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Vitus Ikechukwu Obi

Evaluation of tolerance to brown rot caused by Monilia laxa (Aderhold and Ruhland) Honey, in peach germplasm (Prunus persica (L.) Batsch)

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Tesis Doctoral

EVALUATION OF TOLERANCE TO BROWN ROT CAUSED BY MONILIA IAXA (ADERHOLD AND RUHLAND) HONEY, IN PEACH GERMPLASM (PRUNUS PERSICA (L.) BATSCH)

Autor

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PhD thesis

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Zaragoza, 2018

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Departamento de
Ciencias Agrarias
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INFORME FAVORABLE DE LOS DIRECTORES DE LA TESIS

Los abajo firmantes Dra. Yolanda Gogorcena Aoiz y Dr. Juan J. Barriuso Vargas, como co-directores de la tesis doctoral realizada por Vitus Ikechukwu Obi con el título

"EVALUATION OF TOLERANCE TO BROWN ROT CAUSED BY *MONILINIA LAXA* (ADERHOLD AND RUHLAND) HONEY, IN PEACH GERMPLASM (*PRUNUS PERSICA* (L.) BATSCH)"

Informan favorablemente:

que el contenido de esta tesis doctoral se corresponde con el proyecto de tesis aprobado en su momento.

Los objetivos planteados se han alcanzado satisfactoriamente, y el trabajo realizado cumple los requisitos de calidad y originalidad que establece la normativa vigente.

Y los resultados obtenidos son de interés académico, práctico y científico y las conclusiones obtenidas pueden servir de base para futuras líneas de investigación teórica y aplicada.

Todo lo cual se hace costar a los efectos de la admisión a trámite de la tesis doctoral.

En Zaragoza a 30 de mayo de 2018

Fdo. Dra. Yolanda Gogorcena Aoiz

Fdo. Dr. Juan J. Barriuso Vargas

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El que no ama no ha llegado a conocer a Dios,

porque Dios es amor

(1 Juan 4:8).

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Summary

The peach is one of the most important global tree crops within the economically important Rosaceae family. The crop is faced with numerous pest and disease, especially fungal pathogens that infect stone fruits in the field, on transit and in the store. Over 50 % postharvest, global loss has been ascribed to the brown rot disease of the species of *Monilinia* and in the recent years the disease has been so critical in the orchard that some stone fruits were abandoned. And in Spain, particularly, the disease has been associated with well over 60 % fruit losses after harvest. Although, there exist different control options, the breeding for resistance remains an ideal management option for brown rot disease control considering the uniqueness of its sustainability in the chain of crop production and environmental compatibility. The thesis aims at phenotyping peach germplasm of the Aula Dei-CSIC tolerant cultivars and progenies to brown rot of *Monilinia laxa* with good quality. The study focuses on peach breeding within the Ebro valley of the Mediterranean eco-zone, through the use of tolerant variety or genotypes with good quality characteristics in brown rot disease management. **Chapter I** sets an evaluation method, by optimizing the available protocols, to screen tolerance to brown rot by *Monilinia* spp. in peach germplasm. This was enhanced and achieved with the comprehensive bibliographic review and compilation of information currently available on peach and the resultant effect of interaction (brown rot) with species of *Monilinia* and available management options. **Chapter II** examines the effect of physicochemical factors of pH and titratable acidity (TA) in the host-pathogen interaction of peach and *M. laxa in-vitro* and *in-vivo*. This study encompasses the necessity to know the evolution of fruit maturity in new and old varieties in relation to potential *Monilinia* infection in immature fruits. **Chapter III** is a screening test based upon artificial fruit inoculation to validate on several parental lines of the peach breeding program ('Crown Princess', 'Big Top', 'Andross', and 'BabyGold 9'). In addition, cultivars with different phenolic content and early ('Tebana') or late harvested, as the Spanish traditional non-melting flesh cultivars ('Miraflores', 'Calanda Tardío' and 'Calante'), were included in the study. The correlation of pathogenic factors with their biochemical composition concerning acids, phenolic contents in flesh-fruit is also discussed. **Chapter IV** screens sixty eight progenies from the 'Babygold 9' × 'Crown Princess' population of the breeding program of EEAD-CSIC for susceptibility to brown rot of *Monilia laxa*. Physicochemical traits, such as fruit firmness and soluble solids content were recorded before and after storage. Titratable acidity, pH, and antioxidant composition were also measured at harvest for correlation with pathogenic factors.

Key words: *Prunus persica*; *Monilinia* spp; host; pathogen; stone fruits; crop protection; plant breeding; *ex-situ*; *in-situ*; germplasm; fungus; alkaline; physicochemical; genetic tolerance, bioactive, susceptibility; brown rot; phytochemicals; plant improvement; necrotrophic fungi; infection; introgression; genotypes; firmness.

Resumen

El melocotonero es uno de los cultivos frutales, de la familia de las rosáceas, de mayor importancia económica del mundo. Existen numerosas plagas y enfermedades que afectan a este cultivo, especialmente hongos patógenos de fruto que son infectivas en el campo, durante el tránsito y en el almacenamiento. Más del 50 % de la pérdida global en poscosecha se ha atribuido a la enfermedad de podredumbre parda causada por especies del género *Monilinia*, y en los últimos años la enfermedad ha sido tan acusada en el cultivo que ha producido el abandono de la producción de algunas variedades de fruta de hueso. En España, la enfermedad se ha asociado con más del 60 % de pérdidas de fruta después de la cosecha. Aunque existen ciertas opciones de control y tratamiento, la selección genética para la resistencia sigue siendo una alternativa ideal de manejo para el control de la enfermedad producida por la podredumbre parda, teniendo en cuenta su sostenibilidad y la compatibilidad ambiental. Esta tesis tiene como objetivo principal el fenotipado del germoplasma de melocotón existente en la Estación Experimental de Aula Dei-CSIC y de cultivares y progenies para la detección de tolerancias a la podredumbre parda producida por *Monilinia laxa*. El estudio se enfoca en la mejora de los cultivos de melocotones, en la Valle de Ebro de la zona ecológica mediterránea, a través del uso de variedades tolerantes o genotipos con características de buena calidad en relación a la enfermedad de la podredumbre parda. **El Capítulo I** establece un método de evaluación, al optimizar los protocolos disponibles, para detectar más fácilmente la tolerancia a la podredumbre parda por *Monilinia* spp. en germoplasma de melocotónero. Esto se logró con una revisión bibliográfica exhaustiva y la compilación de la información actualmente disponible sobre melocotón y el efecto resultante de la interacción (podredumbre parda) con las especies de *Monilinia* y las opciones de control y manejo disponibles. **El Capítulo II** examina el efecto de los factores fisicoquímicos del pH y la acidez titulable (TA) en la interacción huésped-patógeno entre el melocotón y *M. laxa* tanto *in-vitro* como *in-vivo*. Este estudio abarca la necesidad de conocer la evolución de la madurez de la fruta en las variedades de melocotones verdes y maduros en relación con la posible infección por *Monilinia* en frutos inmaduros en precosecha. **El Capítulo III** es una prueba de detección basada en la inoculación artificial de fruta para validar en varias líneas parentales del programa de mejora genética de melocotón ('Crown Princess', 'Big Top', 'Andross' y 'BabyGold 9'). Además, se incluyeron en el estudio cultivares con diferente contenido fenólico y precoces ('Tebana') o tardíos, como los cultivares tradicionales españoles de carne no firme ('Miraflores', 'Calanda Tardío' y 'Calante'). También se discute la correlación de los factores patogénicos con su composición bioquímica con respecto a los ácidos y los contenidos fenólicos en la pulpa. **El Capítulo IV** examina sesenta y ocho progenies de la población 'Babygold 9' × 'Crown Princess' del programa de mejora de EEAD-CSIC por susceptibilidad a la podredumbre parda de *Monilia laxa*. Los rasgos físicoquímicos, tales como la firmeza de la fruta y el contenido de sólidos solubles, se registraron antes y después del

almacenamiento. La acidez titulable, el pH y la composición antioxidante también se midieron en la cosecha para obtener la correlación con los factores patógenos.

Palabras clave: *Prunus persica*; *Monilinia* spp; huésped; patógeno; fruta de hueso; protección de cultivos; *ex-situ*; *in-situ*; germoplasma; hongo; alcalino; fisicoquímico; tolerancia genética; bioactivo; susceptibilidad; podredumbre parda; fitoquímicos; protección; mejora; hongos necrotróficos; infección; introgresión; genotipos; firmeza.

General introduction

Review

Peach brown rot: Still in search of an ideal management option

Preprint. Peach brown rot: Still in search of an ideal management option. [Agriculture (forthcoming), doi: 10.20944/preprints201806.0269.v1)]

Abstract: The peach is one of the most important global tree crops within the economically important Rosaceae family. The crop is faced with numerous pest and disease, especially fungal pathogens that infect it in the field, on transit and in the store. Over 50 % postharvest global loss has been ascribed to the brown rot disease especially in late-ripening varieties and in recent years the disease has been so unembroidered in the orchard that some stone fruit were abandoned before harvest. In Spain, particularly, the disease has been associated with well over 60 % fruit losses after harvest. The most common management option available for the control of this disease include: chemical, biological, and physical approaches. The effects of these treatments, especially the biochemical fungicides (BCAs and conventional fungicides) on the environment, human health, and strain fungicide resistance, incline to over cloud the intended efficacy of these control strategies. This review has intended to, comprehensively, compile information currently available on peach and the resultant effect of interaction with species of *Monilinia* cum available management options. The breeding for brown rot resistance remains an ideal management strategy for brown rot disease control considering the uniqueness of its sustainability in the chain of crop production.

Keywords: *Prunus persica*; *Monilinia* spp; host and pathogen; stone fruits; crop protection; plant breeding.

1. Introduction

The peaches [*Prunus persica* (L.) Batch] and nectarines [*P. persica* var. nectarina (Aiton) Maxim], are economically important members among the hundreds of species of *Prunus* including the cultivated almond [*Prunus dulcis* (Mill.) D.A Webb], the apricot [*P. armeniaca* (L).], the European plum [*P. domestica* (L).], the Japanese plum [*P. salicina* (L).] and the cherry [*P. avium* (L)].

Available information from FAO [FAOSTAT, 2016] in 2016 indicates important countries (Figure 1) for the production of peach and nectarines. China was leader in production (14.47 MT), followed by Spain (1.53 MT), Italy (1.43 MT), USA (0.93 MT), and Greece (0.85 MT). And for areas (hectars) under cultivation in the EU-28, Spain is the largest (86,896 ha), followed by Italy (69,005 ha) and Greece (44,271 ha).

There are numerous fungal pathogens that infect the peach both in pre-and postharvest states. Prominent ones include *Rhizopus nigricans*, *Mucor* spp., *Botrytis cinerea*, *Geotrichum candidum*, *AIternaria* spp., *Aspergillus* spp., *Penicillium* spp., and *Monilinia* spp. (Usall et al., 2015). But for the purpose of this work we shall be dwelling on the principal casual pathogens of brown rot in peaches. Species of *Monilinia* are associated with brown rot, which is the most economically important, disease of stone fruit worldwide (Rungjindamai et al., 2014; Villarino et al., 2013). Brown

rot incidence in peach greatly varies during fruit development (Mari et al., 2003). Fruits are less susceptible to brown rot at the early stage of formation becoming resistant in correspondence to pit hardening and increase susceptibility afterwards (Guidarelli et al., 2014; Obi et al., 2018).

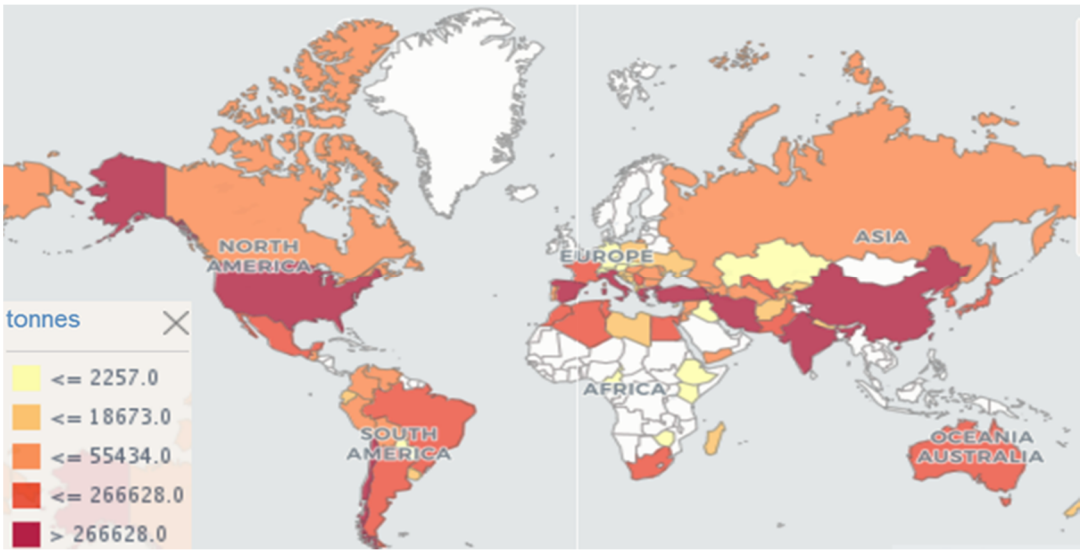


Figure 1. Global map of peach and necterine production by countries [1]
Source: http://www.fao.org/faostat/en/#data/QC/visualize (acessed on 3rd June, 2018)

Brown rot is a polycyclic epidemic (Seem, 1984) hence various secondary or monocyclic components of the brown rot infection sequence are generated throughout the annual growth cycle of the host (Schumann and D`Arcy, 2006). The fungus survives the winter (transmitted from year to year) in several structures such as mummified fruits (Casals et al., 2015), in canopy or on the ground (Hrustić et al., 2013), fruit peduncles (Ritchie, 2000), cankers on twigs, spurs and branches (Villarino et al., 2013; Melgarejo et al., 1986; Kreidl et al., 2015). These propagules infest materials, (Gell et al., 2009), serve as sources of primary inoculum to infect blossoms, buds and young shoots, to establish source of secondary inoculum.

Consequently, the universal annual losses from the epidemic have been estimated at 1.7 thousand million Euros (cited in Oliveira-Lino et al., 2016). And in Spain, particularly, the disease has been associated with up to 80 % of incidence fruit losses after harvest (Egüen et al., 2015; Usall et al., 2015; Teixidó et al., 2018), mostly under favourable environmental conditions for the commencement and growth of the diseases in the orchard.

There are various control and management strategies for brown rot epidemic in peach cultivation. These options include: biological, conventional, chemical, physical, botanicals, and host resistance techniques (Rungjindamai et al., 2014). In the present review we will focus on peach and the resultant effect of interaction with species of *Monilinia* cum available management strategies with a view to developing sustainable peach breeding scheme.

2. The Peach

The peach is the third most important global tree crop after apples (*Malus* spp.) and pears (*Pyrus* spp.) within the economically important Rosaceae family and the largest producer is China, followed by European countries (Spain and Italy), and the United States (FAOSTAT, 2016). The origin of peach is traceable to the Eastern Asian continent of Western part of China, where it could have been cultivated for 4,000 years and subsequently dispersed to Europe, Africa and America (Byrne et al., 2012). Documentation of the first cultivated peach was recorded in Chinese manuscripts as early as the 10th Century BC. In China the species presents the greatest richness in germplasm and has the largest collections of peach germplasm with wild peaches still growing today (Byrne et al., 2012; Desmond and Bassi, 2008).

In the EU agriculture, the fruit sector weight 6.7% of its agricultural output, being peaches the third after apples and oranges (EUROSTAT, 2018). In this zone the cultivation / and or production is concentrated in the countries of the Mediterranean region, including Spain, Italy, Greece and France, owing to the fact that the potential risk of damage from frost (Charrier et al., 2015) is less here than in the countries of the Northern part of the EU. Hence most plant disease models are known to use different climatic variables and operate at a different spatial and temporal scale than do the global climate models (Chakraborty et al., 2000).

2.1. Geography and ecological requirement of peaches

There are the local or modern peach cultivars depending on whether the cultivar is originally indigenous or foreign (Font i Forcada et al., 2013) in the domain of cultivation. Geographically, global commerce has brought peach tree cultivation into both the Northern and Southern hemispheres (Byrne et al., 2012) which experience contrasting summers and winters allowing for year round availability. Peach trees require wet winters and hot dry summers and will not flourish in Oceanic climates (Byrne et al., 2012). Therefore, the Spanish peaches are cultivated under the Mediterranean climate and found concentrated within specific regions (see section 2.3). Among the available varieties of peach, the yellow-fleshed varieties such as the famous Elberta, Redhaven, and Halford are preferred in North America (USA), while both yellow- and white-fleshed types are popular in Europe (Byrne et al., 2012; Font i Forcada et al., 2014).

Often times the cropping practices employed in a region for the production of peach is principally determined by the different environmental and nutritional requirements (Byrne et al., 2012). This plantation crop, though cultivated mainly in temperate zones, between 30 and 45º latitude N and S, is not very resistant to cold, and it requires up to 400 to 800 cold-hours for flowering and good fruit set (Byrne et al., 2012). They are intolerant of severe cold and, therefore, cannot be grown successfully where temperatures normally fall to -23 and -26 ^{o}C (Byrne et al., 2012). On the other hand, they do not grow satisfactorily where the winters are too mild, and most

varieties require some winter chilling to induce them to burst into growth after the annual dormant period.

Edaphically, the peach does well on various soil types but in general it grows best on well-drained sandy or gravelly loams. Hence, peach seedling is susceptible, to both calcareous and waterlogged soils (Egilla and Byrne 1989; Byrne et al., 2012). Nevertheless, the search for iron chlorosis tolerant rootstocks, using peach × almond hybrids, has led to selection of highly vigorous rootstocks such as 'GF 677' (Bernhard and Grasselly, 1981), which was widely adopted in the Mediterranean basin countries, including Spain. By the way, there exist in Spain some tolerant rootstocks to calcareous and waterlogged soil (Pinochet et al., 1999; Jiménez et al., 2008). Nitrogen-rich soils exceptionally support performance of peach crops (Gil-Albert, 1991). This improved performance is achieved when soil acidity is maintained above pH 6.0. Consequently a soil pH below 5.5 is deleterious to peach tree growth, fruit yield and size, and tree longevity (Cummings, 1989). However, such deleterious effects of soil pH below 5.5 have been associated to the toxicity of Aluminium (Al) or low Calcium (Ca) ion availability (Cummings, 1989), an issue managed by lime application to raise the soil pH.

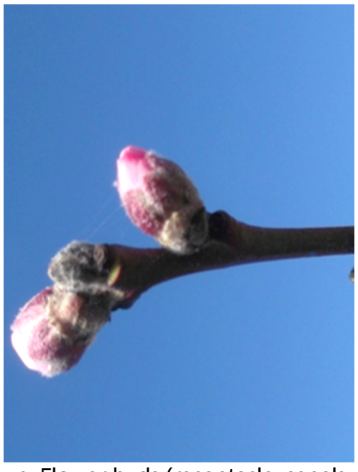
2.2. Botany and susceptibility of peach

The peaches could be classified according the flesh type (non-melting or melting) flesh colour (yellow, orange, white or yellow/orange), fruit type (peach or nectarine), and stone type (clingstone or freestone) (Font i Forcada et al., 2013). Clingstone peaches have flesh that adheres firmly to the stone and the freestones have stones that separate easily from the ripe flesh. Most yellow-fleshed peaches are clingstone varieties while white-fleshed peaches fall into the freestone category (Font i Forcada et al., 2013). The greatest difference between the two is really about texture and taste. Clingstone varieties tend to lean on the side of extremely juicy and flavour, making them very suitable for baking and canning while freestone varieties are generally less succulent and thus chosen for fresh eating in most regions. The Spanish peach industry, hitherto, was based on yellow, non-melting fleshed and clingstone types; however, the replacement of the Spanish traditional varieties by introduced ones, mostly from North America, has induced the domain of the melting flesh cultivars (Font i Forcada et al., 2014).

Peaches under commercial cultivation (Figure 2) are usually kept between 3 and 4 metres by pruning, without which they can reach 6.5 metres in height (Bassi and Moneté, 2008; Encyclopaedia Britannica, 2017),. The leaves are glossy green, lance-shaped, and long pointed; they usually have glands (Font i Forcada et al., 2013) at their bases that secrete a fluid to attract ants and other insects, enabling pollination. It is self pollinative and has an impressive blossoming (Figure 2 d). The flowers, borne in the leaf axils, are arranged singly or in groups of two or three at nodes along the shoots of the previous season's growth. The five petals, usually pink or pink-salmon (Font i Forcada et al., 2013), but occasionally white five sepals, and three whorls of stamens are borne on the outer rim of the short tube, known as the hypanthium that forms the base of the flower that can be showy or no-showy (Font i Forcada et al., 2013).

a: Fruit harvested peach orchard in the summer

b: Flowering peach orchard at early spring



c: Flower buds (receptacle, sepals, and petals intact

d: Flower in blossom (petals, stamens and pistil exposed)

e: Flower petals and stamen shrinking and about to fall off

f: Young peach fruits exposed as petals and

g: Hundred percent fruit setting stage

h: Physiologically matured peach fruits

i: Commercially matured peach fruits

Figure 2: The evolution of peach from one season to the other (bare orchard to commercial maturity) at the Aula Dei peach germplasm

The fruit is a large drupe with a thin epidermis, a pulpy mesocarp and a woody endocarp containing the seed, more or less globose, with a longitudinal groove, and a cavity around the peduncle. Ordinarily the skin of most ripe peaches is downy or fuzzy; however, the nectarines are class of peaches with smooth skins (Bassi and Moneté, 2008; Encyclopaedia Britannica, 2017).

