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Review

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# Brown Rot Strikes *Prunus* Fruit: An Ancient Fight Almost Always Lost

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**ABSTRACT:** Brown rot (BR) caused by *Monilinia* spp., has been an economic problem for the stone fruit market due to dramatic losses, mainly during the postharvest period. There is much literature about basic aspects of *Monilinia* spp. infection, which indicates that environment significantly influences its occurrence in the orchard. However, progress is needed to sustainably limit this disease: the pathogen is able to develop resistance to pesticides, and most of BR resistance research programs in plant models perish. Solving this problem becomes important due to the need to decrease chemical treatments and reduce residues on fruit. Thus, research has recently increased, exploring a wide range of disease control strategies (e.g., genetic, chemical, physical). Summarizing this information is difficult, as studies evaluate different *Monilinia* and *Prunus* model species, with diverse strategies and protocols. Thus, the purpose of this review is to present the diversity and distribution of agents causing BR, focusing on the biochemical mechanisms of *Monilinia* spp. infection both of the fungi and of the fruit, and report on the resistance sources in *Prunus* germplasm. This review comprehensively compiles the information currently available to better understand mechanisms related to BR resistance.

**KEYWORDS:** *Monilinia spp., brown rot, Prunus, QTL*

## 1. INTRODUCTION

The genus *Prunus* has hundreds of species with some economically important members, including the cultivated almond, peach, plum, cherry, and apricot. The five most important countries for the production of these fruits are China (10.7 MTon), the United States (2.9 MTon), Italy (1.9 MTon), Spain (1.4 MTon), and Greece (0.8 MTon).[1]

Different cropping practices are employed for the production of this variety of fruit, according to their different environmental and nutritional requirements. In addition, the broad range of pests has to be controlled to reach a high-quality final product. This latter point is a crucial issue in current fruit cropping, because of the demand for fresh fruit with reduced residual quantities, and the regulation of fungicide use has become stricter in European Union (EU) countries, after the release of European Directive 2009/128/EC, which indicates the use of integrated pest management (IPM) as mandatory.[2,3] In the United States, the government has strongly promoted IPM to reduce chemical pesticide input with the creation of Regional IPM Centers, resulting in progressive decreases in pesticide use and toxicity for humans.[4,5] Reduced pesticide applications have been advised in China,[6] Brazil, and other countries.[7]

Among the plethora of pathogenic agents attacking *Prunus* crops (and other Rosaceaeous), brown rot (BR) is the economically most important disease of stone fruits.[8] *Monilinia* spp. are able to infect various plant organs, causing blossom blight, twig blight, and BR in immature and mature fruits, the latter being the most sensitive host phenological phase. The relatively long period of incidence, extending from bloom to postharvest, the multiplicity of climatic and cropping factors favoring disease spread, the occurrence of diverse fungicide resistances in some BR agents, and the poor availability of host resistance, result in severe, unavoidable, and sometimes unpredictable losses in the fruit market.[7] According to Martini and Mari,[9] the worldwide yearly value of *Monilinia* losses is 1.7 thousand million Euro; in the United States, yearly losses are estimated to be 170 million USD for peach, cherry, and plum production;[10] and in Australia, yearly losses are estimated at 1 million AUD for peach and apricot crops.[11] Under laboratory conditions, BR can result in losses of >60% of peaches and nectarines after 5 days of infection at room temperature.

To avoid these damages, *Monilinia* spp. diseases are controlled by chemical methods. Fungicide applications are necessary to diminish BR damage in humid seasons, but lead to sustainability challenges in pome and stone fruit cropping, as there are many fungicide-resistant strains (*Monilinia fructicola*, see below). An important research field has been dedicated to the epidemiology of BR, as well as aspects related to traditional chemical control and emerging alternative control strategies (e.g., tree management),[12,13] compatible with IPM and organic

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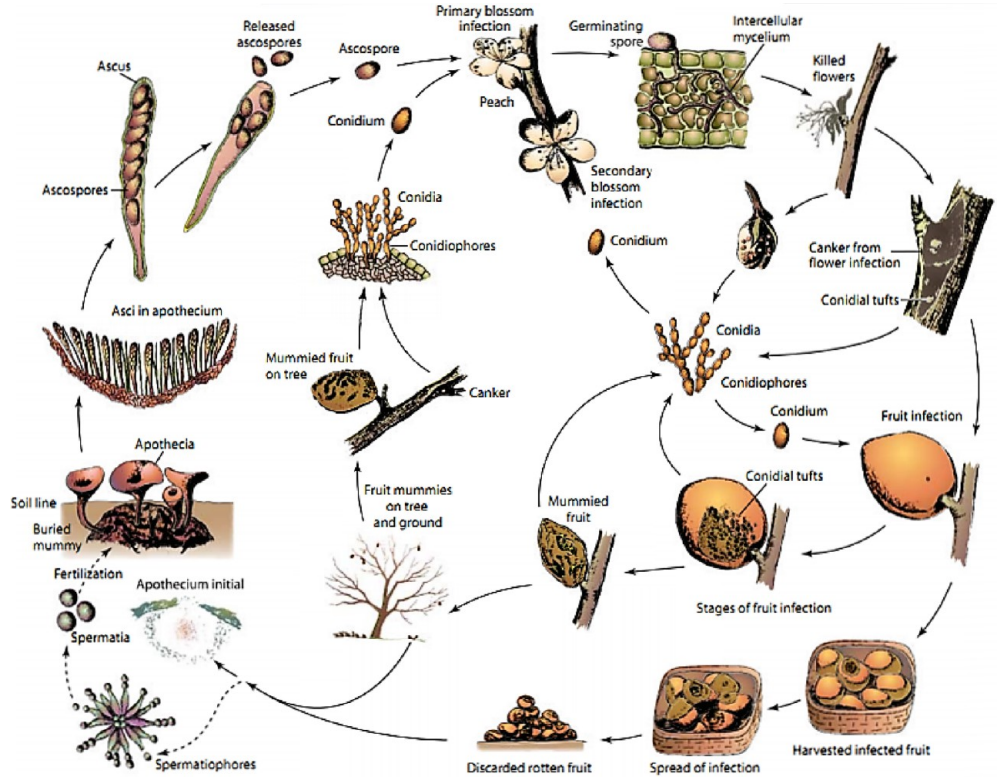


**Figure 1.** *Monilinia* spp. life cycle. Reprinted with permission from *Plant Pathology*. Copyright 2005 Elsevier Limited.

agriculture (biologic agents, postharvest biochemical−physical agents). These topics are thoroughly reviewed and discussed in recent publications.[14,9] Significant efforts are being invested to characterize and enhance fruit resistance to BR for the generation of new varieties with reduced requirements of application of exogenous methods for BR control. These have been included as important objectives of international collaborative initiatives for new cultivar development around the world, such as the Fruit Breedomics European project and the ROSBREED American initiative.

In the present review, we will focus on stone fruit characteristics conferring resistance to BR. For this aim, we compiled information from peer-reviewed papers, congressional acts, and unpublished data obtained over years of work on this topic. After a brief description of the taxonomy, morphology, and geographic distribution of *Monilinia* species, we will focus on fruit features representing points for the start of infection. We will examine the steps of infection development and discuss the main biochemical and molecular host factors for BR resistance in fruit. To finish, we will describe the breeding programs aimed at enhancing BR resistance in stone fruit, generating knowledge for the genetic dissection of fruit BR resistance.

## 2. *MONILINIA* SPP. FUNGI CAUSE BROWN ROT

**2.1. Taxonomy.** The agents causing BR are polytrophic fungi belonging to the phylum Ascomycota, class Leotiomycetes, order Helioteliales, family Sclerotiniaceae, genus *Monilinia*. They attack members of the Rosaceae and Ericaceae families.[15,16] The generic name *Monilinia* includes those members of *Sclerotinia* that produce moniloid conidia and pseudosclerotia.

Of the 35 species of the genus *Monilinia* Honey, three are the main species that are pathogenic to pome and stone fruits: *M. fructicola* (G. Winter) Honey, *Monilinia laxa* (Aderhold & Ruhland) Honey, and *Monilinia fructigena* (Aderhold & Ruhland) Honey.[17] At least two species have been described to be important pathogens of Ericaceae: *Monilinia vaccinium-corymbosi*, causing mummy berry of blueberry,[18] and *Monilinia oxycocci*, causing cottonball of cranberry.[19] According to phylogenetic analyses based on rRNA sequences of *Monilinia* and *Sclerotinia* species, the separation of the genus in two sections is consistent: Junctoriae, attacking Rosaceae hosts, and Disjunctoriae, attacking Ericaceae hosts;[15,20,21] moreover, partial congruence found in the branching topologies of hosts and pathogen phylogenies led to the suggestion of cospeciation between them.[15] In this review, we will focus on *Monilinia* spp. and BR in stone fruits.

The disease cycle of *Monilinia* species is represented in Figure 1. Primary inoculum sources in the spring are overwintering BR fruit mummies either on the tree, which produce asexual fruiting structures (sporodochia) and spores (conidia), or on the orchard floor, which produce sexual fruiting structures (apothecia) and spores (ascospores). The spores are dispersed by wind and rain to susceptible host tissues and germinate under favorable wetness and temperature conditions. In general, blossom blight reduces the crop load in fruit crops, but it can destroy the crop at flowering in susceptible almond cultivars. The infections of blossoms typically remain attached, and the infection spreads into the peduncle and down into the twig. The infection continues with the formation of a twig canker that often develops a gumdrop as a host response. Conidia form on infected tissue and serve as secondary inoculum for infection of immature and mature fruit.[22] Infections on immature fruit, after the endocarp


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lignification, may give place to conidia, providing additional inoculum.

**2.2. Differentiation of *Monilinia* Species.** By observation with the naked eye, it is possible to identify the differences between the three agents of monilioses in fruit in orchard conditions.[23] *M. fructigena* has color ranging from white to light beige, large (1.5 mm on average) conidiospore tufts, and disposition in concentric circles in the fruit. *M. fructicola* has brown-colored, medium-size (1 mm on average), conidiospore tufts and 10% black spots. *M. laxa* can be distinguished by greenish-gray conidiospores tufts <0.5 mm on average that cover the whole infected surface. However, the differentiation in fruit between *M. laxa* and *M. fructicula* may sometimes be difficult, and the use of molecular techniques is required (Figure 2).


