., 2021; Cássia da Silva Linge et al., 2015; Quilot et al., 2004). Our study detected 6 QTLs for FW on different chromosomes; however, they were not showing stable peaks. Linge et al. (2015) reported 11 QTLs for FW and other fruit shape traits, with minor effects. It can be concluded that this trait is heavily affected by environmental and cultural factors. Fruit weight is affected by many cultural and environmental factors.

Fruit hairiness. Our QTL analysis detected only one major QTL at the end of Chr 5, confirming previous studies (Eduardo et al., 2011; Pirona et al., 2013; Vendramin et al., 2014).

The localization of the nectarine (G/g) trait has been reported previously as monogenic and mapped on the same Chr 5 (Vendramin et al., 2014). In our investigation, all nectarines were medium to highly susceptible to BR in the field.

The trichomes of peaches have been studied previously and found to be coated with a thin cuticular layer comprising 15% waxes and 19% cutin, and packed with polysaccharide material (63%) including hydroxycinnamic acid derivatives and flavonoids (Fernández et al., 2011). The presence of trichomes and the phenolic acid, flavonoids and triterpenoids on the skin provide essential protection, implying the resistance trait(s) in fruit is lost with the absence of trichomes. More details were discussed in chapter 3 about the role of phenolics and triterpenoids.

5.5. Conclusion

Brown rot resistance in peach fruit is a complex trait controlled by multiple genes probably with minor effects and affected by the environment, disappointing breeding efforts. Data were not stable across years, confirming the impact of environmental factors. However, the genome-wide QTL analysis showed interesting regions relevant to disease resistance, mainly the QTLs found on chromosomes 2 and 4, which may be candidates for future validation of MAS approaches.

Several other QTLs were detected for different fruit quality traits, including maturity date, solid soluble content, and fruit weight. Date of maturity and soluble solid content QTLs are co-located on chromosome 4 and might co-segregate with resistance genes.

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CHAPTER 6. Concluding remarks

Brown rot disease resistance in peaches necessitates a lot of work and an interdisciplinary approach. This thesis attempted to illuminate some important parts of recent advances in genetic background of the inheritance of brown rot (BR) resistance in peach fruit.

Monilinia spp. are necrotrophic fungi requiring several factors to infect stone fruit successfully. The second chapter describes in detail the different steps of fruit infection assays. The objective was to highlight best practices and further improve phenotyping protocols for brown rot susceptibility. Phenotyping is the crucial part of breeding for BR; however, methodology variations across the described approaches, limit the ability to compare results from different studies. All steps of the phenotyping protocol are crucial to ensure good infection performance, from fruit sampling to inoculum preparation and application.

The second objective was to investigate the antifungal activity of some important phenolic acids, triterpenoids, and fruit surface compound (FSC) extracts of peach fruit (Chapter 3). *In vitro* experiments revealed a limited direct antifungal effect of the studied phenolics, and triterpenoids present in peach fruit skin. However, *Monilinia* gene expression data strongly suggest that chlorogenic acid and other compounds play a significant role in the fungi-plant interactions during the infection process.

The main objective of this thesis was to evaluate a population for BR susceptibility that consisted of three F_2 progenies obtained by selfing three F1 selections. They were derived from the peach interspecific cross of 'Contender' x 'Elegant Lady' cultivars. Single-Primer Enriched Technology (SPET) and a newly constructed 18K SNP array were used for genotyping the whole progeny. The genome-wide QTL analysis revealed some interesting locations that might be significant for BR disease resistance, particularly on chromosome 2 and 4 could be candidates for future Marker-assisted selection (MAS) applications. Other QTLs associated

with fruit quality traits discovered in the study include maturity date, soluble solid content, and

fruit weight, all of which can be investigated further genetically.