The degree of susceptibility to infection by *Monilinia* spp. is variable throughout fruit development. Susceptibility is high during the early stages of fruit development, decreases during the green fruit or pit hardening stage and increases again during the ripening period (Lee and

Bostock, 2006; Obi et al., 2018;). Phenologically, peach fruit development generally undergo four stages (S-1 to S-IV) from flowering to maturation: fruit set (S-I), characterised by cell division and elongation, also referred to as the exponential growth phase; pit hardening (S-II), the endocarp hardens to form the stone and scarcely any increase in fruit size; the pre-climacteric phase (S-III), resumption of rapid cell division and fruit size enlargement, another exponential growth phase; and the climacteric stage (S-IV), final cell division, cell expansion and ripening/maturation (Lombardo et al., 2011; Gogorcena et al., 2012).

Peach nutrient has been found to be at its peak in the first stages of fruit formation and gradually reduces as the fruit develops (Xu et al., 2001). Therefore, the period of highest brown rot susceptibility conversely coincides with the lowest peach nutrient contents.

2.3. Economic significance of peach

China has the largest area of peach cultivation and the largest output of peach yield in the world, with an annual peach cultivation area of 4.520×10^5 ha and yield of 4.600×10^9 kg in 2016 (FAOSTAT, 2016). Production of peaches and nectarines in 2017/18 for the EU-28 is estimated at 4 million MT, 6 % higher compared to the previous cropping season 2016/17 (Table 1), and this is attributable to an expected increase in most of the major producers, hence the EU has remained a net exporter of peaches and nectarines with total exports largely exceeding imports for three seasons running (Figure 3). However, there was a steady slop on this EU export for the three years of 2014/15 to 2016/17 (Figure 3), undoubtedly suggesting an increase in the percentage of internal consumers of this precious product within the EU-28 during this period.

Table 1: Production and estimation of peaches and nectarines (MT) in the EU-28 main producing countries in different campaigns.

Country	2015/16	2016/17	2017/18
Spain	1,581,510	1,475,849	1,487,444
Italy	1,408,504	1,262,127	1,362,749
Greece	777,160	788,120	910,000
France	217,146	207,004	214,800

Source: USDA, (2017); MAPAMA, (2017)

And in Spain, the peach is one of the most important stone fruits in commercial production. The mean production for the period of 1994-2014 was 1.10 MT followed by almond with 0.23 MT (FAOSTAT, 2016). As earlier highlighted in section 2.1, the cultivation of this plantation crop (peach) is principally localized in the Mediterranean arc which includes in Spain Cataluña (27 %), Aragon (25 %) and Murcia (22 %), as the first three largest regional producers accordingly. Other upcoming commercial producers are: Andalucía (9 %), Extremadura (8 %) and the Valencia Community (7 %) (MAGRAMA, 2016). According to a special global report (USDA, 2017) Spain has become, in the last

4 seasons, the largest peach and nectarine producer in EU-28. The reasons for this feat were attributed to factors including: stead performance from the country's most important regions, (Aragón, Cataluña and Murcia), together with the improved productivity in Extremadura, Andalusia and Region of Valencia; increase of early and mid-season peaches, mainly due to good flowering and fruit set; introduction of newer varieties in recent years (USDA, 2017). According to the Spanish Ministry of Agriculture, MAPAMA (2017), peach and nectarine production in Spain for 2017/18 is projected to reach almost 1.487 MT accounting for almost 40 % share of the total EU-28 peach and nectarine production. This is 0.7 % higher compared to the previous season due to favourable weather conditions that resulted in a production with very good quality and calibres.

Globally, the utility of peaches are unlimited ranging from fresh eating, poaching, baking, grilling and processing into jams, syrups, ice creams and preserving in syrup (Cropotova, 2013). They are also utilized for fresh fruit salads, savoury salads, appetizers and for desserts such as cakes and pies. Peaches, especially yellow-fleshed varieties, are chiefly rich in vitamin A. In summary, peaches are high-antioxidant foods that have anti-inflammatory and microbial properties; hence, peach nutrition offers a bunch of impressive health benefits (Abidi et al., 2011; Infante et al., 2011; Stojanovic et al., 2016). Incidentally the optimum peach commodity production is hindered by brown rot epidemic with significant economic consequence. Orchards have been abandoned for the severity of this disease in recent times (Rungjindamai et al., 2014). Consequently, the universal annual losses from the epidemic have been estimated at 1.7 thousand million Euros (Oliveira-Lino et al., 2016). And in Spain, particularly, the disease has been associated with as high as 80% fruit losses after harvest (Egüen et al., 2015; Usall et al., 2015; Teixidó et al., 2018), mostly under favourable environmental conditions for the commencement and growth of the diseases in the orchard.

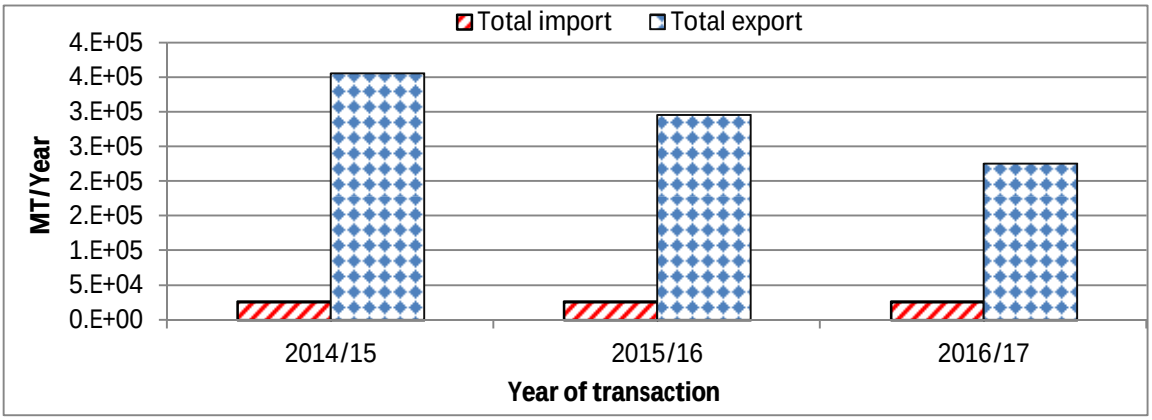


Figure 3: Total import and export of EU-28 of fresh peaches and nectarines during three seasons (2014-2017). Source: USDA, (2017)

3. Brown rot

3.1. *Monilinia* spp.

Monilinia belongs to the group of necrotrophic fungi (Ascomycota) in the order of Heliotiales (Leotiales), a large family of inoperculate discomycetes which includes both human and plant pathogens (Dugan, 2006). The teleomorph genus is *Sclerotinia* spp. (Position in classification: Sclerotiniaceae, Helotiales, Leotiomycetidae, Leotiomycetes, Pezizomycotina, Ascomycota) (http://www.indexfungorum.org/). The species of *Monilinia* are among major causal organisms of brown rot disease on various orchard tree crops including: (a) stone fruits (Hrustić et al., 2015; Rungjindamai et al., 2014; Žežlina et al., 2016) such as the apricots (Pascal et al., 1994; Walter et al., 2004), peach (Villarino et al., 2013, 2016; Obi et al., 2017, 2018), nectarine (Obi et al., 2017), cherry (Holb et al., 2013), and plum (Pascal et al., 1994); (b) the almond (Cimen et al., 2007) and occasionally, (c) some pome fruits (Holb, 2008; Poniatowska, 2013) such as the apple (Holb and Scherm, 2007), pear (Xu et al., 2001) and quince (Hrustić et al., 2012).

Monilinia laxa (Aderhold and Ruhland) Honey is one of the most important species of *Monilinia* globally associated with the brown rot in stone and pome fruits (Rungjindamai et al., 2014). *M. fructigena* (Honey), *M. fructicola* (G. Winter) (Villarino et al., 2013) and *M. polystroma* (G. Leeuwen) (Obi et al., 2017; van Leeuwen et al., 2002) are other important species. The disease is highly destructive on peach from fruit formation to storage. Additional losses are caused by blighting of flowers and twigs.

Hitherto, *M. laxa* and *M. fructigena,* were reported to be the two most important particularly in Spain until 2006. Then, *M. fructicola* was detected for the first time in peach orchards in the Ebro valley, Lerida, Spain (Villarino et al., 2013), and increased in population displacing *M. laxa*, a supposedly indigenous pathogen (de Cal et al., 2014), to the same level in frequency of occurrence (de Cal et al., 2014). Nowadays, in Spain, both *Monilinia* species (*M. laxa* (Aderh.et Ruhl.) and *M. fructicola* (G. Wint.) Honey) coexist in the field (Rungjindamai et al., 2014; Villarino et al., 2013). It can be inference that *M. laxa* and *M. fructicola* have similar epidemiological physiognomies (Bernat et al., 2017 b), for such inherent ecological coexistence. Incidentally, the epidemiology and management of *M. fructicola* has been most extensively studied, whereas the equally important *M. laxa* has had less attention (Rungjindamai et al., 2014).

Changes in the frequency of occurrence of different fungal pathogen species may also be due to fungicide resistance. Egüen et al. (2015) suggested that fungicide resistance of the *M. fructicola* population is co-acting among other factors as an adaptation in the pathogen to change the frequency of occurrence of the three *Monilinia* species in Spain. The displacement of *M. laxa* by *M. fructicola* has been also attributed to its conjugational potency related to sexual exchange and ability to produce ascopores from pseudosclerotial mummified fruits and their gradual process of a sexual propagation in Spain (de Cal et al., 2014). However, *M. laxa* is not known to produce

apothecia (de Cal et al., 2014), while *M. fructicola* does from which ascopores can easily be disseminated in the spring for possible infection in fruiting season (Holtz et al., 1998).

3.2. Geographical distribution of species of *Monilinia*

The occurrence and distribution of the species of *Monilinia* (Figure 4), are global having been detected in virtually all the continents of the world (Rungjindamai et al., 2014; Oliveira-Lino et al., 2016), where potential host are cultivated. This assertion is exceptionally established by their presence as detected, especially when peach fruit is moved (imported) from one country of origin to the other (Rungjindamai et al., 2014). Presently, six closely related species of brown rot fungi have so far been reported, particularly on stone and pome fruit, including *M. laxa* (Aderh. & Ruhland) Honey, *M. fructicola* (G. Winter) Honey, *M. fructigena* Honey, *M. polystroma* G. C.M. van Leeuwen, *M. yunnanensis* M. J. Hu & C. X. Luo, and *M. mumecola* Y. Harada, Y. Sasaki & T. Sano (Oliveira-Lino et al., 2016; Zhu et al., 2016; Vasić and Vico, 2018).

The geographic distribution of these species differs across the world. Hence *M. laxa* and *M. fructicola* are found more globally distributed (Rungjindamai et al., 2014; Oliveira-Lino et al., 2016; Zhu et al., 2016; Vasić and Vico, 2018), across the six continents of the world. *M. fructigena* is mostly restricted to European countries (Rungjindamai et al., 2014; Zhu et al., 2016; Vasić and Vico, 2018). It is a quarantine pathogen for Canada, the USA, Australia, and New Zealand. Its presence has also been reported in Africa (Morocco and Egypt) but it is not present in South and North America (Ivic et al., 2014). *M. polystroma* (van Leeuwen et al., 2002; Petróczy and Palkovics, 2009) have been reported in Japan, Hungary, China, Croatia, and Slovenia (Zhu et al., 2016); *M. mumecola* in Japan and China (Zhu et al., 2016), while *M. yunnanensis* (Hu et al, 2011) is only domiciled in China (Zhu et al., 2016). *M. mumecola* is not present in Europe (Hrustić et al 2013). Consequently, it is no longer relevant to affirm that the different species of *Monilinia* are distributed in specific regions considering the obvious ubiquitous pattern of spread.

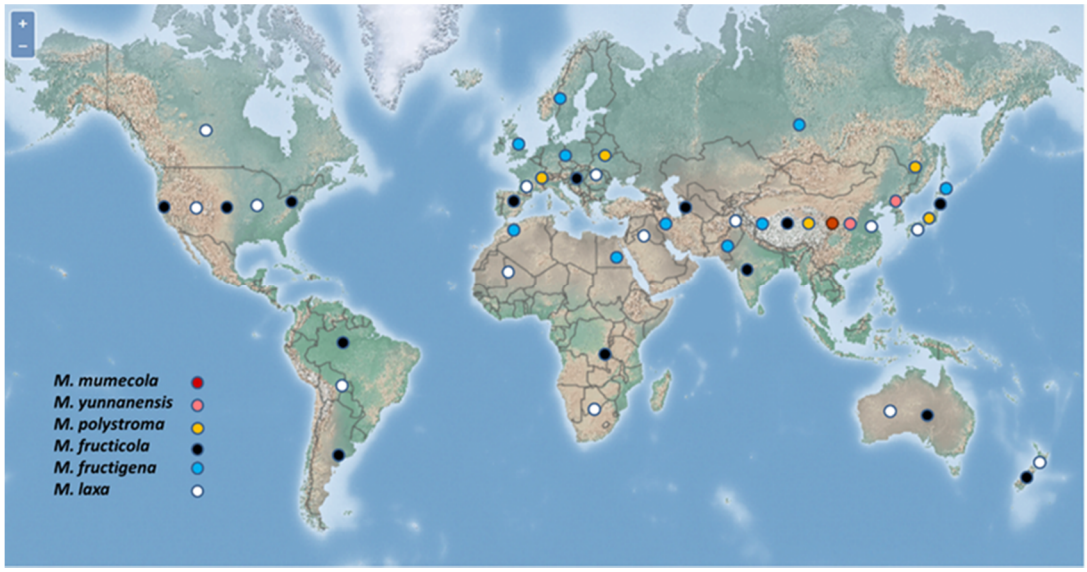


Figure 4. Global map showing the present continental distribution of the species of Monilinia spp.: Source CABI (https://www.cabi.org/isc/ (accessed on June 15, 2018)

3.3. Life cycle of species of *Monilinia*

The species of *Monilinia* as polycyclic pathogen (Seem, 1984) produces numerous secondary cycles throughout the annual growth cycle of the host (Figure 5). The fungus survives the winter (transmitted from year to year) in several structures such as mummified fruits (Casals et al., 2015; Adaskaveg et al., 2008; Janisiewicz et al., 2013), in canopy or on the ground (Hrustić et al., 2013), fruit peduncles (Ritchie, 2000), cankers on twigs, spurs and branches (Melgarejo et al., 1986; Kreidl et al., 2015). These propagules infested materials, according to Gell et al., (2009), serve as sources of primary inoculum and when weather conditions are suitable spores can infect blossoms, buds and young shoots, now establishing source of secondary inoculum (Gell et al., 2009)

So far, the main primary inoculum in Spanish orchards is mainly the mycelium and conidia present in the mummies found in affected trees or on the orchard floor (Villarino et al., 2010). It has also been shown that there is a positive correlation between the number of mummies in the trees and the incidence of postharvest fruit rots (Gell et al., 2009; Villarino et al., 2010).

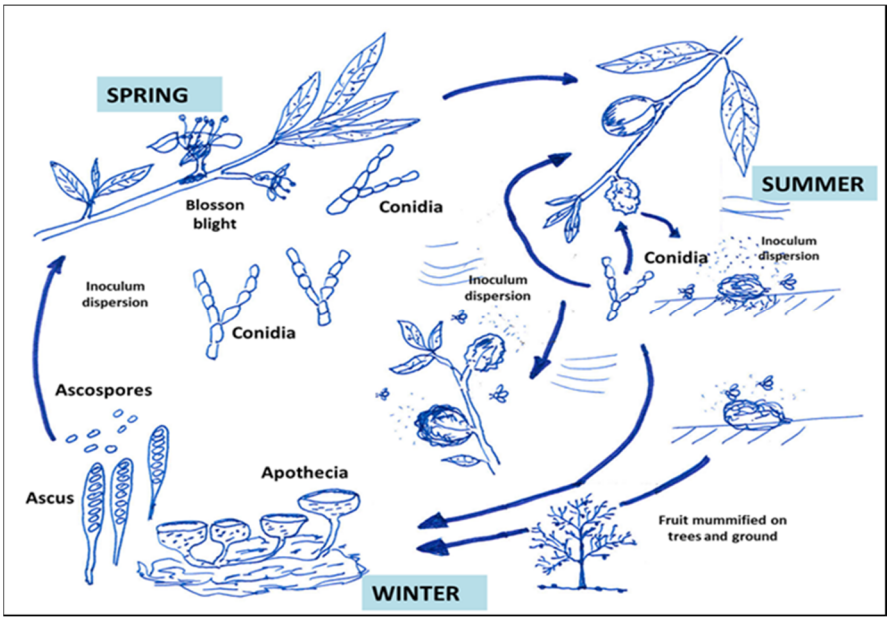


Figure 5: Brown rot disease cycle

It is also very important to note here that brown rot propagules are practically everywhere during the fruit-ripening period. In addition, infection is almost certain to occur if the weather is moist and extended over time (Ritchie, 2000), and if the fruit skin is bruised in any way (Xu et al., 2007).

Brown rot is spread by the dispersal of *Monilinia* propagules, through other microorganisms (Byrne et al., 2012), wind and water (Nagarajan and Singh, 1990), insects, birds, and man (Bosshard et al., 2006). They are also transferred by systems as from rain / overhead irrigation splashes

(Ritchie, 2000). Finally, insect and hail wounds, fruit cracking, limb rubs, twig punctures and a variety of picking and packing injuries are predisposing factors that greatly increase the losses due to brown rot (Ellis, 2008). Young uninjured fruits are thus, always fairly, safe from infection. Hence a clarion call for adequate orchard sanitary (Hong, 1997; Ritchie, 2000) observation at all times, especially at the onset of spring. And special care should be taken during harvesting and packing to prevent puncturing or bruising of ripe fruit. In addition, wild or neglected stone fruit trees that serve as collateral reservoirs for the disease must always be removed (Ellis, 2008).

For *M. fructicola*, an occasional source of inoculum can be sexual spores (ascospores) (Rungjindamai et al., 2014). At about blossom time, a mummified fruit produces up to 20 or more small, tan, cup-like structures on slender stalks that are called apothecia (sg. apothecium). As an apothecium matures, it becomes thicker and the cup opens to a bowl-like disc 3 to 12 mm in diameter across the top. The inner surface of each of this bowl-like disc is lined with thousands of spore-containing sacs (Asci). At this stage, the slightest disturbance or air movement will cause an apothecium to effectively discharge millions of spores. If a film of water (either from dew, rain or irrigation, especially the overhead) is present for 5 hours or longer, the spores can germinate and penetrate the plant / or plant parts (Rungjindamai et al., 2014).

Infected blossoms soon wilt and tan-gray tufts, composed of masses of another type of asexual spore (conidia), develop on the outside of the flower shuck (Villarino et al., 2010). If the infected blossom does not drop off, the fungus soon grows through the pedicel to the twig initiating a canker. Masses of conidia are soon produced on the newly cankered twig surface during moist periods throughout the early part of fruit development period. Entering the summer period, spores are easily detached and, like the ascospores, are mainly wind-borne. And the brown rot disease cycle continues as indicated in Figure 5. However, when conditions (weather) are unfavourable, the infection can remain latent (EPPO, 2010) until fruit maturity, the optimal time of disease development (Bernat et al., 2017 b; Thomidis, 2017).

M. laxa as a mycelium overwinters in twig cankers, blighted blossoms parts, peduncles, and mummified peach fruits in canopy or on the ground (Hrustić et al., 2013). And in the spring these mycelia propagules begin to sporulate and produce abundant conidia which initiate infection on close contact with susceptible tissues such as blossoms, spurs and twigs (Fazekas et al., 2014). In continuation of the disease cycle fruits are susceptible to *M. laxa* infections particularly in the time of fruit maturing which may lead to a disease epidemic by harvest and infected fruits mummified (Fazekas et al., 2014).

3.4. Ecological requirements of *Monilinia* species

Epiphytologically, the knowledge on the ecological requirement of *Monilinia* species is important information in the development of a predictive model to comprehend the epidemiology of brown rot and proffer adequate disease management strategies (Tian and Bertolini, 1999; Xu

and Robinson 2000). Important abiotic factors determining the potential for conidia germination and the growth of *Monilinia* propagules on the host exterior includes: temperature (T), water availability (Water activity a_w) and period of wetting (W) (Gell et al., 2008). These are in addition to the strong influence played by the moisture content of the spore, age of the spore and inoculum concentration on pathogenicity at the period of any conducive environmental factors. According to Casals et al. (2010), more than 80 % of viable conidia can germinate at 25 ^oC and 0.99 a_w within 2 h for the species of *M .fructicola*, *M. fructigena* but 4 h for *M. laxa.* The authors observed that the three species can germinate at a temperature range of 0 to 35 ^oC under 0.99 - 0.95 a_w. The optimum temperature for *M. fructicola* and *M. laxa* performance is reported to be 24.5 ^oC and 19.8 ^oC, respectively (Angel et al., 2017). The estimated maximum temperature for lesion development I higher for *M. fructicola* (30 ^oC) than for *M. laxa* (10 ^oC), inferring that *M. fructicola* is favoured by warmer weather than *M. laxa*. Hence, Bernat et al., (2017 b) reported that *M. fructicola* is better adapted to high temperatures, whereas *M. laxa* is better adapted to low temperatures. These authors observed that under optimal conditions *M. laxa* is as aggressive as *M. fructicola* on peach fruit. *M. laxa* unlike the others (*M. fructicola* and *M. fructigena*) has the potential to germinate in the absence of free water (a_w) in the host, making it relatively more virulent. The minimum germination temperatures estimated for *M. fructicola* and *M. laxa* are 4.7 ^oC and 0 ^oC, respectively (Obi et al., 2017). Nevertheless, conidia of *M. laxa* have been reported to germinate even at -4 ^oC (Tian and Bertolini, 1999).