**Figure 2.** Peach fruit infected by three different *Monilinia* species.

Studies to identify the *Monilinia* species reported that, in culture medium with potato dextrose and agar (PDA) at 22 °C, *M. laxa* is characterized by concentric rings of mycelium with lobbed margins, whereas in *M. fructigena* it is possible to observe fragmented radial colonies. Differences in colony growth rates between the three species were observed (20−25 °C). The highest growth rate on PDA was found for *M. fructicola*, followed by *M. fructigena* and *M. laxa*, respectively. However, *M. laxa* showed the biggest lesion growth rate on peach fruit.[8] In culture medium it is possible to analyze characters as conidial size and germ tube morphology. These methods have been used since 1920, and their simplicity makes them useful still.[22] Differences in conidium size among the species are reported. On average, the conidium size of *M. laxa* is smaller compared to that of *M. fructigena*, 13 × 9 and 22 × 12 μm, respectively. *M. fructigena* produces one or two germ tubes per conidium, and *M. laxa* and *M. fructicola* isolates consistently produce only one germ tube per conidium.[8]

Several molecular biology techniques (mostly based on the polymerase chain reaction, PCR) have been used to develop reliable and sensitive methods to identify and detect *Monilinia* species. Fulton and Brown[24] proposed the study of the small subunit of rDNA (rDNA) to differentiate *Monilinia* isolates from the three major species. Many PCR protocols for *Monilinia* spp. identification, based on the comparison of internal transcribed spacers, the sequence between the 18S small and the 28S rDNA subunits of *Monilinia* genes, have been proposed.[22,25,26] Ma et al.[27] and Hu et al.[8] reported a detection and identification method of *Monilinia* fungi based on species-specific microsatellites.[8,27] Identification methods based on amplified fragment length polymorphism (AFLP) are also reported.[28,29] In addition, molecular techniques have been developed for species identification on quiescent fruit infections of stone fruit[30] and for the early detection of infections in cherry fruit.[31] In Banks et al.,[32] monoclonal antibodies are reported to be useful for the identification and detection of *Monilinia* spp. in pome and stone fruit.[32] Some of these approaches have set the basis for several studies about morphological and molecular diversity of *Monilinia* spp., describing the geographical distribution and host range of the three main species of *Monilinia* that caused BR of stone and pome fruits.[33−35]

**2.3. Host Range and Distribution of *Monilinia* spp.** *M. fructigena* is an economically important BR agent that has been associated with European BR of pome fruits.[15,36] However, its occurrence in stone fruits has also been well documented in Europe,[37,38] Brazil,[39] and China.[6]

*M. laxa* has been historically associated with European blossom blight and BR of stone[36,38] and pome fruit.[40,41] However, in the past two decades it has been also reported in different regions of the world, including Brazil,[39,42] the United States,[43−45] China,[6] and Iran.[46]

*M. fructicola* (G.Wint) is the most widely distributed species, occurring in Asia, North and South America, New Zealand, and Australia.[7,47] In Europe, it was a quarantine pathogen until early 2014, when it was removed from the European quarantine pest list due to its current spread in the following countries: France,[48] Hungary,[34] Switzerland,[49,50] Germany,[51] Czech Republic,[52] Slovenia,[53] Italy[54,55] Austria (subsequently erradicated),[56] Poland,[57] Slovakia,[58] Serbia, and Spain.[35]

The low genetic diversity found in Spanish and French populations of *M. fructicola*, compared with American or New Zealand diversity indicates few and recent introduction events of the pathogen to Europe.[59] In addition to its wide distribution, *M. fructicola* has been reported to infect other hosts such as Cornelian cherry[60] and others that do not belong to the Rosaceae family, for example, grapes[61] and dragon fruit.[62]

These three species share high levels of DNA similarities. *M. fructicola* and *M. fructigena* exhibited 97.5% sequence identity, whereas *M. laxa* and *M. fructigena* displayed >99.1% for the *Cyt b* gene.[63] In this way, we may expect that part of the knowledge acquired from one species may be extrapolated to the other members of the *Monilinia* genus.

A fourth species, *M. polystroma* (also called "Asiatic brown rot"), is native to Japan, where it had been formerly confounded with *M. fructigena*. It was described as a new species after finding significant biological and morphological characteristics with respect to European isolates of *M. fructigena*.[64] Molecular differences between European and Japanese isolates of *M. fructigena* were previously demonstrated, on the basis of the ITS region of rDNA.[65] *M. polystroma* has been reported to occur in pome and stone fruit orchards from China,[66] Poland,[57] and Hungary.[67]

Two other less-distributed *Monilinia* species are described. *M. mumecola* was reported to infect *Prunus mume* in Japan[68] and to be the causal agent of the BR of papaya in Hubei, China, in 2009.[8,69] Finally, *M. yunnanensis* has been recently designated as a new species causing BR in Chinese peach orchards and, on the basis of the DNA sequence similarity analyses of marker genes, was found to be very close to *M. fructigena*;[8] this species is also able to infect fruits of *Crataegus pinnatifida*.[70]

In summary, it is no longer relevant to affirm that the different BR agents are distributed in specific regions. Indeed, all three main *Monilinia* species are present in almost all stone and pome fruit-producing countries,[71] likely due to open trade around the world. The worldwide distribution of *M. laxa* is very well illustrated in Rungjindamai et al.[14] In the same way, the fact that *Monilinia* species have the ability to colonize fruit of virtually any *Prunus* or *Malus* host suggests a relatively wide


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host range of these agents. A few studies of host specificity in *Monilinia* spp. have been reported to date, among which proteomic analysis conducted by Bregar et al.[72] showed a host-specific expression of some proteins between apple and apricot *M. laxa* isolates.

## 3. PENETRATION SITES IN RELATION TO FRUIT GROWTH

As stated before, in this review we discuss only aspects of fruit infection. Different biologic mechanisms may be involved in the pathogenesis of fruit and flowers by *Monilinia* spp., suggested by an absence of correlation between blossom bight occurrence and fruit rot impact, after artificial inoculation of *M. fructicola*, in Brazilian cultivars and selections of peach.[73] In fruit, *Monilinia* spp. have often been considered as opportunistic fungi that may enter in the tissue only via naturally occurring entry points. Therefore, many studies have focused on these entrances or employed infection tests injuring the fruit first. Although in most of the cases the fungus penetrates using "open doors" (Figure 3f), most of the species may also be able to penetrate fruit through intact surfaces, after the establishment of latent or quiescent infections.

For example, the penetration of *M. fructicola* in immature apricot fruit was reported to occur through wounds, stomata (Figure 3b,c), and intact cuticle or via trichoma bases (Figure 3a).[74] The same way in peach, hyphae infect fruits by either degrading the cuticle and epidermal tissue[75] or directly entering through pre-existing skin microcracks (Figure 3d,e). Fungus incidence is greater if the fruit has small cracks or wounds.[76] It has been reported that *M. fructigena* infects fruit via wounds only, in contrast to *M. laxa* that may infect both healthy and wounded fruit.[77] Indeed, infection may depend on which site is most frequently encountered by fungal germ tubes. The penetration site may also depend on the developmental stage of the fruit. For example, stomata are the preferred sites in the case of unripe peaches only. Curtis[78] found that apricots were penetrated through cuticle and stomata, plums via stomata, and nectarines through the cuticle. Sharma and Kaul[79] described the penetration of apple under laboratory conditions by *M. fructigena* through lenticels.

**3.1. Fruit Susceptibility Evolves during Fruit Development.** The stages of development of fruit are very important to understand the occurrence of BR, because the dramatic changes in fruit physiology and biochemical composition are in sync with changes in the susceptibility to BR infection.[76,80,81]

The first stage starts after ovule fertilization, petal fall, and ends when the stone starts lignifying. In this stage the fruit is photosynthetically active, displays intense transpiration activity, and shows the highest nutrient content,[82] resulting in a high susceptibility to BR, probably due, in part, to the fact that stomata are active and offer an entrance opportunity to the pathogen.[78]

The second stage, also known as "pit hardening", is the stage most resistant to infection by *Monilinia* spp.[76,83] This stage is characterized by intense metabolite activity of secondary compounds, such as catechin, epicatechin, and phenolic compounds, associated with the lignification of the endocarp, occurring in this stage. To find genes whose expression is involved in the synthesis of compounds conferring pathogen resistance, Guidarelli et al.[84] compared gene expression profiles obtained by microarray analysis of susceptible phase (stage S1) and resistant phase (S2) RNA samples from peel fruit, finding dramatic changes in the expression of phenylpropanoid and jasmonate-related genes and thus supporting a potential role of these compounds in BR resistance during fruit development.

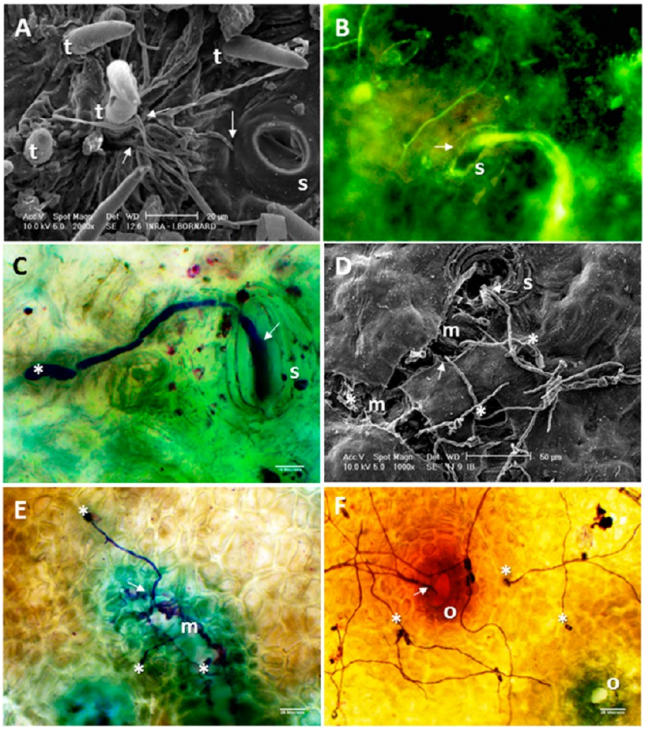


**Figure 3.** Sites of fungi penetration: (a) Scanning electron microscopy examination showing the development of fungi in apricot surface 8 h postinfection (hpi). The fungi develops on the fruit surface, twists around trichomes (t) and moves to the stoma (s) direction and trichomes basis (arrows). (b) Fluorescence photomicrograph 24 hpi. In this image it is possible to see a hyphae entering through an open stomata (arrows). (c, e) Light microscopy images showing infection on the surface of a commercial nectarine 'Magique' at maturity, colored with Toluidine blue, 0.5%, 15 (hpi). (c) Beginning of spore germination (asterisks) and penetration through stomata aperture (arrow). (d) Electron microscopy image showing a strong concentration and germination of spores (asterisks) fungi around the fruit cracks of cv. 'Magique' 15 hpi. (e) Spore germination and development of mycelium in micro crack (m) direction. (f) Infection of nectarine surface at maturity colored with Toluidine blue, 0.5%, observed with light microscopy at 15 hpi. It is possible to note the distribution of spores (asterisks) and their germination. This image illustrates a chaotic germination of spores (asterisks) and the colonization of surface by hyphae. The arrow shows the penetration of hyphae in an epidermis aperture (o). In all images fruit were infected with a drop of 10 $\mu$L and $10^5$ spores/mL of conidia concentration.