Though the lowest storage temperature for stone fruit is 0 ^oC, conidia of the species of *Monilinia*, in general, could have the potential to germinate at temperature range of 0 ^oC to 35 ^oC from 2 h of initial exposure *in-vitro*, especially when the value of free water (a_w) or relative humidity (RH) under equilibrium condition is under 0.99 - 0.90a_w. Hence the pathogen on intimacy with peach skin can germinate under 0 - 40 ^oC at 100 - 80 % RH (Villarino et al., 2010). However, an optimum temperature range for brown rot (Moniliosis) initiation in peach at commercial stage is 22.5 - 25 ^oC, with which more than 79 % of fruits could be infected under a wetting periods of 12 h minimum (Gell.et al., 2008).

3.5. Characterization and identification of *Monilinia* species

Monilinia species, including *M. laxa* (Aderhold and Ruhland) Honey, *M. fructigena* (Honey), *M. fructicola* (G. Winter) and *M. polystroma* (G. Leeuwen) appear difficult to differentiate from one to another. However, relative distinction is possible through the use of "CMM", which is observation and combination of cultural / morphological and molecular methods (EPPO, 2009). The main characteristic on morphological variance (classical) between *M. laxa* and the other three related EU-28 species in culture are presented in Table 2.

3.5.1. Classical methods

As it is indicated in Table 2, classical qualitative or quantitative characterization and identification in species of *Monilinia* is possible by combining cultural physiognomies (van Leeuwen and Kesteren, 1998), such as growth rate, growth pattern and colony colour index (CCI), with morphological data, such as conidial dimensions and the length of the germ tube (de Cal and Melgarejo, 1999).

An *in-vitro* epidemiological study, according to Villarino et al., (2010), the conidia of *M. laxa* could germinate only after 4 h of incubation as against the 2 h with *M. fructicola* and *M. fructigena* at the same temperature and available water (a_w) conditions. Though the lowest temperature for peach storage is 0 ^oC, a potential pathogenic activity of *M. laxa* even at -4 ^oC has been reported (Tian and Bertolini, 1999). On potato dextrose agar (PDA) the colonies of *M. laxa* are greyish-brown while the colonies of the *M. fructigena* are yellowish or creamy (EPPO, 2003).

Table 2: Main characteristic variance between *Monilinia laxa* and the other three associated EU-28 species in culture

Characteristics / Pathogen	***M. laxa***	***M. fructicola***	***M. fructigena***	***M. polystroma***	**Source**
Conidia dimension	11-13 x 8-9.5 µm	12.5-14.5 x 8-10 µm	17.5-20.5 x 10.5-12.5 µm	13-17 x 9-10.5 µm	EPPO Bull., (2009); van Leeuwen et al., (2002)
Number of germ tube	1/conidia	1/conidia	2/conidia	2/conidia	EPPO Bull., (2009); van Leeuwen et al., (2002)
Form of germ tube	Short and twisted	Long and straight	Long and straight	Long and straight	EPPO Bull, (2009); van Leeuwen et al., (2002)
Size description	Smaller	Larger	Similar to *M. laxa*	Similar to *M. fructigena*	EPPO Bull., (2009); van Leeuwen et al., (2002)
Length of germ tube (> 18 h at 22^oC)	150-350 µm	750-900 µm	600-900 µm	700-1000 µm	EPPO Bull., (2009); van Leeuwen et al.,(2002)
Sporulation	Delayed and sparse	Quick, intense and abundant	Sparse	Sparse	EPPO Bull., (2009); van Leeuwen et al., (2002)
Sporulation range*	0 - 3.7	2.8 - 5.3	-	na	Hu et al., (2011)
Mean sporulation*	1.8	3.9	na	na	Hu et al., (2011)
Colony colour	Hazel/Isabelline (greyish-brown)	Hazel/ Isabelline (greenish-brown)	Pale luteous (yellowish/creamy)	Pale luteous (yellowish/creamy)	EPPO Bull., (2003); Petróczy et al., (2009); Petróczy et al., (2012)
Mycelium in distinct layers /colony rosetted	Resetting (mycelium in distinct layers on top of each)	No/rare	On distinct tufts; rings of aerial mycelium	Intense formation of black, stromatal plates initiated after 10-12 d incubation	van Leeuwen et al., (2002); EPPO Bull., (2009); Petróczy et al., (2012)
Colony rosette with black arcs	Yes	No	No	No	EPPO Bull., (2009); van Leeuwen et al., (2002)
Concentric ring of spores	No	Yes	Sometimes	Sometimes	van Leeuwen et al., (2002)
Colony margins	Serrulate/lobed	Not lobed but entire	Not lobed but entire	Not lobed but entire	van Leeuwen et al., (2002); Petróczy et al., (2012)
Range of colony growth rate (mm / 24h)	2 - 11	9 - 20	0 - 12		de Cal et al., (1999); van Leeuwen et al., (2002)
Mean colony growth rate (mm / 24 h) (in continuous darkness)	6	13	3.7	7	EPPO Bull., (2003); Hu et al., (2011); Petróczy et al., (2012)
Growth rating scale	Low	High	Low-moderate	Moderate	van Leeuwen et al., (2002)

*Log transformed number of conidia per cm, (Hu et al., 2011); nd = not detected, na = not available.

Though with some certain similarities, particularly between *M. laxa* and *M. fructicola*, in germ tube per conidia and colony colours they exist peculiar and pronounced characteristics in culture of the former (*M. laxa*) from the rest including: the possession of the smallest conidia dimension (11-13 x 8-9.5 µm), short and twisted germ tube, shorter length of germ tube (150-350 µm) at 22 ^{o}C of more than 18 h of incubation. In addition colony formation in *M. laxa* is in distinct layers on top of each other (resetting) with black curvatures and never associated with concentric ring of spores. Also, in the *M. laxa* the colony margins are serrulate or lobed with low growth rating scale. In general identification in culture of *Monilinia* species is often difficult because appearance varies from isolates to isolates within the same species (Côté et al., 2004 b). However, the aforementioned quantitative methods are overlapping screening system and therefore, the need for a standardized conditions (molecular method) starting with pure cultures (Figure 6).

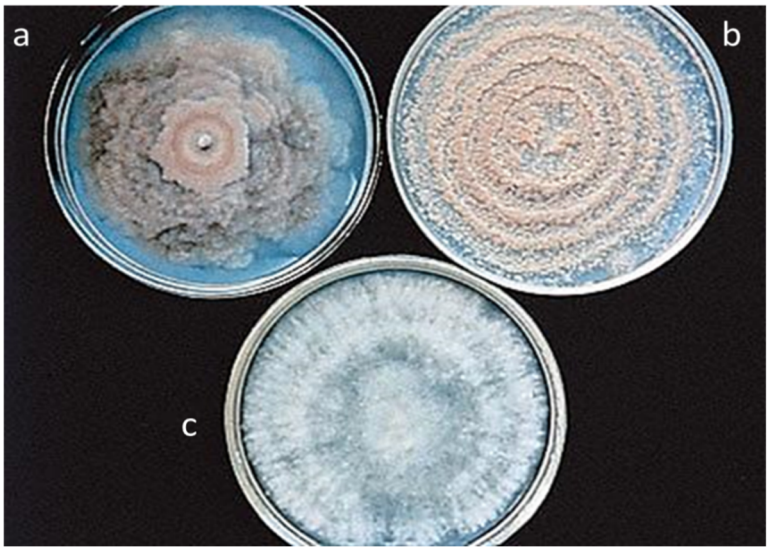


Figure 6: Pure cultures (PDA) and morphologies of the three major species of *Monilinia* at 10 days (22 ^{o}C), of incubation: (a) *M. laxa*, (b) *M. fructicola* and (c) *M. fructigena*.
Source: http://pbt.padil.gov.au/pbt/index.php?q=node/15&pbtID=79 (9th April 2018)

3.5.2. Molecular methods

Information exists on several molecular characterization and methods used since the first decade of this century to distinguish *Monilinia* species (Rungjindamai et al., 2014; Oliveira-Lino et al., 2016; Côté et al., 2004 b; Gell et al., 2007; van Brouwershaven et al., 2010). Most of the molecular methods for disease detection includes the Polymerase chain reaction (PCR) which is an *in-vitro*, primer directed, enzymatic reaction capable of exponential amplification of DNA (Oliveira-Lino et al., 2016). A recent review, by Balodi et al., (2017) revealed that the molecular techniques includes many PCR variants [PCR, nested PCR (nPCR), cooperative PCR (Co-PCR), multiplex PCR (M-PCR), real-time PCR (RT-PCR); DNA fingerprinting), and fluorescence *in-situ* hybridization (FISH)] all largely involving the DNA as principle nucleic acid.

The *Monilinia*-specific DNA primers of the internal transcribed spacer (ITS region) method was employed by Forster and Adaskaveg, (2000) in the detection of early brown rot infections in cherry fruits. With the same technique (ITS) Ioos and Frey, (2000) used the end point PCR to study the genomic variation within *M. laxa, M. fructigena* and *M. fructicola,* and designed primers to successfully achieve directly on the diseased fruits. This method is considered the standard test, according to Riccioni and Valente, (2015). Species-specific detection of *M. fructicola* from California stone fruits and flowers was developed by Boehm et al., (2001) using the PCR technique in 2001. Côté et al., (2004 b) worked on the characterization and identification of *M. fructigena, M. fructicola, M. laxa*, and *M. polystroma* using a Multiplex-PCR.

Later, Gell et al., (2007) utilized two different PCR approaches for universal diagnosis of brown rot and identification of *Monilinia* spp. in stone fruit trees combining a set of universal primers with the inclusion of an internal control for diagnosis of brown rot, caused by the three more important species. Recently, RT:PCR developed by van Brouwershaven et al., (2010) validated against all four brown-rot causing *Monilinia* (*M. laxa, M. fructicola, M. fructigena* and *M. polystroma*). More recently, Guinet et al., (2016) have used a multiplex RT-PCR to detecting and discriminating the three common species of *Monilinia*, including *M. laxa, on Prunus and Malus.* And, Papavasileiou et al., (2016) applying the high-resolution melting (HRM) techniques distinguished the six different species of *Monilinia* in peach (*M. laxa, M. fructicola, M. fructigena, M. mumecola, M. lithartiana* and *M. yunnanensis*) analysing the melting curve of amplicons of two universal primer pairs.

In another development, Garcia-Benitez et al., (2017 b) compared the overnight freezing-incubation technique (ONFIT) and RT-PCR / quantitative polymerase chain reaction (qPCR)-based methods modified to detect latent brown rot infections and, subsequently, distinguishing between the *Monilinia* spp. in flowers and peach fruit. The same authors (Garcia-Benitez et al., 2017 b) later validated the method in reference to test performance accuracy, analytical specificity and sensitivity, repeatability, and reproducibility, as defined by standard PM7/98 of the European Plant Protection Organization (EPPO) for detection of *Monilinia* spp. being more sensitive, reliable, and quicker than ONFIT for detecting a latent brown rot infection.

Another established method is the fast multiplex quantitative / real-time polymerase chain reaction (qPCR / RT-PCR method) developed by van Brouwershaven et al., (2010), modified and validated by Garcia-Benitez et al., (2017 b) in reference to test performance accuracy, analytical specificity and sensitivity, repeatability, and reproducibility, also as defined by standard PM7/98 of EPPO for the detection of *Monilinia* spp.

Rapid, accurate and reliable (Fazinic et al., 2017) detection of *Monilinia* latent infections (Garcia-Benitez et al., 2017 a) is needed to prevent and control dispersion of *Monilinia* spp. in infected localities and non-infected countries (Papavasileiou et al., 2016). The overnight freezing-incubation technique (ONFIT) is one of the established methods for detecting latent brown rot infection, but the test time-cost is between 7 to 9 days (Bernat et al., 2017a). The advantage of the qPCR-based

method for detecting a latent *Monilinia* infection in peaches are its high sensitivity, its ease and rapidity of execution, the low number of handling steps, and reduced personal costs. However, its disadvantages includes the high cost of consumables and reagents, which are much greater than those of ONFIT, and the occurrence of false positives due to detection of nonviable fungal DNA (Garcia-Benitez et al., 2017 b).

Merits of DNA-based detection methods include: reliability, time-saving, higher sensitivity and specificity, when compared to the processes of artificial cultivation, the traditional and serological assays techniques as found documented (Guinet et al., 2016; Garcia-Benitez et al., 2017 b). For example the molecular technique of multiplex RT-PCR assay (one step) developed by Guinet et al., (2016) is efficient and prompt in characterising the three major species of *Monilinia* responsible for brown rot (*M. laxa, M. fructicola* and *M. fructigena*). These authors also inferred that the exceptional reliability of their results are of paramount importance in the framework of phytosanitary regulations, considering the fact that the performance data were generated and the assay fully validated in accordance to the EPPO guidelines (EPPO, 2010).

In summary, molecular biology based methods are progressively providing the means for timely identification of quarantine plant pathogens including some species of *Monilinia*. The method does not necessitate isolation of the particular species of *Monilinia* and, therefore, significantly accelerates the identification process compared with methods based on quantitative characteristics. Finally, these methods can potentially be invigorated to directly and specifically identify the species infecting peach commodity. Its prominence, no doubt, is also necessitated by overlapping classical screening system.

3.5.3. Limitations of molecular methods

There are indisputable limitations associated with molecular technique assays, among which includes: potential existence of genetic similarity between different species of pathogens at primer / probe binding sites due to mispriming; possibility of inept estimate of the viability of pathogen and the likelihood of contamination of commercial reagents with target sequences leading to, apparently positive but, a deceptive result. The formation of non-specific products due to mis-priming and formation of artefacts in form of primer dimers adds more complexities to the probability of correct interpretation (Balodi et al., 2017). Other authors, Guinet et al., (2016) have observed that under less stringent reaction conditions, the test might be able to stand limited experimental errors or equipment drift (e.g., pipetting errors or thermic issues with the thermal cycler).

In the last decade, several protocols did not distinguish among some *Monilinia* species, although modern RT-PCR has been modified to overcome this (Zhu et al., 2016; Garcia-Benitez et al., 2017 b;). The works of Förster and Adaskaveg, (2000); and Côté et al., (2004 a), were found unreliable because some isolates of *M. fructicola* lack a group I intron in their nuclear rDNA small subunit. The

methods of PCR primers and protocols for *M. fructicola* as documented by Förster and Adaskaveg, (2000); Boehm et al., (2001) and Ma et al., (2003), though discriminate *M. fructicola* from *M. laxa*, have not been validated for distinguishing *M. fructicola* from *M. fructigena*.

The characterization methods of Ioos and Frey, (2000); Miessner and Stammler, (2010), and Hily et al., (2010), reliably differentiated three species of *Monilinia* (*M. fructigena, M. fructicola*, and *M. laxa*) within themselves, but practically unable to distinguish *M. fructigena* from *M. yannanensis*. Similarly, the methods developed by Ioos and Frey, (2000); and Ma et al., (2003, 2005) did not discriminate between *M. mumecola* and *M. laxa*. Also, the method developed by Hily et al., (2010) did not differentiate *M. mumecola* from *M. fructicola,* and the methods of Miessner and Stammler (2010) and Hily et al., (2010) also could not discriminate between *M. yunnanensis* and *M. mumecola*. However, some investigators (Garcia-Benitez et al., 2017 a) in their study of RT-PCR detection of latent *Monilinia* spp. infection in nectarine flowers and fruit could only be able to prevent cross-detection by stringently including an allelic discrimination step (extra cost) in qPCR runs, to enable the differentiation between *M. fructicola* and *M. laxa* (Garcia-Benitez et al., 2017 b).

Finally, though results from molecular assay, particularly multiplexed / quadruplexed (Lowe et al., 2016), have demonstrated that it remains sensitive and specific without compromising the reliability of the results.

In addition, other investigators (Ivic et al., 2014; Riccioni and Valente, 2015) have recommended that for optimum performance and accurate identification, particularly with PCR tests, and regardless of DNA extraction method, manufacturer of chemicals, thermocyclers or staining methods, there is the exigency for careful selection of species-specific primer pairs for molecular diagnosis of species of *Monilinia*. We refer to Raja et al., (2017) for more information on merits and demerits of the use of "Molecular Tools" in fungi detection and identification**.**

4. Host-pathogen interactions

Brown rot is the pathologic result of a parasitic interaction between the species of *Monilinia* and the peach (EPPO, 2010; Garcia-Benitez et al., 2016). In such association, and depending on the region of first contact (blossom, out growth, stem, fruit), the pathogen initiates and encourages flower blights, spurs, twigs / branch death and fruit (brown) rot in the field and in postharvest (Gell et al., 2007; Papavasileiou et al., 2015). Hence the pathogen`s activity on the host is highly destructive from flowering stage via peach formation to storage (Thomidis and Exadaktylou, 2010), thereby creating an infection chain as exponent in Figure 7. Brown rot is a polycyclic epidemic (Seem, 1994) hence various secondary or monocyclic components of the brown rot infection sequence are generated throughout the annual growth cycle of the host (Schumann and D`Arcy, 2006). This biological proficiency conversely pertain grave impairment on the harvest, storage and commercial shelf life of the product (Gell et al., 2007; Sisquella et al., 2013). Interestingly, as it is found in any pathogen-host association, the growth and development of brown rot is influenced by

different physicochemical conditions such as temperature and water activity (Pascual et al., 1997; Xu et al., 2001), light, aeration and pressure (Maharshi and Thaker, 2012), pH and titratable acidity of fruit (Holb,2004; Romero-Arenas et al., 2012; Obi et al., 2018).

a: Mummified peach b: Blighted blossoms c: Infected peach fruits

Figure 7: Chain of brown rot infection in peach

During such impending epiphytotics the aforementioned physicochemical factors influence the microbial activity determining either the growth and reproduction or the inhibition of activity and the inactivation of the pathogen. And in particular, the pH and titratable acidity (TA) are interrelated concepts of organic acids (Dirlewanger et al., 1999; Tyl and Sadler, 2017) controlling physicochemical factors that act in an additive and interactive mode to inhibit pathogen metabolic pathway (Romero-Arenas et al., 2012). These two physicochemical components, though complementary in nature, are statutorily different. TA refers to the total concentration of free protons and undissociated acids in a fruit juice that can react with a strong base and be neutralized, hence it is any amount of base needed to neutralize such acidity and bring its pH to a neutral (pH 7), or slightly alkaline (pH 8.1) value, and pH represents the free hydrogen ion activity in the fruit juice (Lobit et al., 2002) or a means of expressing such H^+ ions concentration in a substrate.

In peach fruit acidity is an important genetic quality (Dirlewanger, 1999) which influences both perception of sourness and sweetness found in varying proportions depending on the cultivar and the ripening stage (Lobit et al., 2002). The influence of pH and TA on fungal-host interaction is documented (Yamanaka, 2003; Obi et al., 2018). Some fungal species favour neutral to slightly alkaline conditions (Maharshi and Thaker, 2012). However, the *Monilinia* species, in general, are acidophilic and therefore prefer acidic conditions for their growth (Maharshi and Thaker, 2012). Results of the fruit experiment by Holb, (2004), have shown that healthy peach fruits are quite acidic (pH < 3.5), but that pH rapidly increases in inoculated fruits reaching pH 4.6-5.4 depending on cultivar and fungus isolate. Unsurprisingly, species of *Monilinia* can acidify the host tissue in peaches and nectarines from pH 4.50 and 4.45, to pH 3.75 and 3.90, respectively (de Cal et al., 2013).

5. Other management strategies to control brown rot in peach

Weather conditions have been reported to influence the percentage of latent infection on peach and nectarine flowers and fruits (Thomidis, 2017) and remain a significant factor that must be considered on the effective management of this disease. Latent infection is a pathologic situation where fruit infected can remain asymptomatic and visual decay symptoms only develop during the late ripening period and during postharvest (Bernat et al., 2017 b), particularly when there is an enabling ambient condition. Most stone fruit with a latent brown rot infection caused by *Monilinia* do not develop visible signs of disease until the arrival of fruit at the market or consumers' home. Rapid, accurate and reliable (Fazinic et al., 2017) detection of *Monilinia* latent infections (Garcia-Benitez et al., 2017 b) is needed to prevent and control dispersion of *Monilinia* spp. in infected localities and non-infected countries (EPPO, 2010). The overnight freezing-incubation technique (ONFIT) is one of the established methods for detecting latent brown rot infection, but the test time-cost is between 7 to 9 days (Bernat et al., 2017 a).

Another established method is the fast multiplex quantitative / real-time polymerase chain reaction (qPCR / RT-PCR method) developed by van Brouwershaven et al., (2010), modified and validated by Garcia-Benitez et al., (2017 a) in reference to test performance accuracy, analytical specificity and sensitivity, repeatability, and reproducibility, as defined by standard PM7/98 of the European Plant Protection Organization (EPPO) for detection of *Monilinia* spp. is more sensitive, reliable, and quicker than ONFIT for detecting a latent brown rot infection. The advantage of the qPCR-based method for detecting a latent *Monilinia* infection in peaches are its high sensitivity, its ease and rapidity of execution, the low number of handling steps, and reduced personal costs. However, its disadvantages includes the high cost of consumables and reagents, which are much greater than those of ONFIT, and the occurrence of false positives due to detection of nonviable fungal DNA (Garcia-Benitez et al., 2017 a). In all, it has been recommended (Garcia-Benitez et al., 2017 a) that additional investigation of new primers and probes for characterization of the species of *Monilinia* should be conducted to make identification more transferable among qPCR platforms and laboratories.

There are various control and potential management options available for brown rot epidemic in peach, which include: biological, conventional, chemical, physical, biofungicides, and host resistance (Rungjindamai et al., 2014).

5.1. Biological control

Evidence abounds on the practical and biological control possibilities against diseases of *Monilinia* species (de Cal et al., 1990; Larena and Melgarejo, 1996). Biological control also refers to the use of formulations of living organisms (Biofungicides) to control the activity of plant pathogenic fungi and bacteria (Dicklow, 2017). An insight into its utility could find EPS125 (*Pantoea agglomerans*, a gram-negative bacterium) (Bonaterra et al., 2003), effective in preventive

treatments for control of stone fruit rot on several stone fruit crops, including peach, cultivars. They observed its ability to colonize, rapidly grow and survive in wounds, the fact that the main mechanisms of action is mediated by cell-to-cell interaction, and inferred that the absence of major toxicological effects, constitute interesting traits for an effective use as a biofungicide under fully commercial conditions.

Larena et al., (2005) carried out seven field experiments in peach orchards located in three different countries (Spain, Italy and France), for two years to develop an effective and practical method of controlling brown rot disease caused by *Monilinia* spp. by pre-application of *Epicoccum nigrum*. The result of their work demonstrated that *E. nigrum* applications alone or in combination with fungicides treatment to peach trees in the field reduced brown rot at postharvest. *Bacillus* spp. are among the most recommended bacteria to use against plant diseases, including brown rot, and marketed as commercial products, QST 713 strain, [(Table 3), Rungjindamai et al., (2014)]. Yánez-Mendizábal et al., (2012) used *Bacillus subtilis* (CPA-8) strain to control *Monilinia* spp. in peaches and indicated that fengycin-like lipopeptides play a major role in the biological potential of *B. subtilis* CPA.8 against *M laxa* and *M. fructicola*. Also, antagonistic microorganisms such as *Penicillium frequentans* (Guijarro et al., 2008) and *Bacillus amyloliquefaciens* (Gotor-Vila et al., 2017) are examples of practical pre-harvest and postharvest control measures, respectively, in peach commodity.

Just recently a practical potential of *B. amyloliquefaciens* CPA-8 as alternative or complementary strategies to control *Monilinia* spp. has been highlighted (Gotor-Vila et al., 2017). In this study carried out under field conditions, the authors confirmed the potency of the bioagent considering their observation that the population dynamics of CPA-8 on treated peach fruit surface remained after treatment application, at harvest and at postharvest shelf-life.

According records, the number of new biopesticide registrations in the EU-28 is growing steadily but they currently represent only 2.5 % of the pesticide market (EUROSTAT, 2018). Although several large agrochemical companies are now actively engaged in the development of biological control agents (BCAs), small-medium sized enterprises (SMEs) account for most of the activity in BCA development. Parenthetically, the EU-28 has commissioned a large collaborative project on developing and commercialising BCAs in recent times (Rungjindamai et al., 2014).

However, increasing concern about the effects of biochemical fungicides (BCAs and conventional fungicides) on the environment (Giobbe et al., 2007), human health (Papavasileiou et al., 2015), and strain fungicide resistance (de Cal et al., 1988) still views new alternatives, such as host resistance, as one of the most cost effective and environmentally safe strategies for disease control. For example, Giobbe et al., (2007) found that a biofilm-forming strain of *Pichia fermentans* proved to be most effective in controlling brown rot on apple fruit but pathogenic on peach fruit when co-inoculated into artificial wounds with a phytopathogenic isolate of *M. fructicola.* The authors, therefore, emphasized the need for a thorough risk assessment before allowing any deliberate

release ofBCAs, considering associated potential effects such as displacement of nontarget organisms, allergenicity to humans and other animals, toxicity and pathogenicity, and genetic stability. Hence, any minimal potential biohazard is inherent to any application of biocontrol agents (Giobbe et al., 2007) in peach commodity.

5.2. Conventional fungicide treatments

A major control option of this phytopathogen (species of *Monilinia*), especially in the Spanish peach management, has been the use of preventive and systemic fungicides (Table 3**)** such as thiophanate-methyl, iprodione and cyproconazole (Egüen et al., 2015). Synthetic fungicides like benzimidazoles, dicarboximides and demethylation inhibitors (Egüen et al., 2015) have been used to control the disease in the orchards. In Spain, however, there are only two active substances for the control of this fungus in warehouses, or in post-harvest product store: fludioxonil, with two different formulations (Table 3) [fludioxonil 23% (SC) P/V and fludioxonil 60% (SC) P/V] with current registration until 31/12/2019 and pyrimethanil 30% (GE) P/P, vigent until 30/04/2019 (MAPAMA, (2017); USDA, 2017).

Table 3: Chemical and biological formulations used in stone fruit production for *Monilinia* management of crop production in Spain.

Formulation	Nº of comercial products	Life limit
Sulphur 80% + Cyproconazole 0.8% [WG] P/P	1	31/12/18
Cyproconazole 10% [WG] P/P	1	17/06/18
Cyprodinil 37.5% + fludioxonil 25% [ESP] [WG] P/P	1	31/10/18
Copper (II) hydroxide 35% (expr. in Cu) [WG] P/P	23	31/10/18
Iprodione 75% [WG] P/P	1	31/10/18
Mancozeb 20% + Dicopper chloride trihydroxide 30% (expr. in Cu) [WP] P/P	20	31/01/19
Mancozeb 75% [WG] P/P & 80%	35	31/10/18
Thiophanate-methyl 50% [SC] P/V; 70% [WG] P/P	4	31/10/18
Mancozeb 8% + Cuprocalcium sulphate 20% (expr. in Cu) [WP] P/P.	4	31/10/18
Myclobutanil 4.5% [EW] P/V	1	31/05/21
Dicopper chloride trihydroxide 11% (expr. in Cu) + Cuprocalcium sulphate 10% (expr. in Cu) [WP] P/P	1	30/10/17
Copper(I) oxide 40% (expr. in Cu) (01) P/P	12	28/09/19
Copper (I) oxide 50% (expr. in Cu) [WP] P/P & 52%	47	10/06/19
Copper (I) oxide 75% (expr. in Cu) [WG] P/P	16	10/06/19
Tribasic copper sulphate 40% (expr. in Cu) [WG] P/P	14	27/11/19
Tebuconazole 25% [WG] P/P	11	31/08/19
Fenbuconazol 2.5%/5% [EW) P/V	2	30/04/22
Bacillus subtilis (CEPA QST 713) 15.67% (5.13 X 10E10 UFC/G ESP) [WP] P/P	1	30/04/18
Total active ingredients applicable in preharvest peach and nectarine biofungicidal control in Spain (18)		
Fludioxonil 23% [SC] P/V/ Fludioxonil 60% [SC] P/V	2	31/12/19
Pyrimethanil 30% [GE) P/P	1	30/04/19
Total active ingredients applicable in postharvest peach and nectarine biofungicidal control in Spain (2)		
Total of number of commercial biofungicidal products allowed in Spanish peach market (198)		

Source: MAPAMA, (2017)

In furtherance, from more than 84 biofungicidal formulations, registered in the national pesticide guide web of the "Ministerio de Agricultura, Pesca, Alimentación & Medio Ambiente de España", are recapitulated only eighteen active ingredients authorized for use in the control of brown rot of peach in Spain (Table 3). Life limit (deadline) for fungicide use in Spanish stone fruit production is also provided. The deadline for use ranges currently between October 2017 and April 2022. However, the manufacturers would always like to extend this period or deadline, only when appropriated request is made by the responsible body and on the condition that certain applicable costs are paid for such permission. The same substance or formulation can be applicable to both pre- and post-harvest treatment but with the corresponding restriction in each case.

5.3. Limitations in the use of conventional fungicide

The increase in the demand for fresh fruit with reduced residual quantities (Rungjindamai, et al., 2014) has placed to interrogation the continuous use of conventional fungicides (CF) in peaches. CF treatments often times alter the micro-ecosystem and in extension modify disease severity by altering the interactions among microorganisms (de Cal et al., 1988). Moreover, the toxic chemical residues pose additional ecological issues. In furtherance, recent reports have confirmed that the differential resistance to CF in *Monilinia* spp. is evolving and could be modifying the frequency of occurrence of fungicide sensitive and fungicide-resistant *Monilinia* spp. (Egüen et al., 2016). The evolution of tolerance in species of *Monilinia* to certain CF (Katan and Shabi, 1981; Cañez-Jr and Ogawa, 1985), the increasing cost of chemical control and post application clean up (Pimentel, 2005) and the threat of regulatory restrictions are all yearning need for sustainable and endearing management measure to control brown rot (Zehr, 1982).

The regulation of CF use, however, has become stricter in EU countries, especially after the release of European Directive 2009/128/EC (EPPO, 2009). There is also the global intensification in the number of countries advocating for the reduction / waning regarding conventional chemical uses (Oliveira-Lino et al., 2016). Particularly, the use of CF is becoming more unfashionable because of consumer demands for residue-free fruit (Usall et al., 2015). Add to all the aforementioned includes the fact that contamination of the environment should be avoided (Liu et al., 2012). Finally, the steady rise in development and occurrence of CF resistant to *Monilinia* strains, worldwide, has been reported (Katan and Shabi, 1981; Elmer and Gaunt, 1994; Liu et al., 2012; Chen et al., 2013; Egüen et al., 2016). All these adverse implications put together it is, therefore, pertinent to search for alternatives with lasting effect, enhancing consumer acceptability and at the same time friendly with environmental sustainability.

5.4. Botanical fungicides

Plants provide a wide range of secondary metabolites and essential oils, which have an array of properties including antimicrobic, allelopathic, bioregulatory and antioxidants (Solomon et al., 2005; Suleiman et al., 2009; Khaled-Khodja et al., 2014). This class of plant derivatives are

collectively referred to as biopesticides, including the botanical fungicides (Yoon et al., 2013). Information, both review (Parveen et al., 2016) and practical (Wilson et al., 1997; Ziedan et al., 2008), on the biopesticidal efficacy and utility against phytopathogens abounds (Kouass et al., 2010; Hassani et al., 2012). Some of these bioactive substances have also been assayed *in-vitro* and *in-vivo* (Goncalves et al., 2010) and found potent enough against brown rot of *Monilinia* species (Ganchev, 2007; Carović-Stanko et al., 2013).

In an overview, Hassani et al., (2012) evaluated, *in-vivo*, the antifungal activities of four different [*Thymus vulgaris* (L.), *Eugenia caryophyllata L.*, *Cinnamomum zeylanicum* (Blume) and *Carum copticum* (L.)] plant extracts against two postharvest pathogens (*M. fructicola* and *Botrytis cinerea*) of stone fruit. And precisely, in reference to our target (peach, *Monilinia* and brown rot), Lopez-Reyes et al., (2013) screened, *in-vivo*, the antifungal efficacy of plant essential oils on postharvest control of brown rots on different stone fruits, including the peaches. Just recently, Cindi et al., (2016) have recommended the thyme oil for postharvest handling as biofumigants in peaches after indicating that thyme oil vapours effectively reduced the incidence of brown rot caused by *M. laxa*.

In furtherance, Goncalves et al., (2010) backing their work said that the results obtained in postharvest control of brown rot and *Rhizopus* rot in plums and nectarines using carnauba wax (*Copernicia prunifera*) (Mill.) H.E.Moore was promising because the experiments were carried out under conditions of elevated inoculum pressure for mature fruit. Cindi et al., (2016), also propose that thyme oil fumigation treatment can be considered as a good alternative treatment due to the low concentration used in brown rot decay control in peaches.

Nevertheless, some authors have observed certain limitations in the evaluated biofungicides and made adequate recommendations accordingly. For example, Cindi et al., (2016) observed that there was the need to conduct further studies on the effect of thyme oil fumigation on fruit quality (sensory parameters) in naturally infected peaches after low temperature storage and retail shelf conditions. Thyme oil influences on peach volatile compounds, such as alcohols, aldehydes, carboxylic esters, ketones and esters need to be investigated (Cindi et al., 2016).

5.5. Physical treatments to control brown rot in peach

There are various types of physical control srategies in the management of brown rot (BR) in peaches (Table 4). This includes: hot water dipping (Jemric et al., 2011); dry heat (Liu et al., 2012); wet heat treatment curing with chitosan or in combination with *Bacillus* CPA-8 (Casals et al., 2012); use of radio frequency, water immersion and air exposure, (Sisquella et al., 2013); and hydro cooling techniques (Bernat et al., 2017 a).

Some authors (Cañez-Jr and Ogawa, 1985; Jemric et al., 2011) have indicated that it is possible to control postharvest BR on peach using hot water dipping (HWD) at 48 ^{o}C for 12 min and on nectarine using HWD at 48 ^{o}C for 6 min without a significant loss of fruit quality. The authors,

however, recommended for optimization of the method according to cultivar since too long exposure of fruit to a high temperature can cause the loss of acidity.

In the study of Liu et al., (2012), the use of heat (wet and dry) showed that both the direct inhibition of pathogen and elicitation of defence response in fruit contributed to the significant reduction of decay in peach. The investigators associated the control effect to the inhibition of *M. fructicola* germination and growth, intracellular ROS accumulation, mitochondrial impairment leading to a reduction in ATP, and the induction of defence-related enzymes in peach. Casals et al., (2012) used heat to control peaches and nectarines from brown rot by exposing fruit to 50 ^{o}C for 2 h and 95-99 % relative humidity (RH), which should markedly eradicate the pre-existing *Monilinia* spp., infections (from the field), in combination with the application of chitosan at 20 ^{o}C for 1 min or the antagonist *B. subtilis* strain CPA-8.

Table 4: Physical treatments to control brown rot in peach, conditions, period and effects.

Treatment	Temperature	Period of exposure	Effects	Reference
Hot water dipping (HWD)	48 ^{o}C	6 / 12 min	Reduced brown rot (BR) incidence and no significant loss of fruit quality	Jemric et al., (2011)
Heat treatment (HT)	40 ^{o}C	5 / 10 min	Significant reduction in peach BR	Liu et al., (2012)
Heat treatment (HT) 95% RH	50 ^{o}C	2 h	Proposed as potential strategy to control brown rot on peaches and nectarines	Casals et al., (2012)
Radio frequency (RF) of dipping in hot water (HT)	60 ^{o}C	20 s	A 100% BRI reduction at 6 to 12 h after inoculation and 85.7%. BRI reduction at 0 to 48 h after inoculation as compared to untreated fruit	Spadoni et al., (2014)
Radio frequency (RF) at 27.12 MHz of water immersion	20 ^{o}C	9 min	Controlled brown rot without adverse external and internal damage in both peaches and nectarines	Sisquella et al., (2013)
Radio frequency (RF) at 27.12 MHz of exposition in air	20 ^{o}C	18 min	Brown rot incidence significantly reduced in both peaches and nectarines of different fruit size.	Sisquella et al., (2013)
Radio frequency (RF) at 27.12 MHz of water immersion	40 ^{o}C	4.5 min	Reduced BRI in stone fruits inoculated (0 - 48 h) before treatment and at all maturity levels evaluated in both peaches and nectarines without impaired fruit quality	Sisquella et al., (2014)
Hydro cooling (HC) and water dump (WD)	4 ^{o}C	30s / 10 min	Reduced brown rot incidence by 50-77% when treated at 2 / 24 h of fruit harvest	Bernat et al., (2017 a)

In their study on the influence of hot water treatment on brown rot of peach and rapid fruit response to heat stress peach fruit, Spadoni et al., (2014) inoculated fruit with conidia of *Monilinia laxa* and dipped in hot water (HT) at 60 ^{o}C for 20 s (15 min / 48 h post inoculation). Based on achieved positive result, the authors recommended the techniques as an alternative and new perspective in brown rot incidence (BRI) reduction in peach.

Sisquella et al., (2013) in a study to improve RF treatment to control brown rot in stone fruit evaluated the effect of immersion in water for 9 min and exposure in air for 18 min (RF of 27.12 MHz) all at 20 ^{o}C. They observed that RF treatment with fruit immersed in water at 20 ^{o}C for 9 min may provide a potential postharvest alternative for brown rot control in peaches and nectarines.

And a year later, Sisquella et al., (2014) confirmed RF treatment, particularly at 40 °C immersions for 4.5 min, would be very promising and attractive commercial potential for the control of brown rot on peaches and nectarines at postharvest. In furtherance, they reported a significantly reduced BRI in the naturally infected fruit, from 92% among control fruit to less than 26% in peaches and complete brown rot control in nectarines (Sisquella et al., 2014). In addition, before the commercial application of this treatment techniques, the authors rightly suggested, "it is necessary to design specific equipment with water to determine the economic cost of the treatment".

Bernat et al., (2017 a) applied hydro cooling and water dump techniques to reduced BR incidence on peach fruit with recent infections (2 or 24 h before treatment). The techniques were able to reduce BRI in comparison to direct storage at 0 °C, but not when infections have been established (≥ 48 h before treatment).

5.6. Host Resistance and Genetic Management

Apart from the pre-harvest issues with peaches, the length of conservation and commercial shelf life are negatively influenced due to post harvest diseases principally associated with the brown rot (BR) (Sisquella et al., 2013). Consequently, breeders (in: Brazil, California, Italy, and USA) either individually or associated with pathologists have concentrated efforts on obtaining new cultivars resistant to this pathogen. Nonetheless, reports of resistance (Mexican and Brazilian peaches) or tolerance (peaches from Florida, New Jersey, and Harrow programs) to fruit brown rot within peach exist (Gogorcena et al., 2012; Feliciano et al., 1987).

Breeding for disease resistance is one of the most challenging objectives for crop improvement because disease expression is tetrahedral: it is simultaneously influenced by agent, host, environment, and human management (Fresnedo-Ramírez et al., 2017). Notwithstanding, different methodologies and varying protocols have continued to be assayed in breeding programs to evaluate genetic resistance to fungi in stone fruit crops (Gradziel, 1994; Byrne, 2005; Oliveira-Lino et al., 2016; Obi et al., 2017).

6. Breeding for Tolerant Cultivars or Genotypes

The 'Bolinha' peach variety, of Brazilian origin, presents a good resistance mechanism with less susceptibility to brown rot than other varieties and although this variety possesses relatively poor quality characteristics, it has been observed that the resistance to disease is transmitted to their descendance (Feliciano et al., 1986; Byrne, 2005; Oliveira-Lino et al., 2016). Lack of extensive studies on *M. laxa,* in particular (Rungjindamai et al., 2014), especially on the use of tolerant variety or genotypes with good quality characteristics in BR management and crop improvement, calls for academic and practical attention in that area of significant concern.

The use of commercial varieties or genotypes with some level of disease resistance remains one of the surest and long lasting tolerant alternatives within disease protection and improvement

management techniques in crop cultivation (Vallad and Goodman, 2004; Byrne et al., 2012). Host tolerance to plant pathogens is important to cost effective and environmentally safe strategy for BR management. In the same notion, according to Gell et al., (2007) the use of tolerant cultivars in crop improvement is the topmost principle of crop protection as plants and plant products are usually protected (prophylactic) from disease epidemic (Norton, 1976; Dodds and Coleman, 2017) and, often, not cured of diseases (chemotherapeutic). Cultivar significantly influence rot incidence and severity among other potential factors in stone fruits (Tarbath et al., 2014) and, therefore, fits an ideal component in the measures for disease control (Kreidl et al., 2015). Lasting prophylactic treatment of peach, using *M. laxa* tolerant cultivars, means prevention of the pathogenic problems first in the orchard.

Interestingly in recent times, phytochemicals from plants and plant organs, including fruits, have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative and stress associated diseases (Compean and Ynalvez, 2014; Kasote et al., 2015). In addition, several other studies also point to an active involvement of these phytochemicals and quality characteristics in the protective reactions of crops against phytopathogens including fungi, bacteria and viruses (Chérif et al., 2007; Prasad et al., 2010; Sanzani, 2014; Oliveira-Lino et al., 2016). It is essential to note here, there exists many studies showing that certain phenolic compounds have inhibitory effects *in-vitro* and / or *in-vivo* against *Monilinia* spp. (Tomás-Barberán et al., 2001; Lee et al., 2006; Oliveira-Lino et al., 2016). Phenolic compounds including caffeic and chlorogenic acids may have inhibited *M. fructicola* growth and reduce lesion size (Lee et al., 2006). Hence an increase in susceptibility to brown rot infection has been associated with a concomitant decline in concentrations of these antioxidant compounds especially in maturing fruits (Villarino et al., 2011).

Breeding programs all over the world, especially the ones located in humid areas have disease resistance as one of their top priorities, in part, because the consumers' concern about chemical residues on fruits and vegetables has increased considerably. As merits, tolerant genotypes will allow sustainable control with zero residues in fruits safety harvesting and at least decreasing disease problems in commodities in storage leading to better economic benefit. The total absence of treatment residue due to the use of prophylactic tolerant peach cultivar is friendly to enhanced environment compatibility (Usall et al., 2016). However, disease resistant varieties are not readily available in many fruit crops (Spiers et al., 2005) including commercial peach cultivars (Byrne, 2012). Developing peach cultivars tolerant to *M. laxa* requires, in the first instance, identification of existing tolerant/susceptible genotypes by screening from a germplasm (Rubos et al., 2008).