At the third stage, the highest cell expansion is observed and color changes from greenish to yellow to red. This stage ends with physiological maturity. Stone fruits become increasingly susceptible to pathogens as they mature and ripen, enabling quiescent infections to become active and new infections to begin. Associated with this increased susceptibility, structural changes in the fruit surface take place, such as thinning and fracturing of the cuticle, changes in fruit surface chemistry (e.g., production of sugars, decline of phenolic compounds and organic acids, etc.), structure and integrity of fruit mesocarp.[75]

Notably, various works in different *Prunus* species have observed a shift in the latent infection rate across the diverse stages of fruit development.[85−87] However, the results vary among studies, probably due to differences in methodology and


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cultivars used in those studies. For instance, Luo et al.[88] observed that pit hardening of prunes presented the lowest rates of latent infections, differing from other works reporting a minimum rate of latent infections at the embryo growth stage.[85,87]

**3.2. Infection by Direct Penetration of the Cuticle.** After conidial germination, *Monilinia* species are able to develop appressoria to establish a latent infection and ease the penetration of the intact cuticle when fruit maturity conditions allow colonization.[89] This structure allows adhesion of the pathogen to the surface of the host during infection.[90] Direct penetration of *Monilinia* spp. is enhanced by its production of cutinases,[75] the redox-mediated overexpression of which results in an increased fungal virulence of *M. fructigena* in stone fruit.[91] More details about the infection process are given under section 4.

**3.3. Infection through the Trichome Basis.** A dense layer of trichomes covers the surface of the peach fruit. The infection can occur in both pubescent and non-pubescent peach fruit. The role of trichomes in the infection remains controversial. Indeed, trichomes may protect the fruit in two ways: (1) directly, in which exudates from trichome gland may act as fungicide, and (2) indirectly, when the high density of trichomes could prevent the formation of "water film" important to spore germination. In contrast, trichome basis fracture can result in epidermis crack, resulting in points for fungal entrance.[92,93] Smith[94] showed that removing pubescence by means of brushing reduced the time of infection development, suggesting that the spores could reach the fruit surface more directly. Other studies[74] affirmed that *M. fructicola* is able to penetrate apricots at hair bases. Similar results were found on mature peaches.[78,95]

Finally, is not yet clear whether nectarines are more resistant or susceptible to BR compared to peaches. Large variations of trichome density and length and, more generally, of fruit surface between varieties make comparisons between studies and drawing general conclusions a very hard task.

**3.4. Infection through Stomata.** The literature about stomata and their function on reproductive organs is limited, especially for drupe fruits such as peaches.[96] A majority of studies discuss their function and distribution in dry fruit such as nuts, capsules, and pod fruit.[97] They can occur in small numbers or are even restricted to certain parts of the fruit.[98] The number of stomata per fruit is determined before petal fall and remains constant throughout fruit ontogeny.[99] The morphology of the guard cells suggests that they have the same functions as on leaves. In early stages, stomata provide aeration in the gas exchanges for the photosynthetic system; however, fruit stomata are functional only to a certain extent. Due to the development of the fruits, stomata can develop into lenticels and either close or remain open permanently.[98]

In mature peach fruits, the number of stomata could be insignificant compared to the number of microcracks and may no longer be determinant for pathogen susceptibility. In early fruitlets instead, the high density of stomata could be one of the factors (Figure 4), which may explain the susceptibility at this early stage.

Fungal invasion through stomatal apertures into the substomatal cavities was observed in apricots infected by *M. fructicola* under laboratory conditions.[74] The authors reported that the fungus enters via the stomata and penetrates a guard cell through the thin-walled region at the stomata pore. Close examination of serial radial or tangential sections showed that

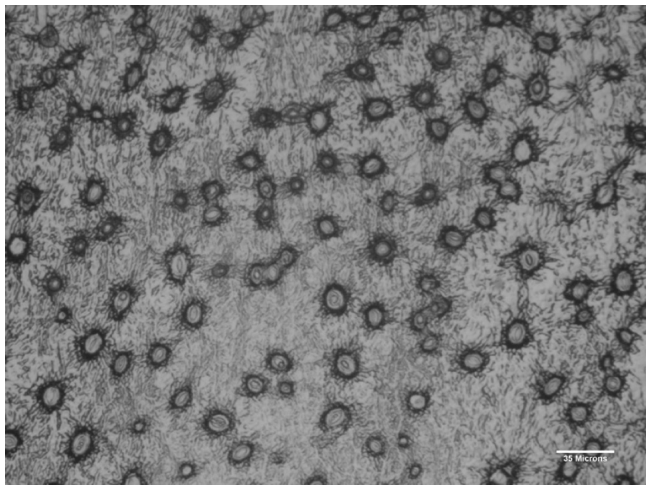


**Figure 4.** Light microscopy image of surface impression of a young nectarine fruit (46 days after full bloom) showing the high density of stomata.

in most cases primary infection was through guard cells. However, in a few cases the lesion center did not coincide with stomata, and initial invasion was through wounds.

**3.5. Infection through Skin Cracks and Wounds.** Cuticular crack is defined as the physical failure of the fruit skin, caused by forces of growth such as turgor pressure within the fruit cells or hydration of fruit fresh acting on the skin.[100] Cuticular cracks on nectarine fruit occur during the final fruit growth stage.[101−103] Microcracks and cracks can develop on the surface of fruit when the growth speed of the internal cells is more rapid than epidermal cell growth. In this case, a time lag between fruit growth and cutin deposit can occur and provoke zones of weakness that may evolve into microcracks. Several factors contribute to fruit cracking, often in interactions, such as unbalanced water flux into and out of the fruit, maximal elastic limit of the cuticle, cuticle strain, and absence of cuticular membrane deposition. Observations of the fruit skin have shown that the cracks are frequently initiated around the lenticels[104] (Figures 3d,e and 5). Larger fruits can present high cuticular crack densities, which may represent >10% of the fruit surface area.[101]

One of the first studies on *M. laxa* penetration in microcracks[105] observed a significant number of cracks and microcracks organized radially around lenticels and noted that germinating conidia of *M. laxa* tended to accumulate in the microcracks in an anarchic pattern and without apparent direct attraction by microcracks, despite the fact that the germ tubes grew inside them. However, Borve et al.[106] demonstrated a clear link between cracking and BR in cherries, by finding significant correlations between the cultivar-specific amount of microcracks and the resulting incidence of BR.

Skin-wounding deprives the fruit of its main barrier to biotic stress agents, as demonstrated in several studies,[77,107] where BR infection rates obtained after infecting wounded regions of the fruit were significantly higher than those of intact fruit regions. The effect of the presence of skin barrier in BR resistance was investigated on apricot, peach, and plum fruit to find resistant genotypes.[108] Injured-fruit infection developed on all fruit with quite similar speed in all species. On the contrary, when uninjured fruit were infected, large variability was observed between genotypes of the same species and between species. These observations suggest that few resistant factors may be


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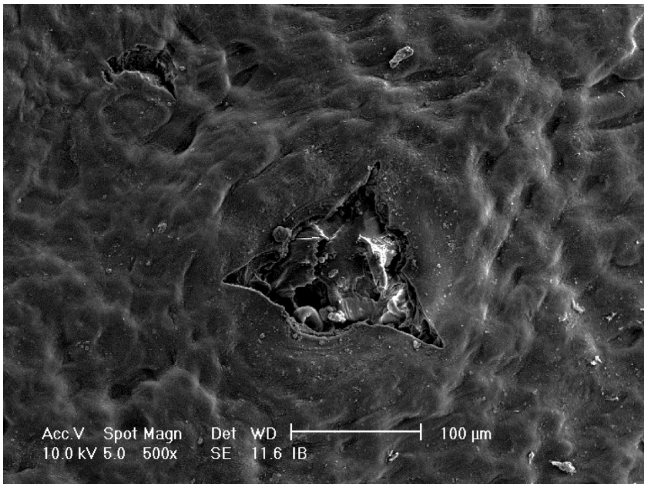


**Figure 5.** Scanning electron microscopy of nectarine cultivar 'Magique'. The image shows the beginning of crack formation around a lenticel at maturity.

expressed at the flesh level and that resistance factors were no more efficient when the fruit was injured. However, Ogundiwin et al.,[109] explored larger genetic diversity by evaluating 81 peach genotypes by infection on wounded and unwounded fruit. The authors observed variability in both cases and suggested that BR resistance is associated with the pericarp or the mesocarp or both, depending on the genotype.[109] Nonetheless, more recently the same group further explored the variability of infection reaction after wounding of a canning peach progeny,[110] concluding that wounding the fruit generally abrogated any resistance to brown rot. Resistance factors at the level of the flesh (wounded fruit) may not provide total resistance to infection but may slightly act on the speed of lesion propagation. To further explore these potential factors of resistance, large trials considering a high replicate number on highly contrasted germplasm panels may be needed.