It has been reported that although greater number of commercial stone fruit cultivars are susceptible to *Monilinia* spp. (Martínez-Gómez et al., 2005; Holb, 2008), there could exist genetic disease control (Martínez-García et al., 2013; Pacheco et al., 2014) to be introgressed in high fruit quality genetic background (Oliveira-Lino et al., 2016). Hence relative tolerance / susceptibility of

fruit to disease has often been used for selecting disease resistant genotype for peach breeding purpose. In addition, considering the recent drive for alternative technologies effective to control postharvest diseases (Spiers et al., 2005; Mari et al., 2015; Teksur, 2015) in particular of stone fruits (Usall et al., 2015), any documentation of composites inhibitory to BR susceptibility would have influence in breeding schemes and particularly useful in the postharvest peach market.

6.1. *In-situ* and *ex-situ* screening methods to evaluate brown rot tolerance

There are two major known systems in screening a germplasm for susceptibility or tolerance to a disease or pathogen. These systems are: *in-situ* / field, which encompass mostly evaluation on flowers, bud, twigs and shoots and *ex-situ* / laboratory, which is mostly assessment on the commercial fruit of a germplasm. A researcher, therefore, can screen for these phylogenetic sensibilities in peach, especially on the *ex-situ* evaluation by: a) assessment of lesion development on wounded, injured or bruised fruit (Gradziel and Wang, 1993); b) assessment of lesion development on intact, uninjured or unbruised fruit (Gradziel and Wang, 1993; Pascal et al., 1994); (c) assessment of spore production from determined lesions (Walter et al., 2004); d) assessment of postharvest biodegradation from natural and latent infection; and e) assessment of cuticle thickness / firmness of commercial ripe fruit (Walter et al., 2004). Table 5 shows some research in the *ex-situ* evaluation from brown rot susceptibility in stone fruits. Some researchers (Gradziel and Wang, 1993; Pascal et al., 1994;) individually used lesion development on both bruised and intact fruit to screen for brown rot resistance in clingstone peach germplasm. Uninjured fruit inoculation test (UFIT) and artificially injured fruit inoculation test (AIFIT) were utilized by Gradziel and Wang, (1993) to evaluate for resistance to *M. fructicola* in peach and Pascal et al., (1994) in apricot, plum and peach to *M. laxa*. Walter et al., (2004) adopted assessment for lesion development, spore production, storage performance and cuticle thickness / firmness in screening apricot fruit for resistance to brown rot of *Monilinia* spp. Many advantages exist in support of *ex-situ* method over the use of *in-situ* method. Manipulation of fruit is easier when evaluation is *ex-situ*. Inoculum load is centrally placed on fruit cheeks, at random including mature and immature sides (Obi et al., 2017).

In furtherance, *ex-situ* methodology ensures that the measurement of pathogenic factors such as lesion and colonization are adequately evaluated on each inoculated fruit. Also, *ex-situ* methodology facilitates the post inoculation evaluation for qualities such as firmness and soluble solid contents (SSC). Some of these operations will obviously be difficult if fruits were to be attached on the tree. *Ex-situ* enables for washing and disinfection of fruit making them pure and uncontaminated for screening against targeted pathogen. Finally, artificial inoculation on unwounded fruits, though seemingly purported to be a reliable method in evaluating for brown rot resistance *in-situ*, has been reported to be not only lengthy and laborious but also affected by season and year variability (Feliciano et al., 1987; Byrne et al., 2012).

6.2. Plant parts and screening for disease tolerance

Different parts have been used to study genetic resistance such as flowers in apricot (Christen et al., 2012) or fruits in peach (Martínez-García et al., 2013; Pacheco et al., 2014; Zeballos et al., 2016). Moreover, evaluations have consistently been performed *in-situ* with attached fruits in natural ambient situation in the field on peaches and nectarines (Paunovic and Paunovic, 1996; Martínez-Gómez et al., 2005) or *ex-situ* in apples (Biggs and Miller, 2005) and stone fruits (Oliveira-Lino et al., 2016); involving detached apricot fruits under controlled systems (Walter, et al., 2014). Considering the fact that several mechanisms are known to be involved in BR resistance, including phenolic concentration, thickness of epidermis components, and flesh texture, selection should be simultaneously based on all known as well as unknown components.

Hence, some authors (Martínez-Gómez et al., 2005; Walter, et al., 2014) went further to highlight certain imperative factors to be considered when breeding for disease resistance as: i) adequate knowledge of the pathogenic agent, including the diversity of virulence; ii) knowledge of the availability, diversity and types of genetic resistance within the breeding program, as well as within the species and close relatives; iii) the handling, developing and improving of screening methods and phenotyping, including accurate selection of the appropriate environment for exhibition of resistance to allow its accurate tracking. The overriding importance of the third point is here reverberated according to Thomidis, (2017) that the knowledge and consideration of host specificity / nonspecificity in disease management is paramount in the selection and preparation of new orchard sites and choice of tree species to be planted, among other salient parameters.

6.3. Procedures for spore production and inoculation *in lieu* of brown rot susceptibility screening

Many different procedures have been adopted in the quest to have spores and mycelia produced in the *Monilinia* for the purpose of artificial inoculation. Hence *Monilinia* isolates could be grown on PDA Petri dishes directly from infected organs: fruits, mummies, twigs (Jansch, 2012) or already prepared cultures. Also, there exists numerous methodologies describing partially similar, and in some cases divergent, protocols concerning spores concentration, inoculum load and associated variables (Table 5) for screening BR susceptibility in stone fruits, peach and nectarine in particular. All the techniques as presented in Table 5 were accomplished *ex-situ* (in the laboratory / controlled environment), except that of Paunovic and Paunovic, (1996) that was *in-situ* (in the orchard / open field). In a situation where effect of inoculum pressure was incorporated in the study, different inoculum densities and corresponding inoculum loads (Biggs and Northover, 1988; Northover and Biggs, 1990) were appropriately evaluated. In general there have been variations in the density and load of inoculum used among different authors.

Table 5: Host inoculation, spore production, and *ex-situ* brown rot (*Monilinia* spp.) susceptibility assessment in stone fruits between 1988 and 2017

Authors	Fruit type	Number of fruit	Method of inoculation	Fruit cheek	Source of inoculum	Inoculum density (cfu)	Inoculum load	Incubation period	Temperature / RH of incubation	Susceptibility variables
Biggs and Northover, (1988)	Peach	NA	UFIT	Randomly	PDA culture	10^6 - 10^3 mL^{-1}	30 µL (30,000 to 30 spores)	144 h	20 °C / 60-95%	Disease severity score
Northover and Biggs, (1990)	Cherry	10	UFIT	Suture	PDA culture	10^6 - 10^3 mL^{-1}	30 µL (30,000 to 30 spores)	144 h	20 °C/60- 95 %	% BRI, lesion diameter
Gradziel and Wang, (1993)	Peach	16	UFIT/ AIFIT	Most matured	PDA culture	2 x 10^4 mL^{-1}	10 µL (200 spores)	72 h	22°C-25 °C / 95%	Lesion diameter
Pascal et al., (1994)	Peach, Plum Apricot.	10	UFIT/ AIFIT	Randomly	Natural fruit	10^6 mL^{-1}	20 µL (20,000 spores)	240 h / 120 h	23°C	% BRI, lesion diameter
Bassi and Cantoni, (1998)	Peach	15	UFIT	Randomly	NA	10^5 mL^{-1}	NA	168 h	25±2 °C / 95-100 %	% BRI, Lesion diameter
Walter et al., (2004)	Apricot	8	UFIT/ AIFIT	Randomly	Natural fruit	1.5 x10^4 mL^{-1}	30 µL (450 spores)	48h / 120 h	Ambient temperature / lightly misted with dH_20	Lesion area, spore counts, storage rot and cuticle thickness
Pacheco et al., (2014)	Peach	10	UFIT/ AIFIT	Sun-exposed fruit cheek	Peach fruit	5 x 10^6 mL^{-1}	10 µL (50,000 spores)	120 h	25 °C / high RH	% BRI, average rot diameter by scores
Obi et al., (2017)	Peach	20	UFIT	Randomly	Peach fruit	25 x 10^3 mL^{-1}	25 µL (625 spores)	120 h	23 °C / 50- 60 %	Lesion diameter, colonization diameter, % BRI, disease severity

Abbreviations: NA (Not available); cfu (Colony forming units); RH (Relative humidity); dH20 (distilled water); % (percentage); BRI (brown rot incidence; UFIT (Uninjured fruit inoculation test (Pascal et al., 1994); AIFIT (Artificially injured fruit inoculation test (Pascal et al., 1994); PDA (Potato dextrose agar).

Several studies have investigated tolerance to BR in existing phenotypic diversity for peach breeding purpose (Biggs and Northover, 1988; Gradziel and Wang, 1993; Bassi et al., 1998; Rubos et al., 2008;) and in most cases relationships with the fruit quality traits (EPPO, 2009) have not been ignored (Pascal et al., 1994; Walter et al., 2004; Oliveira-Lino et al., 2016). Apart from selection within breeding descendant population in peach and nectarine (Bassi et al., 1998; Oliveira-Lino et al., 2016) other stone fruit germplasm include: apricot (Walter et al., 2004), plum (Pascal et al., 1994) and cherry (Holb, 2013).

Parameters usually considered in such test comprises: extent of necrosis and intensity of sporulation (Biggs and Northover, 1988), percentage fruit infection and lesion development (Northover and Biggs, 1990), lesion area, spore count, storage rot and cuticle thickness (Walter et al., 2004). Blighted flowers, twigs and shoots have also been used (Keske et al., 2013; Papavasileiou et al., 2015). However, the use of the fruit for susceptibility screening is widely used among stone fruits (Walter et al., 2004; Obi et al., 2017). Hence, in the use of peach fruit for BR susceptibility screening, BRI (percentage of fruits with lesion) (Keske et al., 2011; Obi et al., 2017) and BR severity (product of the average lesion diameter x proportion of fruit with lesions) (Keske et al., 2011; Kasote et al., 2015) can be synopsized into incidence-severity (I-S) relationships for clear epidemiological understanding and accurate susceptibility screening studies.

7. Conclusions

Despite brown rot importance, there has been relatively little work done on the development of BR resistant peach fruit cultivars probably due to lack of collaborative tendencies among specialist actors including breeders, and phyto-pathologists. Hence, the search for phenotyping protocols to accurately characterize and evaluate brown rot infection is a mission that should always be encouraged by both breeders and pathologists in crop breeding programs.

This review has been able to state clearly the characteristic variance among the three most economic important species of *Monilinia* (*M. laxa, M. fructicola* and *M. fructigena*), in addition to M. polystroma and the chemical formulations used for brown rot management, particularly, in Spain and their life limits made available. The continued increase for healthy fruit by consumers and need for environmental concerns regarding the use of pesticides and associated ecomicrobial destitution require a sustainable alternative measure to combat brown rot in the peach market. Appreciable option, therefore, is the management with the host resistance cultivars which fits into more of the prophylactic than the chemotherapeutic brown rot management available strategies.

In the recent times, several studies have investigated tolerance to brown rot in existing phenotypic diversity for peach breeding purpose and in most cases relationships with the fruit quality traits. These studies have often viewed the use of host resistance cultivars to fit into more of the prophylactic than the chemotherapeutic brown rot management adoptions available. It is our recommendation, therefore, that if adequately and effectively combined with other alternative control schemes (IPM) and especially biological strategies, host resistance could enhance more sustainable peach fruit farming in the future.

8. Compliance with Ethical Standard

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General objectives

The objectives of this thesis were:

1) To have an evaluation method, by optimizing the available protocols, to screen tolerance to brown rot by *Monilinia* spp. in peach germplasm of the Experimental Station de Aula Dei-CSIC.
2) To determine the modulating effect of pH and titarable acidity (TA) to pathogenic activities of *M. laxa*, *in-vitro* and *in-vivo*.
3) To screen eight commercial peach cultivars used as parental for susceptibility to *Monilinia laxa* and rating cultivars' tolerance exploring the genetic approach for breeding purposes.
4) To evaluate for tolerance to brown rot of *Monilinia laxa* within the breeding descendant population of 'Babygold 9' × 'Crown Princess', and to examine fruit quality and phytochemical composition in relation to the tolerance.

General conceptual framework

This thesis follows an article-based structure. It contains three articles published in indexed scientific journals and two manuscripts. The articles are connected in the thematic area, agriculture and the environment, which is the main focus of the thesis, but also by common data regarding breeding in peach for tolerant cultivars to brown rot of *Monilinia laxa* in the Northeast of Spain. The study is a continuation of previous studies developed by the research group. Figure 8 indicates an operational / pictorial framework on the general methodology depicting the *in-situ* and *ex-situ* activities with highlight on the pathogenic and non-pathogenic parameters evaluated. Each thematic objective of the thesis is addressed in each one of the chapters, subsequent to the general review. **Chapter I** sets an evaluation method to screen tolerance to brown rot by *Monilinia* spp. in peach germplasm. This optimization of available protocols was achieved with the comprehensive bibliographic review and compilation of information currently available on peach, brown rot and species of *Monilinia.* **Chapter II** examines the effect of host- pathogen interaction, *in-vitro* and *in-vivo,* using specific physicochemical factors of pH and titratable acidity (TA) in peach and *Monilinia laxa*. **Chapter III** is a screening test on selected parental lines of the peach breeding program with a view to validating and breeding for increase tolerance to brown rot. **Chapter IV** screens sixty eight progenies from the 'Babygold 9' × 'Crown Princess' population of the breeding program of EEAD-CSIC, in three years, for susceptibility to brown rot of *Monilia laxa*. These data were analysed using the SPSS-23 (Statistical Product and Service Solutions Inc., Chicago USA) statistical software.

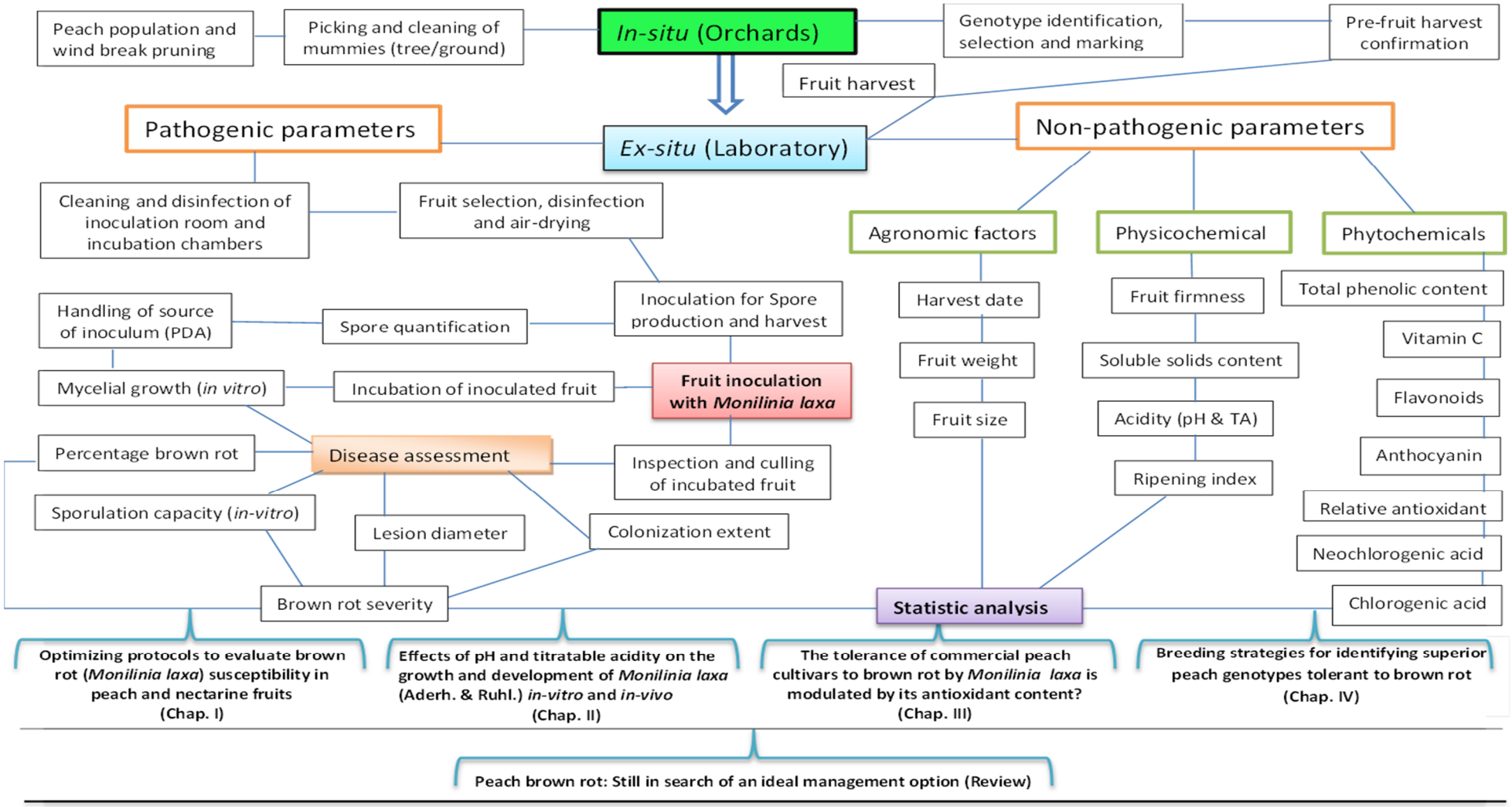

Figure 8: Operational activities in screening for phenotypic susceptibility to brown rot of *Monilinia laxa* in peach germplasm of the Aula Dei -CSIC, Zaragoza

Chapter I. Optimizing protocols to evaluate brown rot (*Monilinia laxa*) susceptibility in peach and nectarine fruits

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Optimizing protocols to evaluate brown rot (*Monilinia laxa*) susceptibility in peach and nectarine fruits.

Abstract

This study assessed and optimized an *in-vivo* method to evaluate the levels of susceptibility/resistance in fruit from the EEAD-CSIC peach germplasm to an isolate of *Monilinia laxa* (Aderhold & Ruhland) Honey from peach. A total of four commercial cultivars and six genotypes, descendants of three families, of peach [*Prunus persica* (L) Batsch.] were superficially inoculated in fruits as "uninjured inoculation fruit test". Inoculum was obtained from artificially infected peach fruit after five days of incubation under a photoperiod of 12 h supplied by fluorescent lighting system. Spores were harvested from the fruit, incubated at 20-26 ^{o}C, of 40-60 % RH, being careful to avoid contamination. Production of inoculum (conidia) was rapid and adequate using these inoculation and incubation conditions (five days at 23 ^{o}C) indicating that the *M. laxa* used was highly pathogenic and inoculation protocol suitable screen the peach material and commercial cultivars. Lesion diameter and colonization extent were measured on inoculated fruits to estimate disease severity (colonization severity and lesion severity) to establish levels of susceptibility/resistance to brown rot. Results from the screening test showed that although all peach genotypes and commercial cultivars screened were susceptible to the *M. laxa* isolate, the lack of sporulation on lesions of 'Calante' cultivar and the significant differences in colonization and lesion severity among the genotypes indicated the existence of genetic tolerance to *M. laxa* infection. The reasons for the differences in tolerance to infection and implications for breeding stoned fruit in the Ebro Valley are discussed.

Additional key words: *Prunus persica; Ex-situ; In-situ; screening germplasm; fungus*.

I-1. Introduction

Peach [*Prunus persica* (L.) Batsch] is the third most important global tree crop after apples and pears within the economically important Rosaceae family (FAOSTAT, 2016). The largest producer is China, followed by Italy, Spain, and the United States (FAOSTAT, 2016). Brown rot is a disease principally associated with the peach fruit. It is caused by *Monilinia* species which include: *Monilinia laxa* (Aderhold and Ruhland) Honey, *Monilinia fructigena* (Honey) and *Monilinia fructicola* (G. Winter) (Hu et al., 2011; Zhu et al., 2014). As polycyclic pathogens they produce numerous secondary cycles throughout the annual growth cycle of the host (Schumann and D`Arcy, 2006) affecting harvest, storage and commercial shelf life of the product (Gell et al., 2007; Thomidis et al., 2008; Sisquella et al., 2014).

Until 2006, peach brown rot in Spain was only caused by either *M. laxa* (Aderh & Ruhl) Honey or *M. fructigena* Honey in Whetzel (Gell et al., 2008, 2009). Of the species, *M. laxa* was the most prevalent (85-90%), followed by *M. fructigena* (10-15%) (Larena et al., 2005). However, since 2006 both species, *M. laxa and M. fructigena*, may have co-existed at the same relative frequency (Villarino et al., 2013; de Cal et al., 2014). The entrance of *M. fructicola* in peach orchards in the Ebro Valley, Lerida, Spain (Villarino et al., 2013) and cohabitation with *M. laxa*, since 2010 led to the displacement of *M. fructigena* (Villarino et al., 2016).

In Spain the disease causes damage of 59-100 % after harvest particularly within favourable environmental conditions (Villarino et al., 2012). Synthetic fungicides like benzimidazoles, dicarboximides and demethylation inhibitors (Egüen et al., 2015) are used to control the disease in orchards in Spain. Also antagonistic microorganisms such as *Penicillium frequentans* and *Epicoccum nigrum* fungi (Guijarro et al., 2008; Larena et al., 2010) are examples of pre-harvest control measures.

Increasing concern about the effects of biochemical fungicides on the environment (Giobbe et al., 2007), human health (Thomidis and Exadaktylou, 2010), and strain fungicide resistance (Ritchie, 1982) views new alternatives, such as host resistance, as one of the most cost effective and environmentally safe strategies for disease control.

In fruit crops different methodologies and varying protocols have been assayed in breeding programs to evaluate genetic resistance to fungi (Gradziel 1994, 2003; Byrne, 2005; Biggs and Miller, 2005). Different parts have been used to study genetic resistance such as flowers in apricot (Christen et al., 2012), twigs and shoots in pears, quince, blueberry (Bell et al., 2004; Polashock and Kramer, 2006), or fruits in peach and citrus (Gradziel and Wang, 1993; Sanzani et al., 2014). Moreover, evaluations have consistently been performed *in-situ* with attached fruits in natural ambient situation in the field on peaches and nectarines (Rubos et al. 2008) or *ex-situ* in apples (Biggs and Miller, 2005); involving detached apricot fruits under controlled systems (Walter et al., 2004).

In fruits, inoculum could be applied superficially on a specific spot (Northover and Biggs, 1990; Walter et al., 2004) or placing mycelium agar plugs on the fruit (Biggs and Miller, 2005); or dipping the fruit into an inoculum suspension (Denoyes-Rothan and Guerin, 1996; Gordon et al., 2005), even though injecting spores beyond the epidermis (Pascal et al., 1994). Finally, different inoculum concentration, treatment load, incubation period and temperature have been assayed for screening in stone fruits: 10 μL load of 2 x 10^4 spores mL^{-1} (Gradziel and Wang, 1993) and 30 μL of different spore density (10^6 to 10^3 mL^{-1}) (Walter et al., 2004; Northover and Biggs, 1990; Bonaterra et al., 2003).

Nowadays, there are numerous methodologies describing partially similar protocols concerning spores concentration and inoculum load for screening brown rot (*Monilinia* spp) tolerance in peach and nectarine (Casals et al., 2012; Kreidl et al., 2015; Cindi et al., 2016). However, little information is available on the genetic screening of peach progenies for tolerance to brown rot of *Monilinia* spp in the Ebro valley region. Casals et al., (2012), used 50 μL of *M. fructicola* at different concentrations of 10^4, 10^5 and 10^6 of spores mL^{-1}; while other authors used 10^4 of spores mL^{-1}; of 10 and 50 μL inoculum load to study *Monilinia* species and *M. laxa*, respectively (Kreidl et al., 2015; Cindi et al., 2016). However, other factors, such as inoculum production, incubation time, etc., are not comparable. Kreidl et al., (2015) used fresh cultures of *Monilinia* grown on PDA media at 25 °C for 7 days (12 h light, 12 h dark) for enhancement of sporulation and conidia production. Casals et al., (2012) and Cindi et al., (2016) used 12 day old of *Monilinia* grown on PDA culture for inoculation.

Using the available methods, longer periods are required for obtaining the source of inoculum and the amount and purity of inoculum are not assured. Moreover, an easy inoculation system would help to screen large populations and controlled environment would be preferable to avoid cross contamination. We have modified the existing protocols by shortening the inoculum production and also ensuring the accurate supply of inoculum. The objective of this study, therefore, was to have an evaluation method, by optimizing the available protocols, to screen tolerance to brown rot by *Monilinia* spp. in peach germplasm. We have evaluated brown rot tolerance on *Monilinia laxa ex situ* inoculated peach fruit by measuring brown rot incidence (%), lesion diameter (mm) and colonization extent (mm). In the Spanish peach collection at the EEAD-CSIC, screening for resistance to brown rot has shown that local germplasm accessions are susceptible although slight differences can be found.

I-2. Materials and Methods

I-2.1. Plant material

A total of two commercial peach ('Calante' and 'Catherina') cultivars, two nectarines ('Venus' and 'BigTop') and six progenies of peach [*Prunus persica* (L) Batsch.] were evaluated (Table I-1). All cultivars, except 'Venus' and 'Calante', were grown in the experimental station of Aula Dei-CSIC. Orchards are all in the Ebro valley (Northern Spain, Zaragoza) under a Mediterranean climate. All progenies are descendant from the crossing of 'Babygold 9' × 'VAC-9510'; 'Babygold 9' × 'Crown Princess' and 'Andross' × 'Calante'. They were grown under standard cultural practices (pruning, collection and adequate disposal of mummies, irrigation and weeding) and chemical spray programs for pest and disease control. In 2012, any fungicide treatment was applied in the field before harvest. The preventive fungicide Teldor® 500 SC was only sprayed after regular irrigation or rainfall on July 16 and August 7.

Fruits were harvested based on optimum maturity (Cantín et al., 2009) (expressed on visual colour change and manual evaluation of firmness, selecting healthy fruit with uniform ripeness and size). Peaches were disinfected by immersion in aqueous solution of 1.6 % sodium hypochlorite (commercial), 0.005 % Tween® 80 (polysorbate surfactant) and 1.6 % ethyl alcohol for 4 minutes, rinsed in sterile distilled water, and spread out on sterile holding stone-fruit cardboard cells for 20 minutes ambient air drying in blossom-stem (upside down) position to avoid any possible percolation at the stem position.

I-2.2. Pathogen culture

The culture of the *Monilinia laxa* (Aderh. & Ruhl.) Honey used in this study was supplied by the Post-harvest Pathology Unit, IRTA Lerida, and it was isolated from peach from the Cataluña region of Spain (isolate number: CPML02). The strain was maintained at 4 ^{o}C in the dark on 39 gL^{-1} solid potato dextrose agar (PDA) medium (Panreac, Spain). The fungal culture was replicated biweekly and maintained at 23 ^{o}C under continuous darkness during the periods in which the experiments were conducted. There was also periodic inoculation on peach fruit for preservation of pathogenicity according to Koch-Pasteur's postulates (de Cal et al., 1990).

I-2.3. Conidia production and inoculation

To ensure sufficient conidia production (Kreidl et al., 2015) for the susceptibility studies, the isolate was inoculated onto surface sterilized peach fruits (Catherina cv.), to avoid surface contaminants, by wounding the fruit (1 x 2 mm) with a sterile surgical blade and transferring mycelium plug from the PDA culture to the wound site using a sterile inoculating needle. We have used wounded non-inoculated fruits as control to determine the existence of latent infections. The commercial cardboard box hosting the fruits was covered with sterile transparent polythene sheet, with the ends slightly sealed with sellotape. Fruits were incubated at 20-26 ^{o}C, of 40-60 % RH and photoperiod of 12 h supplied by a mixture of two fluorescent lighting systems (Sylvania Gro-Lux, F36W / GRO and Ogram Daylight, F36W / 840DL of the Fridger Growth Chamber, Spain) for 4 to 6 days.

Conidia were efficiently harvested into a solution of sterile distilled water and Tween® 80 (0.0005%) surfactant by mildly rubbing over the infected regions of the fruit using a sterile dental brush. This involves superficial submerge of sample in the harvest solution and relative slight spore wash with the brush (restrained harvest as against profound harvest method). The spore solution was stirred at 1500 r.p.m. for 3 min, and then filtered through a sterile fourfold cheese cloth to remove mycelia and inert particles. Quantification of conidial suspension was determined on a hemocytometer (Neubauer new improved chamber) and the density adjusted to 25 x 10^3 mL-1 for inoculation purposes.

To evaluate tolerance to brown rot, 20 disinfected fruits from each genotype were inoculated with the isolate of the virulent pathogen. On the equatorial position of each unwounded fruit, 25 μL spore load (one drop) of the 25 x 10^3 mL^{-1} conidial suspension of *M. laxa* was deposited. Five fruits used as control were loaded with 25 μL sterile water accordingly.

I-2.4. Incubation and brown rot evaluation

Inoculated fruits were incubated for 5 days in darkness at 23 ^oC and 40-60% RH (Climatronic 2132-model growth incubator, Germany). Brown rot incidence was assessed using the fraction infected over total number of inoculated fruits. Lesion diameter and colonization extent were measured across perpendicular sectors (longitudinal and latitudinal fruit sectors) with a digimatic venier caliper (Mitutoyo CD-15 DCX, Tokyo Japan). These parameters were used in the determination of brown rot disease severity for genotype tolerance rating in the study according to (Martínez-García et al., 2013). Lesion severity (LS) was calculated by the percentage of brown rot incidence (% BRI) x lesion diameter (LD) /100 and colonization severity (CS) by the percentage of colonization (% C) x colonization extent (CE)/100. Mean, standard errors (SE) and Pearson correlations were carried out with SPSS-19 statistical software (Statistical Product and Service Solutions Inc., Chicago USA). Statistical significance was judged at the level $P < 0.05$, and the Duncan test was used for mean comparison (ANOVA test).

Table I-1: List of accessions, harvest time, brown rot incidence and mean ± SE of the pathological parameters evaluated in 2012

Accession name	Fruit type	Harvest date (Julian days)	Brown rot incidence (BRI) (%)	Colonization extent (CE) (mm)	Colonization severity (CS) (mm)	Lesion diameter (LD) (mm)	Lesion severity (LS) (mm)	Source
1_(B9×VAC)	Peach	208	100	51.50 ± 3.6 **c**	51.50 ± 3.6 **d**	57.10 ± 1.4 **b**	57.10 ± 1.4 **c**	EEAD
2_(B9×CP)	Peach	219	100	28.78 ± 5.7 **b**	28.78 ± 5.7 **bc**	49.85 ± 3.2 **ab**	49.85 ± 3.2 **bc**	EEAD
3_(A×C)	Peach	219	80	27.07 ± 7.0 **b**	21.65 ± 5.6 **bc**	47.06 ± 2.7 **ab**	37.65 ± 2.3 **b**	EEAD
4_(A×C)	Peach	240	100	49.76 ± 2.4 **c**	49.76 ± 2.4 **d**	55.40 ± 3.5 **b**	55.40 ± 3.5 **c**	EEAD
5_(A×C)	Peach	240	100	49.68 ± 2.6 **c**	49.68 ± 2.6 **d**	61.95 ± 2.1 **b**	61.95 ± 2.1 **c**	EEAD
6_(A×C)	Peach	240	100	50.68 ± 3.9 **c**	50.68 ± 3.9 **d**	62.52 ± 2.8 **b**	62.52 ± 2.8 **c**	EEAD
Big Top	Nectarine	218	100	32.96 ± 8.8 **b**	32.96 ± 8 8 **c**	38.26 ± 8.6 **a**	38.26 ± 8.6 **b**	EEAD
Calante	Peach	291	100	00.00± 0.0 **a**	00.00 ± 0.0 **a**	38.00 ± 5.8 **a**	38.00 ± 5.8 **b**	Alcañíz
Catherina	Peach	208	100	48.56 ± 1.4 **c**	48.56 ± 1.4 **d**	54.33 ± 0.9 **b**	54.33 ± 0.9 **c**	EEAD
Venus	Nectarine	218	60	27.04 ± 6.9 **b**	16.22 ± 4.2 **b**	34.95 ± 6.0 **a**	20.97 ± 3.6 **a**	CITA

SE: Standard error (n = 5-20); source: location of sample orchard. B9: 'Babygold 9', VAC: 'VAC 9510', A: 'Andross'; CP: 'Crown Princess'; 'Calante' was grown in Alcañiz (Teruel) and 'Venus' in orchards of CITA-Aragón. Means with different letters show significant differences at $P \leq 0.05$ (Duncan's test).

I-3. Results

As indicated in Table I-1, there was 100 % brown rot incidence (BRI) in all genotypes except for the 3_(A × C) genotype with 80 % and the 'Venus' cultivar with 60 %. There were significant differences within the mean colonization extent and lesion diameters (Table I-1), as well as in the colonization and lesion severities (Figure I-1).

The 3_(A × C) genotype, and 'Big Top', 'Venus' and 'Calante' cultivars exhibited brown rot severity (BRS) significantly lower than 40 mm (Figure I-1). Lesion severity (LS) was between 62.52 and 20.97 mm, colonization severity (CS) was between 50.68 mm and zero. The 'Venus' and 'Big Top' cultivars and 2_(B9 × CP) and 3_(A × C) genotpes showed colonization severity below 40 mm (16.22 mm and 32.96 mm; 28.78 mm and 21.65 mm, respectively). The 'Calante' was the only treatment without colonization owing to no or restricted sporulation and therefore, zero CS.

The Pearson's correlation coefficients (r) between pairs of traits are shown in Table I-2. Brown rot incidence (% BRI) significantly correlated with all pathologically associated factors [LD (r = 0.293, $P \leq 0.01$); LS (r = 0 471, $P \leq 0.01$); CE (r = 0.349, $P \leq 0.01$) and CS (r = 0.473, $P \leq 0.01$)]. This indicates that the level or frequency of infection will always and significantly influence the lesion diameter and colonization extent including severities in a disease situation. All pathological traits showed significant correlation higher than 0.883. Also harvest date showed a positive and significant correlation with % BRI (r = 0.314, $P \leq 0.01$); LS (r = 0.405, $P \leq 0.01$) and CS (r = 0.313, $P \leq 0.01$).

I-4. Discussion

In this study, we have validated the inoculation method finding differences in the susceptibility to the pathogenic activities of *M. laxa* in two peaches ('Catherina' and 'Calante'); two nectarines ('Venus' and 'Big Top') and six peach hybrids. In order to screen a large germplasm sample we have modified the existing methods. Incubation factors as temperature (20-26 ^{o}C), relative humidity (40-60%) and 12 h photoperiod enhanced colonization and sporulation for substantial conidia production.

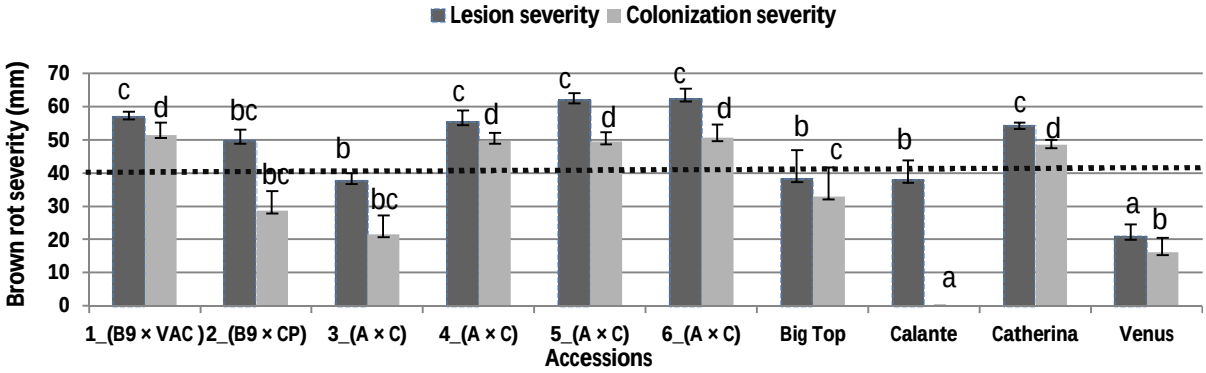

Figure I-1: Brown rot severity (BRS, in mm) on the studied genotypes. Lesion severity = %BRI x Lesion Diameter. Colonization severity = % Colonization x Colonization extent. Bars represent the standard errors (n = 5-20). The dotted line indicates brown rot severity less than 40 mm. Means with different letters show significant differences at $P \leq 0.05$ (Duncan's test).

We have found that the peculiar lighting source (mixture of two fluorescent lighting systems, Sylvania Gro-Lux, F36W / GRO and Ogram Daylight, F36W / 840DL) was positive to obtain enough inoculum in a short period of time. We have also found that artificial injury ensured adequate and prompt infection of host (Casals et al., 2010; de Cal et al., 2013).

Table I-2: Pearson`s correlation coefficients among pathological traits and harvest date

Traits	LD (mm)	LS (mm)	EC (mm)	CS (mm)	Harvest date (Julian days)
BRI (%)	0.293**	0.471**	0.349**	0.473**	0.314**
LD (mm)		0.980**	0.901**	0.908**	0.374**
LS (mm)			0.883**	0.920**	0.405**
EC (mm)				0.989**	0.285**
CS (mm)					0.313**

Units and abbreviations: BRI = Brown rot incidence; LD = Lesion diameter; LS = Lesion severity; CE = Colonization extent; CS = Colonization severity; ** = Correlations significant at $P \leq 0.01$.

We have also found that artificial injury ensured adequate and prompt infection of host (Casals et al., 2010; de Cal et al., 2013). Fruit injury encouraged short period of incubation making available inoculum source within 3 days post inoculation and avoiding cross-contamination of other fungi such as *Botrytis* and *Rhizopus* species. Moreover, the purity of inoculum load was ensured with this method because potential contaminants such as nematodes were avoided during conidia harvest by mildly rubbing over the infected regions of the fruit using a sterile dental brush. Initially enough *M. laxa* inoculum was produced from PDA culture incubated (Memmert CO_2 incubator INCO, Germany) at least 35 days at 23 ^{o}C in the dark. Nevertheless, we tried to reduce the time to obtain inoculum adapting the methods used by Gell et al., (2007) and Jansch et al., (2012). They incubated prepared PDA cultures of *Monilinia* spp in the dark at 20-25 ^{o}C for 7 to 10 days for adequate sporulation. But our preliminary trials (data not shown) indicated poor inoculum production when incubated in the dark even with more than 6 days of incubation at 23 ^{o}C. Finally we have assured effective inoculum in 4 to 6 days after incubation of inoculated peaches under at temperature (20-26 ^{o}C), relative humidity (40-60%) and 12 h photoperiod.

Furthermore, the composition of inoculum for susceptibility screening could involve one pathogenic strain (Gradziel and Wang, 1993) or two or more pathogenic isolates pooled (Biggs and Miller, 2005) to enhance pathogen virulence. A mono-conidial culture was used in our study because our inoculum source was tested for stable virulence and was stable enough to initiate pathogenesis without need for strain synergism. We established effective inoculum density of 25 x 10^3 mL^{-1} with inoculating load of 625 spores per fruit. Pascal et al., (1994) though employed a mono-conidial isolate but higher loading of 20,000 spores / fruit that may be less sustainable.

We have evaluated 20 fruits per genotype as enough sample size to assess representative evaluation considering the large population involved in plant breeding. The number of samples used to evaluate disease tolerance against brown rot in stone fruit varies among researchers from 10 peaches per genotype (Gradziel and Wang, 1993) to 30 fruits in apricot, plum and peach (Pascal et al., 1994). Moreover, the preference for the *ex-situ* evaluation is that the methodology enables for effective and efficient handling of large population and sample material. The *ex-situ* system also enables for thorough selection of healthy fruits and disinfection from insects and potential contaminants. Also essential is that direct influence such as variation in temperature, precipitation and relative humidity are avoidable environmental factors when screening, for brown rot tolerance, under a controlled environment. The germination and other pathogenic activities of conidia are markedly influenced by the interaction of the above mentioned factors (Xu et al., 2001; Casals et al., 2010).

The 3_(A × C) genotype, and Big Top, 'Venus' and 'Calante' cultivars exhibited brown rot severity (BRS) lower than 40 mm (Figure I-1). Paunovic and Paunovic, (1996) have reported similar

susceptibility values in peach germplasm in Yugoslavia. However, their investigation was done *in-situ* involving five pathogens (*M. laxa, M. fructicola, Sphaerotheca pannosa, Tapharina deformans* and Sharka virus).

As shown in Table I-2, brown rot incidence (% BRI) showed positive and significant correlations with all pathologically associated factors (LS, CE and CS). This indicates that the level or frequency of infection will always and significantly influence the lesion diameter and extent of colonization including severities in a disease situation. Also harvest date showed a positive and significant correlation with % BRI, LS and CS. Inferring that genotypes that mature later generally tended to have bigger lesions and would be less tolerant to fungal pathogen. This is the first time to find that the pathological parameters correlate, positively, with Julian days. However, an ANOVA showed that there were scarcely any significant differences in pathological traits between early and late harvested treatments (Table I-1).

There was a particular interest on the 'Calante' cultivar which, though produced lesion, was devoid of colonization (sporulation) (Table I-1), an important factor in disease spread. This feat is biologically and epidemiologically an advantage for plant breeding. Its high level of tolerance may be related to a late harvest or to the agronomical practices performed in Alcañiz. In these orchards, due to the singularity of this late cultivar harvested in October, fruits are protected to pathogen injuries using paper bags. Other authors have found annual variation for brown rot infection in 'Calante' (Obi et al., 2015; Ágreda et al., 2016). It was described that colonization in nectarines and peaches fruit infected by *Monilinia* spp. could be associated with the local acidification of the host tissue (de Cal et al., 2013) and that acidic environment can prevent brown rot colonization on peach (Bonaterra et al. 2003), however, it is not the explanation for our results since the fruit has at maturity the same pH or acidity year by year. Other uncontrolled factors such as environmental instead of genetic may be responsible for non-colonization in 'Calante' harvested in the 2012. Moreover, colonization on host (extensive differentiation of hyphae at the surface of host) is not a genetic trait (Giobbe et al., 2007).

According to this research we have not found differences indicating the impact of hairiness on susceptibility although other authors have proposed that fruit hairiness could encourage susceptibility to disease infection in some stone fruits (Wade and Cruickshank, 1992; Xu et al., 2007; Garcia-Benitez et al., 2016). In our plant material using this protocol we have demonstrated a relatively similar degree of tolerance / susceptibility between peach and nectarine fruits. An example are the low levels of *Monilinia laxa* incidence registered with the 'Venus' nectarine and 3_(A × C) peach genotype or the lower LS found in 'Venus' in comparison with the rest of cultivars.

I-5. Conclusions

In this study an efficient *ex-situ* procedure through artificial skin inoculation to assess the susceptibility to brown rot (*Monilinia laxa*) of commercially ripe fruits was performed in ten peach and nectarine genotypes. We have modified the existing protocols by shortening the inoculum production and also ensuring the accurate supply of inoculum. The methodology has been tested and shall be applied in our breeding program.