In conclusion, it is evident that stomata, lenticels, pores, cracks, and microcracks offer preferential entry sites for *Monilinia* and make fungus colonization easier. The number of stomata, lenticels, and pores may be under genetic control, but structure may be influenced by environment conditions. As for cracks and microcracks, genetic determinism has not been investigated, but studies have demonstrated the effect of cultural practices (e.g., irrigation and thinning) on their density.[101]

## 4. INFECTION DEVELOPMENT

Infection is a term that implies the entry of an organism into a host and the subsequent establishment of a parasitic relationship.[36] The process could be broadly divided in three stages: prepenetration, penetration, and postpenetration (Figure 6). The prepenetration phase concerns the transport of the spores from the inoculum source to the organ host that will be infected. It will not be detailed here.

In general, fungi utilize diverse mechanisms to infect host tissue, which include (i) chemical sensing and oriented growth in response to mechanical contact to optimally position infection structures, (ii) the production of enzymes to degrade host surfaces, and (iii) the formation of specialized structures such as appressoria.[111] Initial events are adhesion to the cuticle and directed growth of the germ tube on the plant surface. At the penetration site, appressoria are often formed that may have

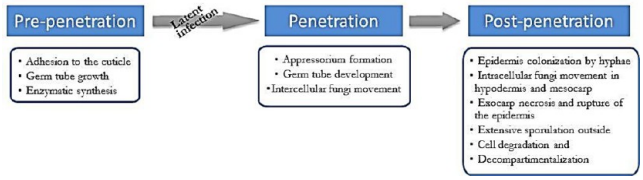


**Figure 6.** Process of *Monilinia* spp. infection.

melanized walls and develop high turgor pressure to support the penetration process. The penetration hypha accumulates components of the cytoskeleton in the tip and secretes a variety of cell wall-degrading enzymes in a highly regulated fashion to penetrate the cuticle and the plant cell wall. As cited in many papers and reviewed by Rungjindamai et al.,[14] the presence of moisture near the fruit is a crucial factor for spore germination and infection development.

**4.1. Adhesion to the Cuticle and Germination.** Conidia and ascospores, which are the main inoculum for BR infections, require free moisture for germination, which is obtained from films or droplets of water and from plant exudates that accumulate on the surface of the host or in damaged tissues.[112] Germination of conidia takes about an hour in the presence of free water, whereas ascospores require 4−6 h. However, the germination process could sometimes last 60 h in the case of dried spores that need time to rehydrate and reactive the protoplast.[36]

**4.2. Latent Infection.** Infections may remain latent when microclimatic conditions and fruit growth stage are unfavorable.[36,88] Latent infection generally happens in immature fruit. A subcuticular infection begins, but growth of the pathogen quickly stops. These quiescent infections may be visible or nonvisible. During fruit growth, *M. fructicola* expresses genes and proteins enabling later successful infection and colonization of the fruit.[91] As the fruit matures, fungal growth restarts and BR develops.[14]

The relationship between the number of conidia on the fruit surface and the incidence of latent infections in orchards or after harvest has been investigated for different fruit species.[77,86,88,89,113] A significant positive link has been reported for peaches.[85] Therefore, early identification of fungal infections is needed to determine pre- and postharvest disease management practices, as well as postharvest shipping strategies. To choose targeted fungicide treatments, molecular methods to identify latent infection of *Monilinia* spp. have been developed.[31]

**4.3. Appressorium Formation and Hypha Penetration.** The formation of appressoria is induced by specific physical or chemical cues provided by the host plant. Irrespective of whether fungi use enzymes or force, or a combination of both, to penetrate, appressoria need to adhere tightly to the plant surface. Appressorium differentiation can be stimulated in *C. gloeosporioides* by wax isolated from fruit of its host plant, avocado, but not by wax isolated from other plants.[114] Careful analyses suggested that nonhost wax contained inhibitors of appressorium development.

High pressure can be generated by turgor within the appressorium and possibly also by the cytoskeleton and pushes the hypha to penetrate through the surface. Penetration is likely to be supported by enzymes that soften the host cell wall. To analyze the contribution of cell wall-degrading enzymes to the penetration process, Dumas et al.[115] used the endopolygalacturonase promoter of *Colletotrichum lindemuthianum* (a necrotro-


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phic fungus like *Monilinia* spp.) to control green fluorescent protein expression. These authors were able to show that the gene is expressed in appressoria prior to penetration. Finally, *C. lindemuthianum*, as other necrotrophic fungi, required pectolytic enzymes not only for tissue maceration during in-planta growth but also to assist forceful penetration.

Appressorium formation by *M. fructicola* on fruit surfaces has been related to BR incidence to fruit surface topography and hydrophobicity, as well as the presence of nutrients and fruit volatiles.[90,116] Appressoria were observed on the stomatal guard cell lips, and germ tubes apparently perceived particular topographical features to trigger differentiation of appressoria. Because appressorium-mediated penetration was observed both by natural openings (stomata) and by direct penetration of intact cuticle (through penetration pegs produced from appressoria), authors suggested that mechanisms may be diverse. In contrast, they did not observe appressoria on mature nectarine fruit. The authors suggested that *M. fructicola* restrains the formation of specialized infection structures such as appressoria to immature tissues and behaves as a saprophyte pathogen when nutrients are readily accessible, as in mature fruit. Also, a role of cAMP as well as the calcium−calmodulin pathway was suggested in the formation of appressoria.[90]

**4.4. Appressoria Melanization Increase Pathogenicity.** Melanins are brown-black pigments, biological macromolecules composed of various types of phenolic or indolic monomers that are produced by fungi and other organisms. Various fungi synthesize melanin from the oxidation of tyrosine. The extracellular dark pigments produced by fungi may be formed from various fungal phenols, usually named heterogeneous melanins.[112,117] The production of melanin by microorganisms has been associated with their virulence, and the melanization of appressoria was considered necessary to different fungal pathogens for infection and disease development. Howard et al.[118] proposed the importance of melanization for surface penetration. These authors exposed appressoria from the rice blast fungus *Magnaporthe grisea* to solutions of high osmotic pressure and observed no melanization and an inhibition of penetration of the leaves. They concluded that melanization is involved in the reduction of porosity of the appressorium wall. According to Dean,[119] this causes the locking of cytosolic solutes efflux and leads to higher appressorium pressure.

Indeed, many fungal pathogens, such as *Venturia inaequalis*, *Magnaporthe gray*, *Pyricularia oryzae*, and *Colletotrichum legenarium*, need melanized appressoria to cause infection and disease development.[117,120] De Cal and colleagues have reported that a melanin-deficient mutant strain of *M. laxa* (albino mutant) was no longer able to induce peach twig blight.[118] They also observed that *M. laxa* treated with pyroquilon, an inhibitor of melanin biosynthesis, could not induce peach twig blight.[121] Finally, they found that in vitro, chlorogenic acid or pyroquilon added to the culture medium of *M. laxa* inhibited melanization of the colony.[122] They concluded that the ability of *M. laxa* to produce melanin is crucial for its pathogenicity.

Rehnstom and Free,[123] however, showed that melanin-deficient mutants of *M. fructicola* are able to infect nectarines, by producing lesions as large as those produced by the wild type. Therefore, they concluded that melanization is not required for the successful infection of host fruit. Nevertheless, their presence could improve the success of the development of fungi and increase their permanence in the field under adverse conditions.

**4.5. pH Lowering Regulates the Expression of Pathogenicity Genes.** Fungi are able to modify the host pH. Preliminary data on *M. laxa*, *M. fructicola*, and also *M. fructigena* indicate that they can reduce host pH during colonization of peach cv. 'Big Top', 'Venus', and 'Tirrenia' by secreting gluconic acid.[76] Analysis of the acidification process in colonized fruit showed that gluconic acid was the main organic acid accumulated at the infection site and under liquid-culture conditions. When compared to a nectarine cv. 'Big Top' and peach cv. 'Plácido' with differing sensitivities to *M. fructicola*, a 250% higher accumulation of gluconic acid was observed in the susceptible peach cultivar than in the less susceptible nectarine cultivar. Under liquid conditions, at pH 3.6−3.7, the relative expression of transcripts of mfpg2 and mfpg3, encoding for two polygalacturonase genes of *M. fructicola*, increased 12- and 6-fold, respectively, suggesting the importance of acidification for the secretion of pathogenicity factors by *M. fructicola*. The authors also emphasized the importance of acidification for the secretion of pathogenicity factors by *M. fructicola*, suggesting that ambient pH created by the pathogen is a regulatory cue that promotes pathogenicity expression. Specific genes contributing to pathogenicity may be expressed as a result of the environmental pH induced by the pathogen.

**4.6. Biochemical Arsenal of *Monilinia* spp.** Studies in past decades ascertained the effects of fungicides on fungus enzymes in buffer extracts of mycelium of *M. laxa*.[124] Thus, they reported large groups of enzymes such as catalases, peroxidases, glutamic dehydrogenases, esterases, and alkaline phosphatases produced by this fungus.

The most important enzymes produced by *Monilinia* spp. may be the cutinases needed to penetrate the intact surface of fruit.[75,91] High levels of these enzymes may result from former activation, as in the necrotroph *Fusarium oxysporum*.[125] In the case of *M. fructicola*, gene expression of the cutinase *MfCUT1*, which is up-regulated in an oxidant environment, contributes directly to the virulence of the pathogen.[91]

Cellulase has been found in all species of *Monilinia*, but its secretion seems to be very restricted. The cellulase secretion was detected in *M. laxa*, whereas for *M. fructigena* a trace of activity was detected in extracts of rotted pear fruits. A very weak cellulase activity for *M. fructicola* in medium was found.[36]

The polygalacturonic acid chain is attacked by three enzymes, which are secreted by all three *Monilinia* spp., namely, (i) endopolygalacturonase (EC 3.2.1.15), which hydrolytically attacks polygalacturonic acid; (ii) pectin lyase or pectin methyl-*trans*-eliminase (EC 4.2.2.10), which attacks a polygalacturonic acid of a high degree of esterification; and (iii) pectin esterase or pectin methylesterase (EC 3.1.1.11), which liberates the methoxyl groups from the carboxyl groups of the galacturonic acid. The optimum pH for each enzyme differs for each species.[36] An important factor for the expression of *Monilinia* spp. polygalacturonases is the presence of calcium in the extracellular environment.[126] Recently, Chou et al.[5] investigated five endopolygalacturonase (endo-PG) genes in *M. fructicola*. They were differentially expressed during pathogenesis and in different culture media. *MfPG1* was the one mainly expressed. Gradziel and Wang[127] observed that an overexpression of *MfPG1* diminished the virulence of the pathogen. The authors suggested that *MfPMG1* expression could be due to the activation of the plant defense by higher levels of reactive oxygen species (ROS) produced in this case.