The artificial injury ensured adequate and prompt availability of inoculum source. Incubation factors as temperature (20-26 ^{o}C), relative humidity (40-60%) and 12 h. photoperiod with two fluorescent lighting systems enhanced colonization and sporulation for substantial conidia production. In addition, this method ensured between 3 to 6 days the optimum conidia purity considering the 'restrained' harvest system we adopted to obtain inoculum.

Finally, it was possible to discriminate between highly and less susceptible peach germplasm at the EEAD. However, we have observed on a few of the genotypes the interaction of firmness and soluble solid compounds with pathologic factors. For further studies we suggest to consider adequately in the screening for tolerance, firmness, soluble solid compounds (at harvest and incubation) and other agronomic and quality traits. On the development of brown rot the effect of the agronomical practices shall be surveyed to ascertain influence on evaluation of the tolerance.

I-6. Acknowledgments

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Chapter II. Effects of pH and titratable acidity on the growth and development of *Monilinia laxa* (Aderh. & Ruhl.) *in-vitro* and *in-vivo*

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Effects of pH and titratable acidity on the growth and development of *Monilinia laxa* (Aderh. & Ruhl.) *in-vitro* and *in-vivo*

Abstract

This investigation examines the effects of pH and titratable acidity on the growth and developments of a strain of *Monilinia laxa* (Aderhold & Ruhland) on seven different pH in Potato Dextrose Agar media and on peach fruit from formation to commercial maturity. The fungi growth was obtained by daily measurement of mycelia on the pH amended Potato Dextrose Agar. The sporulation performance was determined after 30 days of culture incubation. Fruits were inoculated with *M. laxa*, from fruit set to maturity, on weekly basis for brown rot susceptibility. The pathogen development, *in-vitro,* was affected, by the pH (2.4-11.52) amended nutrient media. *M. laxa* exhibited variation in its growth and sporulation capacities on the seven pH amended PDA, preferring relatively moderate acidic conditions for their optimum performance. In the *in-vitro* analysis, there was mycelia growth in pH from 2.40 to 8.84 while pH 11.52 did not support any mycelia growth. There was a continuous and stable increase in weight of fruit as it develops whereas the fruit size increased, decreased at a time and finally increased as the fruit develops. The acidity dynamics exhibited non-sinusoidal waveform through the growth and development of the fruit. In all these characteristic variations, *M. laxa* could not develop infection or shown any brown rot incidence in the fruit until the period of commercial maturity.

Key words: Alkaline; Monilia; Physicochemical; *Prunus persica;* Tolerance.

Abbreviations used:
BRI: brown rot incidence
DAF: days after floration
DAS: days after fruit set
EpHV: expected pH values
FS: fruit size
FW: fruit weight
JDs: Julian days
PDA: potato dextrose agar
PP: polypropylene
RPM: revolutions per minute
RpHV: real pH values
TA: Titratable acid.

II-1. Introduction

Brown rot in peaches (*Prunus persica* (L.) Batsch) is a disease primarily incited by *Monilinia* species which includes *M. laxa, M. fructigena, M. fructicola, and M. polystroma* (Gell et al., 2007; Jansch et al., 2012). The degree of susceptibility to infection by *Monilinia* spp. is variable throughout fruit development. Susceptibility is high during the early stages of fruit development, decreases during the green fruit stage and increases again during the ripening period (Gradziel, 1994). Stages of peach development can generally be considered to occur in four phases which includes, fruit set, rapid cell division, cell expansion and ripening/maturation (Moing et al., 1998; Tutu and Ciornea 2011; Guidarelli et al., 2014).

In a pathogen-host interaction the growth and development of microorganism is influenced by different physicochemical conditions such as temperature and water activity (Pascual et al., 1997; Xu et al., 2001; Moral et al., 2012), light, aeration and pressure (Maharshi and Thaker, 2012), pH and titratable acidity (Pascual et al., 1997; Dirlewanger et al., 1999; Holb 2004; Romero-Arenas et al., 2012). During this process the physicochemical conditions influence the microbial activity determining either the growth and reproduction or the inhibition of activity and the inactivation of the pathogen (Dirlewanger et al., 1999; Tutu and Ciornea, 2011).

The pH and titratable acidity (TA) are interrelated concepts of organic acids (Tyl and Sadler, 2017) controlling physicochemical factors that act in an additive and interactive mode to inhibit metabolic pathway (Dirlewanger et al., 1999). These two physicochemical components, though complementary in nature, are statutorily different. TA refers to the total concentration of free protons and undissociated acids in a fruit juice that can react with a strong base and be neutralized, hence it is any amount of base needed to neutralize such acidity and bring its pH to a neutral (pH 7), or slightly alkaline (pH 8.1) value, and pH represents the free hydrogen ion activity in the fruit juice (Lobit et al., 2002) or a means of expressing such H^+ ions concentration in a substrate (Tutu and Ciornea, 2011). In peach fruit acidity is an important genetic quality (Dirlewanger et al., 1999) which influences both perception of sourness and sweetness found in varying proportions depending on the cultivar and the ripening stage (Lobit et al., 2002).

The behavior of microorganisms-host interaction shows variation in their growth and sporulation on different levels (Maharshi and Thaker 2012). Most microorganisms, especially non fungi, grow best around neutrality (pH 7), while fungi in general prefer slightly acidic conditions for their growth (Alexopoulos, 1952; Yamanaka, 2003; Agrios, 2011; Maharshi and Thaker, 2012). Some fungi species, however, favour neutral to slightly alkaline conditions (Maharshi and Thaker, 2012). *Monilinia* spp. can acidify the host tissue in peaches and nectarines from pH 4.50 and 4.45, to pH 3.75 and 3.90, respectively (de Cal et al., 2013). For *M. laxa*, information related with the effect of pH on growth and development *in-vitro* and *in-vivo* is hardly available. *M. laxa* can infect flowers, resulting in blossom blight, as well as both healthy and wounded fruit, resulting in brown rot (Rungjindamai et al., 2014); a disease able to produce millions of spores on a single fruit that can spread quickly within and between orchards, locations, and hosts (Fazekas et al., 2014).

The general objective of this experiment was to determine the modulating effect of pH and titarable acidity (TA) to pathogenic activities of *M. laxa.* This included (a) determining the effect of pH on the mycelia growth, sporulation and development of *M. laxa* on solid PDA, and (b) to determine the effect of pH and TA evolution on the brown rot incidence (BRI) depending on the growth and development of peach fruit.

II-2. Materials and methods

II-2.1. Culture pH media preparation, inoculation and incubation

The cultivation medium, potato dextrose agar (PDA), was prepared in line with the manufacturer's instruction following which 7.8g was measured into seven Erlenmeyer flasks (500 mL) and 200 mL sterile water added to the different flasks and contents slightly heated in a micro wave for proper dissolution of mixture. They were sterilized at 121^{o}C for 15 minutes and in a laminar flow chamber (aseptic conditions), known quantity of H_2SO_4 and KOH chemicals as previously determined by titration, were added with a pipetman into the flasks (pH 5.30) marked with the expected pH values

(EpHV) at warm (45°C-60°C) temperature. The solution was agitated with a magnetic stirrer at 110 rpm for about 30s for homogeneity.

Known quantity of 20mL was separately and aseptically decanted into 50mL transparent polypropylene (PP) jar and pH stripe indicator cut and dipped into the jars to observe the associated pH readings. The molten PDA was later measured with the pH meter to obtain the real pH value (RpHV) before pouring out into Petri dishes of 90mm diameter at 20mL / plate for inoculation with *M. laxa*. The desired pH was adjusted with 1mole Hydrogen sulphate (H_2SO_4) and 5Mole Potassium hydroxide (KOH) for acidic, neutral or alkaline values using the pH meter. Known quantity of H_2SO_4 or KOH was, aseptically, added to the sterilized PDA (in 500mL Erlenmeyer flask at warm (45°C-60°C) temperature. The pH amended PDA were poured out at 20mL / Petri dishes of 90 mm diameter (5 Petri dishes / treatment). Active growing (7 days old) *M. laxa* mycelia (6.5 mm) from a PDA culture was centrally plated in the Petri dish containing the real pH values (RpHV) and incubated at 23°C. And later the mycelia growth and extent of sporulation were determined.

II-2.2. Measurements of mycelia growth rate and sporulation density

Measurements of mycelia growth were taken daily using a digital Venier meter (Mitutoyo CD-15 DCX, Tokyo Japan) at a cross section. This was done until a Petri dish was fully covered with the mycelia extension. Rate of evolution and influence of pH on pathogen activity were determined using the mycelia growth. There were five replications for each treatment.

After the data collection on mycelial growth, the culture was subsequently evaluated for sporulation activity at the 30th day of incubation. Distilled water (5 mL) was added to each of the Petri dishes containing 25µL Tween® 80 and the mycelia colony rasped with a sterile laboratory metal spatula. The rasped colony together with the Petri dishes was placed on a mechanical shaker at 175rpm for 30minutes. Each treatment was filtered through a 4 fold of cheese cloth into premarked test tubes. From this suspension, 25µL was pippeted onto a hemacytometer (Neubauer Cell Counting Chamber) and examined under a microscope. Two different spore loads (40 counts / load) were made in each replication of the seven treatments.

II-2.3. Plant material, fruit size and weight determination

Two peaches (*Prunus persica* (L.) Batsch) were the source of plant materials. The plant materials were 'Babygold 9' and 'Crown Princess' from the collection of the Aula Dei-CSIC, Zaragoza. There were three trees per cultivar. Number two (middle tree) was marked and labeled after fruit setting for use in the evaluation. The marked tree was not thinned to enable enough fruits for sample harvest. Numbers 1 and 3 trees were however given the normal orchard treatments (e.g. fruit thinning). Fruits selected for analysis were all of similar maturity and size at each developmental stage. Inoculation with *M. laxa* was completed on 'Babygold 9' and not on 'Crown Princess' after observing that there was no infection at the early stage of inoculation. And the aim was to preserve enough samples for other essential analysis due to fewer available fruits on the 'Crown Princess'.

The range of complete floration and fruit settings in the two cultivars (Table II-1) occurred in the early to mid-season of spring between 10/03/2014 and 11/04/2014; and the range of harvest date occurred in the early to mid-season of summer. 'Babygold 9' reached 100 % floration and fruit setting on the 14/03/2014 (72 JDs) and the 11/04/2014 (100 JDs) respectively and harvested on the 21/08/2014 (232 JDs). 'Crown Princess' reached 100 % floration and fruit setting on the 10/03/2014 (68 JDs) and fruit setting on the 11/04/2014 (100 JDs) and harvested on the 18/06/2014 (168 JDs).

Five fruits per cultivar were used for evaluations at weekly basis. Fruits of visually uniform size were harvested each period. Fruit size (mm) was considered by measuring the two diagonal sections with a digimatic venier caliper (Mitutoyo CD-15 DCX, Tokyo Japan). The fruit weight (g) was determined on a precision weighing machine.

II-2.4. Development of pH and titratable acidity

TA and pH were determined as explained in previous studies (Cantín et al., 2009; Abidi et al., 2015). In brief three fruits were used per cultivar for this purpose. At weekly bases, after setting, fruits were harvested, cleaned with tap water, peeled and cut into thin slices. Five grams was weighed out into an automatic titrator tube and mashed dry with a polytron machine (Ika T-18 Ultra Turrax Digital High Speed Homogenizer, Germany) and later 45mL of distilled water measured into the titrator tube. The pH automatic valuator machine was first calibrated using a buffer (for pH 4 and pH 8). This is after cleaning and hushing the electrodes with distilled water then filled with electrolyte (KCl) for compensations of any possible loss due to evaporation. The known quantity of the solution (50g) in the automatic valuator was used to determine the pH with TA values accordingly. This was repeated every week until the cultivar reaches commercial maturity date (Larena et al., 2005).

II-2.5. Evaluation for susceptibility to *Monilinia*

Five fruits per cultivar were used for this determination. Fruits were disinfected according to Obi et al., (2017).

Table II-1: Complete floration, fruit settings and harvest in two peach cultivars (gestation period)

Activity	'Babygold 9'	'Crown Princess'	BBCH-scale
Floration (100%)	14/03/2014 (72 JDs)	10/03/2014 (68 JDs)	65
Fruit setting (100%)	11/04/2014 (100 JDs)	11/04/2014 (100 JDs)	73
Harvesting date	22/08/2014 (233 JDs)	12/06/2014 (162 JDs)	87

Using a Pipetman P100, fruits were inoculated with the isolate of *M. laxa* in the method of Obi et al. (2017). In brief each fruit, unwounded, was inoculated with 25μL spore load of the 25 x 10^3 cfu mL^{-1} conidia suspension. Inoculated samples were incubated at 23 ^{o}C and 40-60% RH and duly observed for brown rot incidence for seven days.

II-2.6. Statistical analysis

The size, weight, pH and TA of fruit, including their standard errors were analyzed using the SPSS-23 statistical software (Statistical Product and Service Solutions Inc., Chicago USA). Earlier normality test was realized on parameters with the Kolmogorov-Smirnov ($P \geq 0.05$), enabling the presentation of frequency of histograms. The same statistical software was used for mean standard errors (SE) and Pearson's correlations. ANOVA test was used to compare differences between means and a post hoc test of the Duncan (DMRT) used to measure for separation of specific variability ($P \leq 0.05$) between pairs of means.

II-3. Result

II-3.1. Mycelial growth

Mycelial growth or extent of colonization was evaluated on seven levels of pH. There was mycelia growth in all the pH levels except on pH 11.52. The highest mycelia growth was on pH 6.40 (80.61 mm)

at the rate of 8.96 mm/day. The lowest mycelial growth was on pH 2.40 (11.60 mm) at the rate of 1.29 mm/day (Figure II-1). Mean values varied according to the extent of colonization.

II-3.2. Sporulation capacity

Sporulation capacity was determined using the *M. laxa* colonies of the seven different levels of pH after 30 days of incubation. Sporulation was found to be highest at pH 5.30 with mean conidia concentration of over 1 x 10^5 cfu mL^{-1} and lowest at pH 8.84 with mean conidia of less than 5 x 10^4 cfu mL^{-1} (Figure II-2). There were no sporulation of *M. laxa* at pH 2.40, 3.01 and 11.52. There were, however, significant differences ($P \leq 0.05$, Duncan's test) in the sporulation capacity among pH 4.21, 5.30, 6.40 and 8.84.

II-3.3. Development of pH and TA in 'Babygold 9' and 'Crown Princess'

The evolution of pH and TA in fruits of 'Babygold 9'and 'Crown Princess' cultivars are non-sinusoidal waveform. This waveform is best demonstrated in the 'Babygold 9' (Figures II-3 and II-5) which has a longer period of gestation. In this particular cultivar the pH peak (highest) was at 208 JDs (77 BBCH scale) which corresponds to 136 and 108 days of complete floration and fruit setting respectively. The lowest dip was at 215 JDs (78 BBCH scale) which was equivalent to 143 and 115 days after floration (DAF) and days after setting (DAS) respectively. From this position the pH increased along with the remaining growth and commercial maturity of the fruit.

In all this non-sinusoidal waveform dynamics of pH in 'Babygold 9', the pattern in TA evolution was always in the contrary (Figure II-3), hence the lowest dip was at 208 JDs (77 BBCH scale) which corresponds to 136 and 108 DAF and DAS respectively. The highest peak was at 215 JDs (78 BBCH scale) which was equivalent to 143 and 115 DAF and DAS respectively. From this position the TA decreased along with the remaining growth of the fruit.

II-3.4. Growth in fruit size and weight

The evolution of fruit size (FS) and weight (FW) is presented in Figure II-4. FS was at steady increase for well over 194 JDs (75 BBCH scale). This pattern slightly changed at 201 JDs (76 BBCH scale) where there was a slight slope that picked up and briskly ascended at the 208 JDs (77 BBCH scale) and then a gentle smooth increase in size until the fruits' period of commercial maturity and subsequent harvest on the 232 JDs (79 BBCH scale).

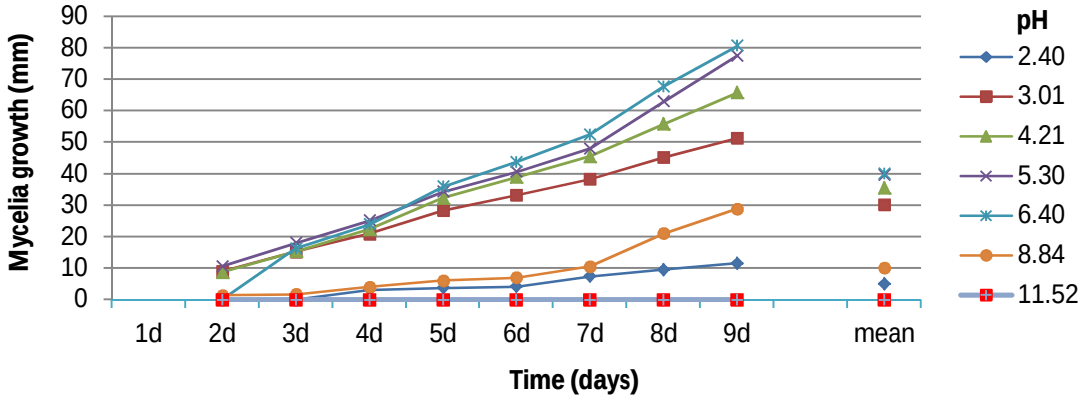

Figure II-1: Mycelia growth rate of *Monilinia laxa* (mm), *in-vitro*, on the 7 different pH. *M. laxa* performed best at pH 6.4 and lowest at pH 2.4. No mycelium at pH 11.52. Each treatment was replicated five times and experiment repeated twice.

In the same vein, FW was at a steady increase for over a period of 201 JDs (76 BBCH scale). This pattern changed at the 208 JDs (77 BBCH scale) when a noticeable rapid jerk up was observed. This increase appeared to have continued until the fruits' period of commercial maturity and subsequent harvest on the 232 JDs (79 BBCH scale).

II-3.5. Effect of pH and TA on brown rot incidence

Figure II-5 represents the effect of pH and TA on brown rot incidence along with the growth and development of the fruit. After the highest increase in pH 4.39 ± 0.10 at 208 JDs (77 BBCH scale) and the lowest dip in pH 3.63 ± 0.10 at 215 JDs (78 BBCH scale), the pH reinitiated and continued to increase until fruit maturity. The reverse was the case with the TA which obviously started to decrease until maturity (pH and TA linked with broken lines at 208 JDS (77 BBCH scale). Hence at the full commercial maturity, the fruit was associated with pH 4.19 ± 0.10 and TA 0.41 ± 0.01 (Mg $100g^{-1}$ FW). From the beginning of fruit inoculation (145 JDs) (65 BBCH scale) with *M. laxa* to 222 JDs (78 BBCH scale) there was no incidence of brown rot until when the fruit was inoculated at the 232 JDs (79 BBCH scale) of commercial maturity.

II-4. Discussion

In general according to the obtained results, in this investigation, there is an ample influence of pH and titratable acidity (TA) in the solid PDA and the peach cultivars inhibiting the growth of the *M. laxa*. According to Tutu and Ciornea (2011) pathogen host interaction involves the process of nutrition which leads to either the growth / reproduction or the inhibition of activity and the inactivation of the pathogen as a result of available acidity factors. In the pH-amended PDA there was no effective pathogen growth/development under a high acidic condition. Similarly, *M. laxa* developed no infection in the fruit until commercial maturity at pH 4.19 ± 0.10. This is relatively a moderate state of non-acidity in the fruit. Previously peach fruits with a non-acid character have been characterized at maturity by a pH higher than 4.0 (Dirlewanger et al., 1999).

Mycelia growth (colonization) and sporulation are the most accurate variables used in plant pathology to effectively compute the degree of disease development (Douds 1994; Gigot et al., 2009; Miles et al., 2009; Burnett et al., 2010; Obi et al., 2017).

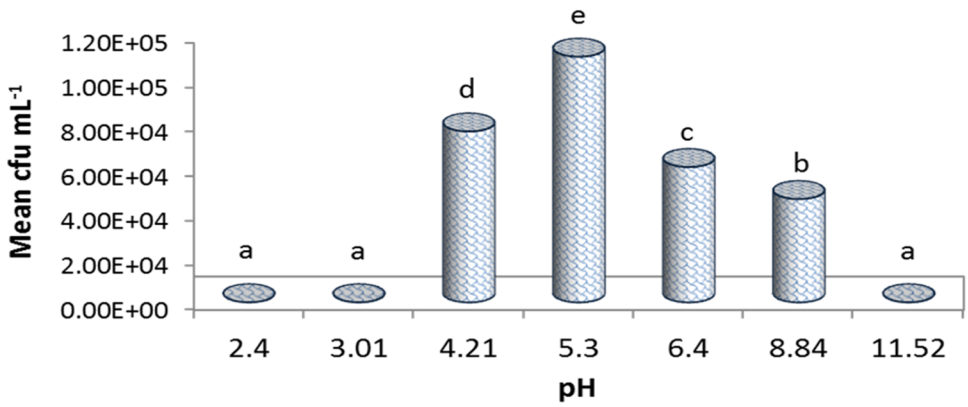

Figure II-2: Sporulation capacity of *Monilinia laxa* on 7 different pH at 30 days of incubation. Different mean letters indicate significant differences (P ≤ 0.05, Duncan's test) among pH. *M. laxa* sporulated highest at pH 5.30. No sporulation at pH 2.40, 3.01 and 11.2. Each treatment was replicated five times and experiment repeated twice.

Consequently, determining the influence of pH and TA on the subsistence and development of *M. laxa,* at both *in-vitro* and *in-vivo* levels, is an avenue to understanding the epidemiology of brown rot and subsequent development of disease management strategies to effectively combat the problem (Tian and Bertolini, 1999; Hong et al., 2000).