Among enzymes degrading neutral sugars (arabinans and galactans) from the host cell wall, α-L-arabinofuranosidase (EC


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3.2.1.55) from *M. fructicola* was found to release monomeric arabinose from arabinans by hydrolyzing the terminal bond.[127] This enzyme is localized in the hyphae of *M. fructigena*, may migrate to the plant plasmalemma, and can be secreted by a process of reverse pinocytosis with involvement of multivesicular bodies.[128] Other enzymes that degrade neutral sugars have been found in *M. fructicola* as β-galactosidase, but have not been studied in detail.[36]

**4.7. Postpenetration.** Once infection is established, the hyphae of the pathogen spread through the host tissues and bring about symptoms such as browning and softening of the tissue in fruit (Figure 7).

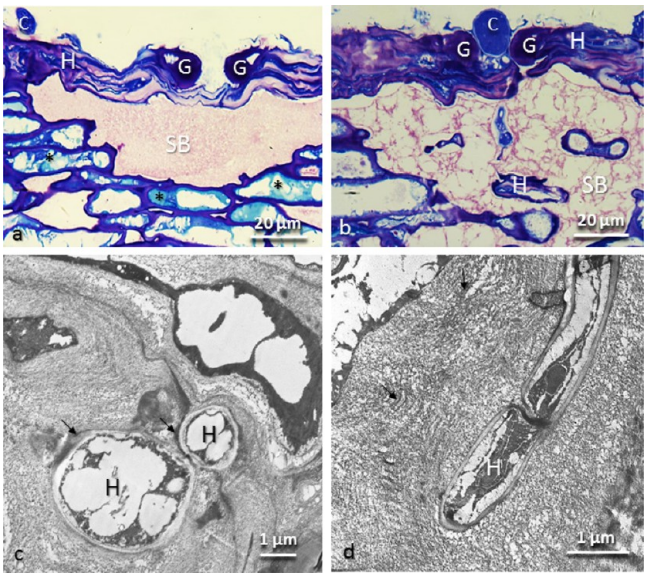


**Figure 7.** The borderline between resistance and susceptibility to *Monilinia laxa* is often faint. In this figure the fungal infection, 48 h after artificial inoculation on fruits from two peach varieties, is illustrated both at light (a,b, Toluidine blue staining) and transmission electron microscopy (TEM, c,d) level. Both resistant BO92038071 (F1 from the cross 'Contender × Elegant Lady', left panels) and susceptible 'Elegant Lady' (right panels) present discrete fungal colonization on the epidermis with stacked hyphae (H) and conidia (C), sometimes germinating over guard cells (G). At this infection stage, the substomatal regions[10] appear digested in both fruit varieties as shown by the pink staining of pectins; however, only in the resistant fruit (a) infection is blocked, possibly by the deposition of plant phenolics (asterisks) in the adjacent cells. TEM images show that resistant fruit hyphae, although able to digest cell walls, are almost encapsulated by electron-dense material (c, arrows), probably of phenolic origin. This material is not present in the fungal−plant interface in susceptible fruit infection (d), where the cell wall matrix has been almost digested and cellulose fibrils (arrows) are completely disaggregated.

The spread of BR pathogens is generally intercellular. It could penetrate and permeate any part of the host. Investigations by Reinganum[129] showed a particular affinity of *M. laxa* for the middle lamella region. Transmission electron microscopy of *M. fructigena* attacking pear fruit also confirmed that hyphae are generally intercellular, although in particular infections they become occasionally intracellular and the dead protoplasts are pushed across the cell lumen.[124]

Changes in the host plasmalemma could occur even if the membrane is intact. In infected tissues, its function could be drastically impaired as shown by conductivity measurements, resulting in leakage of sugars and amino acids from cells. Subsequently, the pathogen has sources of carbon and nitrogen to use. Moreover, if membranes of vacuoles, mitochondria, chloroplasts, or other organelles have been damaged, their contents mix, following a process described as decompartmentalization.[36]

Endopolygalacturonase and pectin esterase activities generate low molecular weight metabolites.[95,130] These secretions cause the collapse of the affected host cell.[90,130,131] Willaman[132] suggested that a hydrophilic gel of calcium pectate is formed from pectin degraded by *M. fructicola*. This gel may help the permanence of the fungus in the fruit mummy.[36]

In fruit, the rate of increase in rot diameter depends on the combination of environment conditions, the host genotype, the pathogen species, and the stages in fruit maturity.[124] After a few days, conidial pustules of the fungus burst through the fruit epidermis and cuticle. Apart from allowing the fungus to perpetuate itself, this bursting leads to the desiccation of the host tissues and often, ultimately, to the formation of a mummified fruit. In the meantime, the pathogen develops a stroma of dense mycelium within the host.[36]

In conclusion, the infection process unfolds differently depending on the growth stage of the fruit. Some steps may be extended and others avoided. The fungi may deploy different strategies mobilizing specific structures (e.g., appressorium), developing processes (e.g., melanization, acidification), and deploying a large arsenal of enzymes. Although many works have identified different elements involved in the infection process, it is still not possible to fully comprehend the successive steps of the infection progress (Figure 8).

## 5. HOST FACTORS FOR BR RESISTANCE/SUSCEPTIBILITY IN FRUIT

Research has long tried to identify host factors contributing to BR resistance. Byrde and Willets[36] listed some of them: flowering date, fruiting habit, gumming of wounds for cherries, duration of flowering for apricots, cork in lenticels, fiber and pentosan contents, parenchyma plugs in stomata, skin thickness, and texture on ripening for plums. However, the authors emphasized the importance of caution because evidence is based on only a few cultivars.

To date there is limited evidence on factors limiting BR in mesocarp, and most research has shown that BR resistance relates to fruit epidermis.[75,116,127,133,134]

**5.1. Constitutive Components of BR Resistance: Plant Cuticle, a Multicomponent Barrier.** The plant cuticle is supposed to constitute an efficient mechanical and chemical barrier against most of the pathogens that colonize the plant surface, as a form of constitutive defense of the plant. The different layers of the fruit surface (waxes, cutin, epidermis cells) and its attributes (trichomes) may each play a role in this barrier, but these roles are not yet well understood. To develop infection, the fungi need to pass mechanical barriers corresponding to the successive barriers of fruit skin. Recent observations are starting to reveal complex inter-relationships between cuticular lipids and immunity, suggesting that the cuticle is not just a physical barrier, because a variety of biochemical compounds localized in different layers or tissues may play a role in the fruit's defense against infection.

The first level is the epicuticular wax layer that covers the cuticle and is a complex mixture of very long saturated, unbranched-chain aliphatics and *n*-alkanes, ranging in carbon number from 21 to 33, depending on the plant taxa.[135−137]


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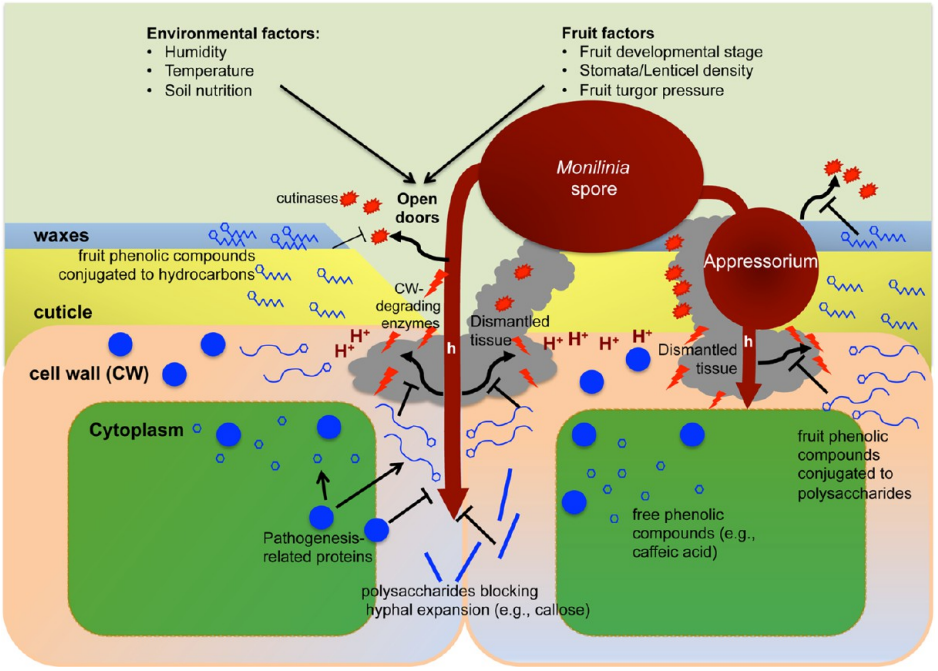


**Figure 8.** Main components of the biochemical warfare between *Monilinia* spp. fungi and *Prunus* fruits. Germinated spores can develop hypha that can (i) enter through open doors (microcracks, lenticels, or trichomes basis) or (ii) penetrate the cuticle after its degradation by fungal cutinases and subsequent appresorium formation. After cuticle breakdown, cell wall degrading enzymes hydrolyze cell wall polysaccharides through cellulases, pectinmethylesterases, and exo- and endopoluygalacturonases, among others, generating dismantled tissue (gray). Fungus-induced organic acid biosynthesis is another process that promotes fungal colonization. Polyphenol substances can be constitutively present or synthesized in response to pathogen colonization, among epicuticular waxes (light blue), cuticle (yellow), and cell wall constituents, or in the cytoplasm. Polyphenol substances stop hyphal colonization by creating a chemically adverse environment that results in a reduction in the gene expression of fungal cutinases or cell wall degrading enzymes. Pathogenesis-related enzymes that constitutively are present in fruit tissues are able to activate the phenylpropanoid pathway as well as peroxide emission. In some cases, cell wall strengthening by callose deposition may block the infection progress.

Waxes can form crystals that enhance water repellence and prevent the formation of the film of water crucial for spore germination. If wounds occur, new wax plates are formed to repair or protect the fruit.

However, factors such as temperature, the health status of the plant, and chemical treatments may interfere with this process.[92] In their review, Reina-Pinto and Yephremov[138] exposed various studies demonstrating that cuticular lipids play a role as messenger molecules in plant−pathogen interactions. For instance, Podila et al.[139] showed that the germination and appressorium formation by *C. gloeosporioides* in avocado is induced specifically by the surface waxes of this host, but not by waxes from other plants.[114] The authors explained this effect by the longer chain in fatty alcohols, the presence of terpenoid components, and the absence of inhibitors that allow the fungus to use the host surface wax to trigger germination and differentiation of infection structures. Some studies reported stimulatory effects of extracted cuticular waxes on the germination and differentiation of *Magnaporthe grisea*, *Metarhizium anisopliae*, and *Puccinia graminis* f. sp. *tritici*.[140−142] Similarly, *Blumeria graminis* f. sp. *hordei* germination was more rapid and greater on the surfaces of intact than dewaxed barley.[143]

On the contrary, it was reported that cuticular waxes inhibit conidial germination of plant pathogens, such as *Podosphaera leucotricha*, on certain varieties of apples.[124] This evidence suggests that the different constituents of waxes may play opposing roles for the pathogens. The extension of the scope of the results exposed above is limited because the quantity and composition of cuticular wax shows great variability among different plant species, different organs of an individual plant, and/or during the ontogeny of individual organs.[144] Unfortunately, with respect to *Monilinia* spp., there is a lack of information on the role of waxes in fruit−fungi interactions and a direct translation of results from other plant−pathogen couples is not valuable. Further specific studies are therefore needed to decipher wax's role in *Monilinia* spp. infection.

The cuticle is the second barrier that the fungi need to cross. This structure consists of hydrocarbon polymers and cutin synthesized exclusively by the epidermal cells. For example, the cuticle of *Prunus persica* fruit has been characterized as a complex of structures with various protective purposes. In this species, cuticle is composed of 53% cutan, 27% waxes, 23% cutin, and 1% hydroxycinnamic acid derivates; trichomes are covered with a thin cuticular layer containing 15% waxes and 19% cutin and filled by polysaccharide material (63%) containing hydroxycinnamic acid derivatives and flavonoids.[93]

The cuticle is structurally diverse among species, but exhibits the organization of a composite material consisting of cutin, a polyester that is partly covered and interspersed with waxes (epicuticular and intracuticular waxes).[145] However, a characterization of the fruit surface of diverse varieties of *Prunus*, to determine cultivar-specific skin features, has not been developed to date. Considering the cuticle as a structure of resistance to pathogen penetration deserves caution, seeing that its proprieties are dependent on qualitative and quantitative chemical composition. Indeed, a complex inter-relationship between the cuticular lipids and the fungus may occur, playing a molecular messenger role in interactions between plant and pathogen. As well as some components of epicuticular waxes,


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they can act as fungal pathogenicity activators or, in contrast, inhibit the infection. Isaacson et al.[146] demonstrated on tomato that cutin plays an important role in protecting tissues from necrotrophic infection by *Botrytis cinerea*. According to Kolattukudy et al.,[114] some pathogens sense plant surfaces thanks to cuticle monomers that may be produced by basal cutinase activity of fungal spores. Sensing of cutin monomers would then induce high levels of cutinase required for penetration.

In conclusion, the cuticle is thought to be a crucial factor in the fungal penetration process. However, as previously mentioned, the cuticle is not a continuous layer. It may display discontinuous sites as secretory tissues, trichomes, stomata, and even pores that could be "open doors" for pathogen colonization, as well as the presence of fractures in the epidermis.

The last barrier in the surface is the epidermis cell wal, which can vary in composition and thickness. The major substance that reinforces the cell wall structure is lignin. The process of lignification could improve the resistance of the cell wall against the action of degradation enzymes and block the diffusion of pathogen toxins and the diffusion of nutrients from the fruit, restringing the process of colonization. Sites around the infection point could also accumulate callose, suberin, tannin, and pectin substances.[147]

**5.2. Phenolic Acids and Their Redox-Mediated Role in Fungal Inhibition.** Early studies of peach phenolic compounds started from the observation that fruit from 'Bolinha' peach cultivar, known to be resistant to BR, displayed high levels of these compounds in their epidermis. This group of compounds became one of the most studied for BR resistance.[127]

Among the phenolic compounds of the epidermis of peach fruit, chlorogenic and caffeic acids have high concentrations, especially in immature fruit and in fruit of peach genotypes with a high level of resistance to *M. fructicola*.[116] In cultures of *M. fructicola*, these phenolic acids did not suppress spore germination or mycelia growth, but they inhibited cutinase activity.[75] Likewise, the presence of caffeic acid in cultures prevented the appearance of two major cutinase isoforms.[75] In addition, a series of cinnamic and benzoic acid derivatives also suppressed cutinase levels in culture.[38] These results led the authors to suggest that chlorogenic acid and related phenolics, in combination with other factors such as iron, could have a role in arresting *M. fructicola* in quiescent infections.[63] Furthermore, they may contribute to resistance by interference with the production of factors involved in the degradation of host polymers. Subsequent studies in vivo confirmed the effects of caffeic acid, chlorogenic acid, or reduced glutathione on infection development. Adding those compounds in conidial suspensions of *M. fructicola* did not inhibit germination on flower petals and fruit, but inhibited appressorium formation from germinated conidia and subsequent BR lesion development.[116]

Further work conducted by the same group showed that antioxidant phenolic acids suppressed mRNA accumulation and enzyme activity of a cutinase.[148] However, other antioxidant compounds also significantly attenuated *M. fructicola* cutinase production, indicating a general effect of antioxidants rather than a specific effect of a given phenolic compound (see section 5.3).[91,148]

Villarino et al.[122] demonstrated that chlorogenic acid and its isomer, neochlorogenic acid, can interfere with the production of melanin in *M. laxa* without any effect on the growth and germination of the fungus (see section 4.4).[149] Even though these results are interesting, the role of the different phenolic compounds in limiting *Monilinia* spp. remains unsolved. Prusky and Lichter[150] have reviewed pathogen quiescence in postharvest diseases and discussed how fruit factors such as high acidity and phenols in unripe fruits can contribute to disease resistance.

**5.3. Active Mechanisms in Response to Pathogen Attack: Defense Proteins.** Although cuticle research has mainly focused on the analysis of cuticular lipids, cuticular proteins may also be of importance. They are referred to as lipid transfer proteins (LTPs), and many have been shown to play an important role in plant defense.[138] They specifically inhibit pathogen and pest enzymes by forming complexes that block active sites or alter enzyme conformations, ultimately reducing enzyme function. They include defensins, amylase inhibitors, lectins, and proteinase inhibitors. Unlike simple chemicals such as terpenoids, phenolics, and alkaloids, proteins require a great deal of plant resources and energy to be synthesized; consequently, many defensive proteins are made in significant quantities only after a pathogen or pest has attacked the plant. Once activated, however, defensive proteins and enzymes effectively inhibit fungi.

On defensins in particular, Nanni et al.[151] investigated the possible role of *Ppdfn1* in peach defense against fungal pathogens. *Ppdfn1* gene expression was analyzed in peach tissues susceptible to *M. laxa*, such as flowers and fruit, and its induction upon pathogen infection was tested. They concluded that *Ppdfn1* displayed an antifungal activity through specific interactions with the membrane lipids of the fungi.

Plants also produce hydrolytic enzymes, such as chitinases, glucanases, or lysozymes, in response to fungus attacks. Zemanek et al.[152] showed increased levels of mRNAs encoded by the $\beta$-1,3-glucanase gene following treatment of a peach cultivar with culture filtrates of the fungal pathogen *M. fructicola*.

The changes in the transcriptional level of genes coding to pathogenesis-related proteins (PR) have also been associated with the BR infection process in European plum fruits (*Prunus domestica* L.). It is well-known that some families of PR proteins are inducers of phenylpropanoid accumulation and other resistance effectors.[153] El-kereamy et al.[154] described differential expression patterns of the PR-10 coding gene among two European plum cultivars with contrasting BR-resistance phenotypes, as well as other transcripts coding to intermediary proteins in the signaling pathway of this PR. The authors observed that after *M. fructicola* artificial inoculation, transcripts of PR-10 and phospholipase D-$\alpha$ (PLD$\alpha$, a cell membrane-phospholipid degrading enzyme, involved in the signaling of stress responses) remained constitutively expressed in the resistant variety (cv. 'Violette'), whereas in the susceptible one (cv. 'Veeblue') these levels increased after pathogen attack. Hydrogen peroxide concentration in fruit tissues correlated with transcript pattern of these genes on both cultivars, with higher but steady levels of the compound in the resistant cultivar, suggesting an inhibitor role for the pathogen.

The same authors demonstrated the antifungal activity of PR-5; its differential expression among plum cultivars was correlated with their BR resistance. Activity showed a pattern similar to PR-10, that is, no significant change in PR-5 transcript levels after infection in resistant cultivars ('Violetta' and 'Stanley') and a rapid increase in susceptible genotypes


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('Veeblue' and 'Victory'). Furthermore, the ectopic overexpression of this protein in *Arabidopsis thaliana* transformants increased resistance to *Alternaria brassicicola*, as well as a higher induction of camalexin biosynthesis, and transcript abundance of genes coding to phenylalanine ammonia-lyase (PAL; a central point in phenylpropanoid and phytoalexin biosynthesis) and to three cytochrome P450s involved in the biosynthesis of some antifungal phenolics.[155] Finally, the same authors,[156] described a very similar expression pattern after *M. fructicola* infection in the gene coding to MYB3 transcription factor of European plums, suggesting an intermediary role of this transcription factor in the hormone-mediated defense responses that result in the induction of PR proteins. The study of the variability of these genes, which have effects in defense pathways, in *Prunus* germplasm collections has a crucial importance in the generation of knowledge for the development of more resistant varieties of fruit species.

**5.4. ROS, Oxidative Stress, and Programmed Cell Death.** The knowledge of virulence mechanisms in BR is still rudimentary; however, recent research reported that ROS play dual roles in plant-host interactions. The production of ROS can either stimulate host resistance or enhance pathogen virulence. Chiu et al.[157] examined the regulation of the gene *Mf CUT1* (that encodes the major cutinase of *M. fructicola*) by redox status. The authors reported that gene expression is down-regulated by caffeic acid (CA) and by the antioxidant glutathione (GSH) and up-regulated by a GSH synthesis inhibitor, the buthionine sulfoximine (BSO). These results indicate that changes in cellular redox status could affect the virulence of BR and suggested that redox cycling is related to this regulation.

Liu et al.[158] investigated the production of hydrogen peroxide, a major component of ROS in peach flower petals, in response to *M. fructicola* and *Penicillium digitatum*, a nonhost pathogen. During the interaction with the host, *M. fructicola* induced hydrogen peroxide accumulation in flower petals, high levels of protein carbonylation, lipid peroxidation, and a significant reduction of hydrogen peroxide accumulation in tissues. They also observed a reduction in the incidence of BR with application of exogenous antioxidants. The presence of *M. fructicola* spores at the surface of intact flower petals induced gene expression and increased enzyme activity of NADPH oxidase, a membrane-bound enzyme complex important to generate ROS and cell wall peroxidase in host tissues. This resulted in the production of hydrogen peroxide, whereas the same tissues inoculated with a nonhost pathogen did not show significant responses.[158] These results suggested that the antioxidant compounds can influence intracellular antioxidant levels in the pathogen and that changes in the redox environment may influence both gene expression and the development of structures used by the pathogen to facilitate infection.[116]

In some cases the fruit can respond by the death of cells around the point of infection, the formation of phellogen at the margin of twig lesions in stone and pome fruit trees,[159] the suberization of walls of surrounding living cells in fruit, and the accumulation of phenolic compounds in cells up to 20 cells around the distant site of initial infection. Despite such responses aimed at limiting the spread of BR; growth of mycelium may continue, although the activities of some enzymes are inhibited. Several penetrations within a small area would produce a greater and more obvious reaction by the host. The results obtained by Jekins and Reinganum[160] with *Sclerotinia fructicola* on stone fruit suggest that sometimes the host response to penetration permanently inactivates the fungus.

The diversity of studies and results published indicates a complex multifactor resistance that may involve different types of defenses localized in different tissues (epidermis and mesocarp). They highlighted the involvement of constitutive factors (mechanical barrier) and active compounds (waxes, cutins, phenolic acids) as well as specific responses to the attack (proteins and enzymes, ROS). However, no generic model of fruit resistance to BR has been proposed.

## 6. BREEDING FOR BR RESISTANCE

Currently, commercial cultivars are more or less sensitive to BR. The peach cultivar known to have one of the highest levels of resistance is the Brazilian cultivar 'Bolinha'.[161,162] Feliciano et al.[163] investigated resistance in peach cultivars and found that 'Bolinha' had fruit with particularly small size and a thick cuticle with high phenolic content. This cultivar has been used as a donor of BR resistance in conventional breeding for canning and low-chill peaches despite its poor fruit quality, high susceptibility to enzymatic browning, reduced fruit size, and high rate of preharvest fruit drop.[134,155,164,165] The case of 'Bolinha' demonstrates the challenge of breeding for BR, as characteristics associated with fruit resistance may conflict with commercial requirements. As mentioned before, Bostock et al.[75] suggested that cuticular characteristics may be involved in BR resistance. Many other fruit traits discussed in previous sections of this review may be implicated in host resistance to BR in stone fruit. However, the statistical and genetic correlations of those traits with the BR phenotype, as well as their genetic basis, are poorly understood.

Apart from cultivar 'Bolinha', from which many studies have developed knowledge about host resistance to BR in peach and stone fruit, few sources of resistance have been discovered (see below), and no commercial cultivar of peach with melting flesh declared to be resistant to BR has been released by any *Prunus* breeding program around the world. Regardless of the lack of sources of BR resistance found in the germplasm of stone fruit, this trait is presently a major objective for breeding programs in different countries for cherries (sour and sweet), apricots, plums, and peaches. Hence, deciphering the genetic control of resistance to BR remains a challenge.

**6.1. Genetic Resources, Breeding Programs, and Phenotyping Strategies.** As mentioned before, some traits associated with host resistance to BR are present in cultivars or accessions of poor commercial and productive quality. Identifying reliable sources of resistance to be introgressed in high fruit quality genetic backgrounds is one of the main objectives of such breeding programs. However, one of the first steps for the establishment of breeding programs or genetic studies for a given trait is the definition of a reliable measurement or phenotyping protocol, to compare afterward the phenotypic variations among a population of genetically diverse individuals (cultivars, accessions or offspring from a cross), and then the identification of interesting breeding materials on the basis of robust phenotypic data. In the case of assessment of cultivar-dependent BR impact on stone fruit, there is a lack of consensus in the employed experimental strategies, and each laboratory has adopted a particular protocol, according to its experimental capacities and/or specific objectives.


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**6.2. Fieldborne Inoculum Assessment.** The simplest system to score BR resistance is to assign to each analyzed accession a resistance level from a subjective scale fixed by the observer, based on the disease impact caused by fieldborne inoculum. Although it is scarcely precise and is highly subjected to the criterion of the evaluator and the environmental and climatic conditions on the experimental orchard, this strategy offers a quick way to evaluate a large number of accessions. The use of this strategy has been reported in the selection of numerous promising accessions with relatively high BR resistance in breeding programs all over the world, mostly for peach and sour cherry.

In the Fruit Research Institute of Cacak (Serbia), preliminary evaluation of BR resistance of indigenous "vineyard" peach accession germplasm was made by the use of a six-level scale, which allowed the identification of 11 evaluated accessions showing higher resistance level (described as "symptoms are not observed") during three years, among a total of 75 genotypes evaluated.[166] In the same research center, but in the sour cherry breeding program,[167,168] a subjective scale from 1 to 9 (1 for no attack, 9 for very strong attack) was used to evaluate 11 advanced selections at the final step of the selection process, as well as 9 landraces from autochthonous germplasm.[72] Advanced selections showed relatively high levels of resistance (scores between 2 and 3), but a slightly higher diversity was found in the local genotypes collection (scores from 1 to 4). Subjective scale scoring was also used in the sour cherry breeding program of the Institute of Plant Breeding in Dresden, Germany, as well as in the beginning of the peach breeding program aiming to develop cultivars adapted to humid and temperate climates at Embrapa in Pelotas, Brazil, from which mid- to high-resistant cultivars such as 'Olympia' and selection 'Conserva 947' have been generated.[169]

**6.3. Artificial Infection Assessment.** BR resistance evaluation can also be scored by artificial infection of harvested fruit under laboratory conditions. This allows the control of many factors that can affect the final result of BR impact in an experiment, such as elimination of fieldborne spores from the fruit surface, presence/absence of skin barrier (wounded/unwounded fruit), spore concentration, and temperature, humidity, and time of incubation before BR impact measurement. It also allows following infection progress by recording the diameter of the BR lesion.

One of the first groups that started to use artificial inoculations of BR was at University of California—Davis, within the cling peach-breeding program.[127,170,171] Researchers considered the average rot diameter 72 h after inoculation (10 $\mu$L drop of a conidial suspension of *M. fructicola* containing $10^5$ spores/mL, on previously diluted sodium hypochloride and ethanol-disinfected fruits), as specified elsewhere.[110] In this way, a large phenotyping effort has been carried out to screen mature fruit for resistance to *M. fructicola* in over 4000 peach genotypes from very different origins: landraces, standard canning peach cultivars, advanced experimental selections with various pedigrees including some with 'Bolinha' heritage as well as some interspecific hybrids generated to introgress BR resistance from almonds. The material selected with this protocol has been useful also for studies of genetic dissection of the BR resistance trait in segregating populations (see QTL of Resistance).

As mentioned in other sections of this review, Pascal et al.[133] evaluated two screening tests for resistance to *M. laxa* in apricots (7 accessions), peaches (12 accessions), and diploid plums (7 accessions of *P. salicina*, *P. cerasifera*, and interspecific hybrids between them) at INRA, Avignon, France. The tests consisted of artificial inoculation of uninjured and artificially injured fruit. Each fruit was inoculated with a 20 $\mu$L droplet containing conidia of *M. laxa* at a concentration of $10^6$ spores/mL. Percentage of infected fruits and rot diameter progression were recorded (Figure 9). The authors observed no correlation between the BR resistance rankings from the uninjured and injured tests. Accordingly, they suggested that epidermal resistance and flesh resistance were not linked processes. This work also highlighted high variability of lesion progression within the uninjured test and very similar rot spread within the injured test, suggesting that no resistance was expressed at the flesh levels in the tested material. At INRA—Avignon, a breeding program focused on pest resistance (including resistance to BR by *M. laxa*) has generated very interesting materials, such as introgression of *Prunus davidiana* resistance to peach materials.[108]

**Figure 9.** Development of brown rot 5 days post artificial infection in nectarines of cv. 'Summergrand' at maturity. Fruits were disinfected in a water bath at 55 °C for 40 s, put in acrylic plastic boxes, and infected with one 10 $\mu$L drop at $10^5$ spores/mL concentration deposited without wounding. Fruits were put in a chamber with controlled temperature (18 °C) and 24 °C, respectively, during dark (8 h per day) and light (16 h per day) storage. High humidity was maintained in the closed boxes.

Material from the breeding program of Embrapa-Pelotas (Brazil) has also been screened with artificial inoculation, and BR resistance results on these breeding materials have been reported.[162] BR screenings were made by monitoring the percentage of infected fruits 72 and 96 h after spraying a solution (containing $10^5$ spores/mL) over intact harvested fruits. The authors observed a significant genetic component when comparing some selections and cultivars. Interesting selections such as 'Conserva 1798', 'Conserva 1596', 'Conserva 1218', and 'Cascata 1493' were identified.[165] Authors evaluated three crosses ('Conserva 672' × 'Maciel', 'Conserva 672' × 'A.334', and 'Leonense' × 'Bolinha') by drop-inoculations. Broad-sense heritability was estimated to be around 80%. Twelve seedlings from these three progenies were determined to be of equal or better resistance than the 'Bolinha' cultivar.

Resistance to *Monilinia* spp. in peach cultivars for the fresh market has been an important objective in the breeding program of University of Milan (formerly at University of Bologna, Italy). Offspring from crosses between melting flesh peaches were selected.[172,173] In these works, mature fruits were


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artificially sprayed with a suspension of *M. laxa* ($10^5$ spores/mL), and the disease impact was registered as percentage of infected fruits after 5 days of incubation at 25 °C and 95−100% relative humidity. Several parental combinations were analyzed. The 'Contender' × 'Elegant Lady' F1 population presented the most interesting results with individuals presenting higher levels of resistance than the resistant parent (cv. 'Contender'). Besides generating prebreeding materials, this population has been useful in the genetic dissection of BR resistant-related traits.[174] This group is currently developing new phenotyping strategies, based on in planta spray of conidial suspension of *M. laxa*, aimed at increasing the capacity of sample analysis in breeding programs bearing high numbers of seedlings, obtaining promising results for scoring BR resistance phenotype.[175]

Studies of BR resistance evaluations in apricots by artificial inoculum have been reported mostly from two breeding programs. At the Regional Council for Agriculture of Rome (CRA-FRU, Italy), several apricot accessions showing high BR resistance have been evaluated by artificial inoculation procedures consisting of fruit disinfection (diluted sodium hypochlorite and ethanol), inoculation with a drop of *M. laxa* conidial suspension ($10^5$ spores/mL) in two points near the peduncle cavity, incubation for 7 days at 22 °C, and registration of affected fruit percentage. Among the evaluated crosses, the authors found remarkable levels of BR resistance: selections such as '485GII37', '493C12III61', and '493 C12 VI 1' (open pollinations of cultivars 'Don Gaetano', 'Fiammetta', and 'Boreale', respectively) showed 0−10% of infected fruits. whereas 'Don Gaetano' F2 seedlings such as '493C11VIII8' or '493C11VIII26' showed very high infection rates (>50%). On the basis of the observed segregations, the authors concluded that BR resistance on the analyzed crosses behaves as a quantitative trait.[30,176,177]

Walter et al.[178] tested several methods to evaluate BR in 'Sundrop' and nine accessions from the 'Clutha' series ('Sundrop' × 'Moorpark'), bred in HortResearch at Clyde research orchard (Alexandra, New Zealand). In this study, the authors analyzed some infection parameters for three seasons: lesion area (artificial drop infections with *M. fructicola* and *M. laxa* spore suspensions in wounded and intact fruits), spore count on lesions, storage rot (natural orchard infection at room temperature and high humidity), and cuticle thickness. The authors determined that the most robust method to evaluate BR resistance in apricot was measuring lesion area on wounded, artificially infected, fruits 72 h after inoculation. However, they recommended combining more than one method for the evaluation of the material. Remarkably, the accession 'Clutha 14/107' showed significantly highest value of resistance to *M. fructicola* (measured as the mean of lesion area obtained in three seasons), the lowest quantity of produced spores per square millimeter lesion, a storage rot rate of <5%, and one of the highest cuticle thicknesses.

BR resistance was screened in several released cultivars and advanced selections from the sweet cherry breeding program at the Pacific Agri-Food Research Centre, British Columbia, Canada.[179] During 4 years, a total of 36 genotypes were submitted to artificial inoculation (25−50 fruits triplicates per accession; ethanol and sodium hypochlorite fruit disinfection; spray of $10^4$ spores/mL; incubation at 13 °C and 95−97% relative humidity; BR impact assessment after 8 and 11 days after inoculation). On the basis of the difference in the percentage of rotten fruits between each genotype and the overall population mean, they established three resistance categories: more resistant than overall mean, close to mean, and less resistant than mean. Although they identified some cultivars showing a high resistance level in two of four years (cultivars 'Staccato', 'Stardust', and 'Sweetheart'), the authors stated that the observed resistance level was not enough to avoid fungicide applications in plants of these accessions and confirmed the results of Brown and Wilcox,[180] demonstrating that there are no sources of high-level genetic resistance to BR in sweet cherry materials.

Although it is difficult to find reliable sources of resistance in stone fruit species, seasonally consistent differences in the tested materials have been observed in all of the works presented in this section. The existence of these differences indicates that exploring wider germplasm and using these sources to introgress resistance in cultivars of high fruit quality could result in new selections with improved BR resistance.[170] The "BR-resistant" cultivars and selections found up to now still have too low resistance levels to allow the suppression of fungicide application; however, the most resistant could already be cropped under integrated pest control strategies, suited to minimize exogenous chemical input in the orchard.

Finally, as can be observed from the cited works, screening for BR resistance in germplasm collections and/or offspring is a very time- and effort-consuming task and often underappreciated because results are frequently hampered by the influence that climatic conditions and agronomical practices exert on the level of resistance and pathogen strength. However, the variability observed between cultivars allowed identifying suitable materials to generate populations segregating for BR resistance and to perform genetic studies for identification of genetic determinants associated with the variation in the phenotype.

**6.4. QTL of Resistance.** To generate new cultivars with less necessity of fungicide inputs, the identification of genes or loci associated with resistance to BR would allow progress in the incorporation of favorable alleles in breeding programs. In addition to functional studies seeking to understand the interactions between the pathogen and its host, genetic studies have been conducted to identify genomic regions associated with BR resistance. Although possible mechanisms of resistance can be inferred from these studies, their principal objective is the discovery and further incorporation of resistance alleles into breeding materials with the use of linked markers. Indeed, high-throughput molecular genetic tools and a high-quality genome sequence have been developed recently for peaches[181] and can now be exploited to radically improve the efficiency of disease resistance breeding in peaches, as well as in other *Prunus* species. Indeed, as commented before, breeding programs aimed at enhancing BR resistance have been impaired by time-consuming procedures for assessing this trait on field-grown segregating trees.

Therefore, an important objective is the generation of new tools for the early selection of seedlings with enhanced BR resistance. Marker-assisted selection is a valuable strategy for these purposes, as it allows the early selection of seedlings bearing favorable alleles at marker loci genetically linked to genomic regions that control the trait of interest. Considering that fruit resistance to BR may be a multifactor system and that each different cultivar may hold only a little part of these factors, dealing at the same time with different sources of resistance may lead to confusion rather than to better understanding. Therefore, association studies have not been


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engaged, and the first studies seeking QTL of resistance had focused on biparental progenies stemming from a cross between a susceptible parent and a potential donor of resistance. This approach may represent the first compulsory step to identify genome regions controlling resistance. Hopefully, the comparison of detected loci between crosses may help identify different factors of resistance from different donors. The final step would then be the combination of these different factors in elite genotypes to confer higher resistance.

To date, two studies exploring genomic regions linked to BR resistance have been published, both using peach host species. Martinez-García et al.[110] performed a QTL analysis using *M. fructicola* resistance phenotypic data of 73 seedlings from the Pop-DF progeny ('Dr. Davis' × 'F8, 1-42'), with parental accessions derived from canning peach and peach−almond backcrossing in the UC—Davis breeding program.[110] A linkage map composed by 1037 SNPs segregating through the population was used for Interval Mapping-QTL analysis. The study revealed three QTLs, two of them in LG1 and one in the LG4 of *Prunus* genome. The genomic region of one of the QTLs in LG1 was significantly correlated with three years of phenotypic evaluation. The region included two potential candidate genes, coding for PAMP-triggered immunity, and effector-triggered immunity (ETI) proteins. SNP markers of this region are promising tools to enhance efficiency of breeding programs using similar genetic background.

The second genomic study based on QTL analysis was performed using 80 melting-fleshed F1 individuals from the 'Contender' × 'Elegant Lady' cross, genotyped with a set of 89 markers (63 SSR and 26 SNP) and phenotyped for two seasons with artificial infections of *M. fructigena*, in the presence and absence of an artificial wound. The aim was to find genetic markers associated with skin and flesh resistance to BR.[174] In this material, the maturity date of seedlings correlated negatively with their BR resistance (late-maturing individuals appeared as less resistant); however, using a multiple QTL model including maturity date as a covariate phenotype, significant genotype−phenotype associations were found between skin resistance and both M1a and EPPISF032 SSR markers (located in the LG2 and LG4 of *Prunus* genome, respectively). Additionally, flesh resistance was correlated with SNPs located in LG3 of peach genome, confirming the previously reported independence between genetically controlled mechanisms for skin and flesh resistance.[132]

Despite the different results obtained in these two studies, probably due to differences in the different genetic background of the studied populations, the pathogenic agent employed, and the different phenotyping approaches, they contribute to the literature regarding the identification of potentially useful genetic markers for assisted selection of new cultivars with enhanced BR resistance.

The research community has invested in the identification of resistance sources and the development of cultivars resistant to BR. Up to now, little progress has been made in this sense. However, advances in terms of phenotyping are noteworthy, and the development of quantitative genetic studies may help to find ways of moving forward.

## 7. CONCLUSION

Understanding BR pathogenesis mechanisms, the biological barriers that *Prunus* fruit can offer to *Monilinia* spp., and the interaction between them is crucial for designing phenotyping strategies able to measure resistance level in a robust way. Such approaches are needed to identify resistance sources across the *Prunus* germplasm and provide tools for breeding new hybrids with enhanced BR resistance that, together with other alternative control strategies, could contribute to more sustainable stone fruit cropping.

In this review we have collected the information available in historic and contemporary literature about the elements involved in the interaction between *Monilinia* spp. and *Prunus* fruit. We conclude that host specificity is not a strict condition for disease impact and infection development and that one of the main causes for the success of pathogen colonization is the relatively high presence of "open doors" in some *Prunus* fruits' epidermis, especially in peaches, cherries, and plums. In the past decade, many works have identified and validated some important elements of the fungal infection and host resistance processes; nevertheless, the scientific community has not assembled these elements to generate a precise BR resistance model that explains the phenotypic diversity among *Prunus* species and their varieties. Finally, the significant influence that environment has in the infection process has been a persistent constraint that hampers a clear identification of such elements, but has to be considered in the generation of new varieties.

These elements constitute valuable information and are useful in the design of new phenotyping approaches for breeding, as well as for testing new alternative methods for BR control at the pre- and postharvest stages. BR-resistant breeds and sustainable pathogen control strategies are being developed and validated.[9,14] In the meantime, stone and pome fruit growers have the difficult task of combating damages caused by BR with lower quantities of synthetic fungicides, as recommended (or imposed) by IPM regulations and initiatives and adopting agronomical strategies and practices to eliminate natural inoculum sources.

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### Notes

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