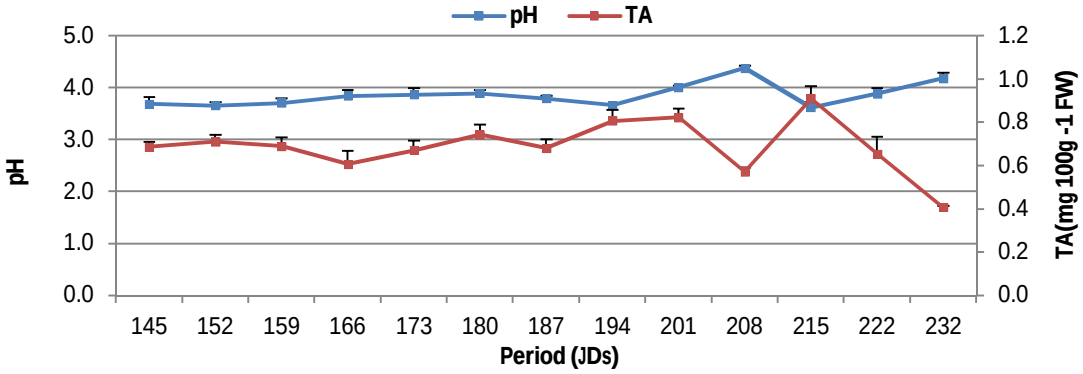

Figure II-3 The evolution of pH and TA plus SE in 'Babygold 9' fruit. Fruits were harvested once a week and each treatment replicated 3 times. There was significant difference (P ≤.0.05, Duncan test) in pH of fruits among weeks of harvest. However, no significant difference in the pH of fruit among 145, 152 and 194 JDs; and among 166, 173,180, 187, and 222 JDs. There was significant difference (P ≤.0.05, Duncan test) in TA of fruits among weeks of fruit harvest. However, no significant difference in TA of fruits among 145, 152, 159 and 187 JDs; and among 166 and 222 JDs. (See supplementary Table II-1 for details).

Sporulation itself is a function of colonization (Douds, 1994). Colonization concerns dimension (size or area) occupied by infection while sporulation deals with population (conidia or spores) involved in an occupied or diseased area. This implies that sporulation is the subsequent effect of colonization due to infection (Xu et al., 2001). However, we have found according to our results that high rate of colonization did not equate to high rate of sporulation. Hence the extent of lesion or colonization does not always equate to the degree of sporulation in a host pathogen interaction but depends on available level of acidity as exponent by this experiment.

For example in our assay the mycelia growth or extent of colonization against pH 6.40 and 5.30 were 80.61 mm and 77.50 mm producing mean conidia concentration of 60800 and 110800 spores mL^{-1} respectively (Figures II-3 and II-4). Though the extent of colonization was higher in pH 6.40 than in pH 5.30 but sporulation was found to be higher in pH 5.30 than in pH 6.40 with about 45.13%. This appears to suggest that sporulation of *M. laxa* in a disease situation increases as the pH increase, reaches its maximum still in the acidic region and then begins to descend as the pH approaches neutral or the alkaline precinct.

We are of the view and assertion, therefore, that *M. laxa* could sporulate at a wide range of pH between 3.5 and 9.5 with the optimum between pH 4.5 and 5.5. This is similar to, and in agreement with, the results of Agarwal and Sarbhoy, (1978) that acidic pH favours fungi growth with best performance within a range of pH 3.5-6.5, and Pascual et al., (1997) that observed pH 4-6 range for optimum growth of their working fungi *Penicillium oxalicum*.

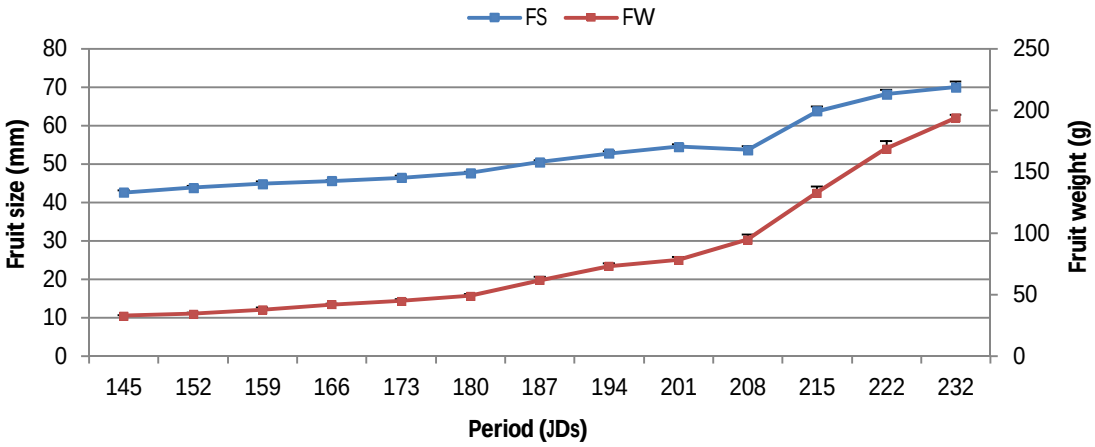

Figure II-4: The evolution of size (mm) and weight (g) plus SE in 'Babygold 9' from setting to fruit maturity. Fruits were harvested at weekly basis and each treatment replicated five times. There was significant difference ($P \leq 0.05$, Duncan test) in fruit size between weeks. However, no significant differences found in the size of fruits harvested at 194, 201 and 208 JDs; 222 and 232 JDs. There was significant difference ($P \leq 0.05$, Duncan test) in fruit weight between weeks. No significant difference found in weight of fruit harvested at 145 and 152 JDs; 194 and 201 JDs (See supplementary Table II-1 for details).

Furthermore, Amiri et al., (2009) found, as most suitable, pH 3.6 to enable selective isolation and enumeration of three *Monilinia* spp. of stone fruits. However, our working pathogen (*M. laxa*) sporulated best under acidic state at pH 5.3 while in the work of Pascual et al., (1997), the fungi sporulated best under a neutral/alkaline range of pH 7-8. Though, on different fungi from *M. laxa*, Gupta et al., (2010) observed maximum growth and sporulation of *F. solani* at pH 5.5. Hence *M. laxa* could be associated with the greater percentage of fungi that grow well in marginally acidic condition. It is equally significant to note here that *M. laxa* has had less attention, notwithstanding the fact that it is as important as *M. fructicola* and *M. fructigena* especially, in the study of epidemiology and management of brown rot (Rungjindamai et al., 2014).

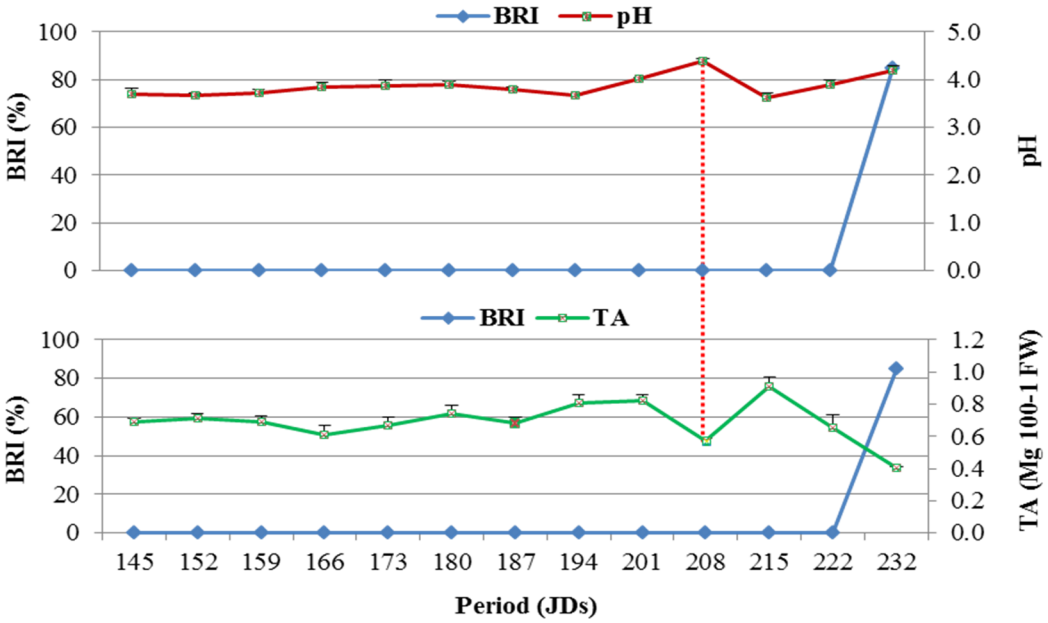

Figure II-5: The effect of pH and titratable acidity (TA) plus standard error (SE) on the susceptibility to brown rot incidence (BRI) of 'Babygold 9' from immature to fruit maturity. Five fruits were inoculated and three fruits evaluated for acidity at weekly basis. The broken line between pH and TA at 208 JDs indicates a clear and unique non-sinusoidal characteristic of acidity in the evolution of peach fruit.

The evolution of pH and TA in the growth and development of peach fruit is non-sinusoidal waveform, contrasting in beats or waves between them (Figure II-3). This is found to corroborate the report of Moing et al., (1998). High pH value gives a corresponding lower content in the TA and vice versa. This is so obvious, for example, at 208 JDs (77 BBCH scale) of 'Babygold 9' (Figure II-3) where the highest pH peak corresponds to the lowest dip in TA value. Also this tends to validate the works of Dirlewanger et al., (1999) that TA and pH in peach are negatively correlated, and Lobit et al., (2002) that pH and TA, the most common measure of acidity with perceived sourness or sugariness in peach fruit, well correlate inversely.

The non-sinusoidal waveform peak of pH reached by fruits at 208 JDs (77 BBCH scale), which corresponds to 136 and 108 days of complete floration and fruit setting respectively, must have occurred at the cell expansion phase. In peach fruit there is usually reduction in organic accumulation, which results in fruit with lower acidity and higher pH as was determined in our work at 208 JDs (77 BBCH scale). This tends to support Moing et al., (1998) that such physicochemical activity occurs far before reaching ripening. Stages of peach development are considered to occur in four phases which includes: fruit set, rapid cell division, cell expansion, and ripening/maturation (Tutu and Ciornea, 2011).

Hence the most resistant period to pathogen infection in peach fruit is during the stages covering pit hardening to pre-harvest (Keske et al., 2011). It could, therefore, be inferred that immature peaches are very resistant to brown rot because of high levels of acidic pH found in the epidermal cells. This high level of pH could have inhibited brown rot incidence at the immature stages in 'Babygold 9'due to acidification activities. Gluconic acid has been reported as the main organic acid associated with the enhancement of peach acidification in host-pathogen interaction (de Cal et al., 2013). *M. laxa* colonized the 'Babygold 9' at the commercial stage of maturity (232 JDs) (79 BBCH scale) when the acidic pH has run down and probably aided by local acidification of the host tissue (de Cal et al., 2013).

It is found worthy to mention, however, that in the inoculation of uninjured 'Babygold 9' with the normal conidia from immature to mature fruit state, the effects of inoculating with mycelia on both injured and uninjured immature fruits were also determined simultaneously. The observations indicate that: at 145 JDs (65 BBCH scale) (pH 3.69 ± 0.13) there was no BRI when fruit was inoculated with 25μL of 25 x 10^3 cfu mL^{-1} spores on artificial injury. There was also no BRI in fruits without artificial injury inoculated with 25μL of 25 x 10^3 cfu mL^{-1} spores. There was, however, BRI in the immature fruit with artificial injury inoculated with a 6.5 mm mycelia PDA. In addition, at 222 JDs (78 BBCH scale) (pH 3.89 ± 0.11), within the fruit colour break, there was no BRI in fruits with intact skin (no artificial injury) when inoculated with a 25μL of 25 x 10^3 cfu mL^{-1} spores. In the contrary, artificially injured fruits showed BRI when inoculated with 25μL of 25 x 10^3 cfu mL^{-1} spores.

The significance of these observations is that the degree of susceptibility to infection by *Monilinia* spp. is variable throughout fruit development (Gradziel, 1994; Guidarelli et al., 2014). Our findings support this assertion because in our experiment at the early stage of growth and development, immature fruit could not exhibit brown rot symptoms when inoculated with conidia, neither through injury site nor on intact epidermis, but could only develop infection when inoculated with mycelium in an injury situation. Furthermore, at colour-break 222 JDs (78 BBCH scale), peach fruit at pH 3.89 ± 0.11 could develop infection with conidia inoculation only through an injury and not possible when there was no injury on the fruit epidermis. Hence skin injury could be responsible for the incident of brown rot on immature peach fruits observed in orchards (Northover and Biggs, 1990; Holb, 2004).

Further, FW at maturity (194.04 ± 1.99) was significantly different ($P \leq 0.05$, Duncan test) from the rest of the FW at development. Also the pH (4.19 ± 0.10) and TA (0.41 ± 0.01) values at maturity were all significantly different from those of the development values (Supplementary Table II-1). Pearson´s correlation shows inverse correlation between pH and all the pathologic activities of peach cultivars at harvest. Harvest date (HD) significantly correlated with fruit size (FS) ($r = 0.912$, $P < 0.01$); fruit weight (FW) ($r = 0.889$, $P < 0.01$); and pH ($r = 0.440$, $P < 0.01$). Also FS significantly correlated with FW ($r = 0.980$, $P < 0.01$). There was a fair and significant correlation between pH and FW ($r = 0.356$, $P < 0.05$). Expectedly, however, pH inversely correlated significantly with TA ($r = -0.604$, $p < 0.01$).

Finally while there was a continuous and stable increase in weight of fruit as it develops, the reverse was the case in fruit size. There was fluctuation in the size as the fruit developed. Fruit size increased, later decreased at a time and finally increased as the fruit developed. The obvious dynamics in pH and TA values also occurred when there were clear changes in fruit size and weight evolution. In addition the pH 4.19 ± 0.1 at which *M. laxa* could infect the peach in this work relatively correlate with the range of pH (3.5-9.5) in the solid PDA considered to support sporulation *in-vivo*. Hence, brown rot infection and expression in peach fruit is dependent on the influence of pH and TA as chemical factors and in extension upon the stage of the fruit growth (Emery et al., 2000; Holb, 2004; Gell et al., 2009). This study encompasses the necessity to know the evolution of fruit maturity in new and old varieties in relation to potential *Monilinia* infection in immature fruits. The knowledge, in addition, could be useful in the determination of the presence of pathogen in latent infection within molecular techniques.

The study has shown that *M. laxa* exhibited variation in its growth and sporulation capacities on the seven pH amended PDA, preferring relatively moderate acidic conditions for their optimum performance. We found, in the *in vitro* analysis, that there was mycelia growth in pH from 2.40 to 8.84 while pH 11.52 did not support any mycelia growth. The pH 5.30 supported the highest sporulation while pH 6.40 encouraged the highest colonization extent or mycelia growth. This is in support of the findings of Holb, (2004) that the most favorable initial hydrogen-ion concentration for mycelial growth occurs between pH 3.5 and 5.5. We found that there was a continuous and stable increase in weight of fruit as it develops, the reverse being the case in fruit size. The fruit size increased, decreased at a time and finally increased as the fruit develops. The pH dynamics exhibited non-sinusoidal waveform through the growth and development of the fruit. In all these physicochemical variations, *M. laxa* could not develop infection or shown any brown rot incidence in the fruit until the period of commercial maturity.

On the basis of this study it can be concluded that pH and titratable acidity have great impacts on the growth activity of *M. laxa* in a host-pathogen association both in solid PDA substrate and in peach fruit growth and development. And as organic acids and genetic traits, pH and titratable acidity could constitute imperative antibrown rot determining elements to be given adequate attention in peach breeding program.

II-5. Acknowledgments:

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II-6. Compliance with Ethical Standard

i. **Conflict of interest**: The authors have declared that there is no conflict of interest exists.

ii. **Research involving Human Participants and/or Animals**: This article /manuscript does not contain any studies with human and /or animal subjects performed by any of the authors.

iii**. Informed consent**: This article is as a result of a general study in the evaluation of peach germplasm for tolerance to brown rot by *Monilinia laxa* at the Aula Dei-CSIC peach collection, Zaragoza. I, Vitus Ikechukwu Obi, testify on behalf of all co-authors (Yolanda Gogorcena and Juan Jose Barriuso) that our article,

Title: "Effects of pH and titratable acidity on the growth and development of *Monilinia laxa* (Aderh. & Ruhl.) *in-vitro* and *in-vivo*",

a) has not been published in whole or in part elsewhere;

b) is not currently being considered for publication in another journal;

c) and that all authors have been personally and actively involved in substantive work leading to the manuscript, and will hold ourselves jointly and individually responsible for its content.

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Chapter III. The tolerance of commercial peach cultivars to brown rot by *Monilinia laxa* is modulated by its antioxidant content?

***Manuscript*:** The tolerance of commercial peach cultivars to brown rot by *Monilinia laxa* is modulated by its antioxidant content?

The tolerance of commercial peach cultivars to brown rot by *Monilinia laxa* is modulated by its antioxidant content?

Abstract

Brown rot, caused by *Monilinia spp*., provokes pre - and post-harvest damages in peach [*Prunus persica* (L.) Batsch] with an economic impact in the industry. With a view to breeding for increase tolerance to this disease, a screening test based upon artificial fruit inoculation was validated on several parental lines of the peach breeding program ('Crown Princess', 'Big Top', 'Andross', and 'BabyGold 9'), during 2014-2015. In addition, cultivars with different total phenolic content, ranging from 4.77 to 18.67 mg per 100 g fresh weight, early ('Tebana') or late harvested ('Miraflores', 'Calanda Tardío' and 'Calante'), were included in the two-year study. All fruit physico-chemical traits recorded at harvest (pH, titratable acidity, firmness and soluble solids content) showed differences among all cultivars. The antioxidant content determined by spectrophotometry (ascorbic acid and antioxidant capacity) and UPLC-MS (phenolic compounds) also revealed important differences among all genotypes. Rate of brown rot lesion following fruit inoculation varied widely among cultivars and it was possible to discriminate between highly and less susceptible cultivars. 'Andross' was the cultivar with the lower lesion brown rot severity (LS = 30.3 mm) and 'Calante' (LS = 50.1 mm) exhibited the highest one. Cultivars with minimal development of damage were identified as germplasm with the desirable allele combination to increase brown rot tolerance in peach breeding programs. Finally, Pearson's correlation coefficients (r) between pairs of traits were calculated searching for any biochemical candidate conferring tolerance. The correlation of pathological traits with the antioxidant composition concerning contents of ascorbic, neochlorogenic and chlorogenic acids and total polyphenols in flesh-fruit and peel are discussed.

Key words: *Prunus persica;* monilia, genetic disease tolerance, ascorbic and phenolic acids.

III-1. Introduction

One of the most important stone fruit crops in Spain is represented by peaches [*Prunus persica* (L.) Batsch] [(1.53 million tons in 2016) (FAOSTAT 2018; http://fao.org/faostat/)] often hindered by the activities of pathogenic fungi. The most important fungal disease agents of peaches and nectarines in Spain are *Monilinia* spp. The most extended species with greater damage is *Monilinia laxa* (Aderhold and Ruhland) Honey, whose occurrence is presently at the same relative frequency of *M. fructicola* (G. Winter) Honey (Egüen et al., 2015; Obi et al., 2017; Villarino et al., 2013). Both species have been associated with about 85-90% brown rot (BR) incidence in Spanish peach commodities (Egüen et al., 2015). The pathogen can initiate infection in peach starting from the flower and developing later in storage. In general, yield losses have been recorded especially after harvest, reaching 80-85% depending of the meteorological conditions (Casals et al., 2010; Villarino et al., 2012) posing obviously a great danger for crop sustainable production.

Common control practices for this phytopathogen, especially in the Spanish orchards, were represented by the use of preventive and systemic fungicides such as thiophanate-methyl, iprodione and cyproconazole (Egüen et al., 2015). However, the use of fungicides is becoming more limited because of consumer demands for residue-free fruit (Usall et al., 2015), including the fact that postharvest treatment have been limited by law until 2016 in Spain, and contamination of the environment should be avoided (Liu et al., 2012). In addition, the steady rise in development and occurrence of fungicide resistant to *Monilinia* strains, worldwide (Chen et al., 2013; Elmer and Gaunt

1994; Holb and Schnabel, 2007; Liu et al., 2012) and in Spain (Egüen et al., 2015, 2016) has also been reported. All these adverse implications put together it is, therefore, pertinent to search for alternatives with lasting effect, enhanced consumer acceptability and at the same time friendly with the environment (Mari et al., 2014). In this direction the availability of genotypes more tolerant and or resistant to *Monilinia* would be a safety solution, in combination with fungicide application and practical measures, for a sustainable peach production.

Nowadays, there exist considerable interest in the development of host-peach resistant cultivars and the identification of appropriate markers that segregate with resistance to *Monilinia* spp. One of the first selection studies did identify high levels of brown rot resistant in 'Bolinha' (Feliciano et al., 1987) and thereafter its tolerance was associated with the high content of phenolic compounds ´with respect to the brown rot susceptible cultivars (Gradziel and Wang, 1993; Bostock et al., 1999). The involvement of secondary plant metabolites such as polyphenols in defense during fruit development has been well established (Lantazio et al., 2006) and widely studied in peach brown rot tolerance (Gradziel and Wang, 1993; Bostock et al., 1999, Mari et al., 2003; Lee and Bostock, 2007; Thomidis et al., 2007; Villarino et al., 2011; Guidarelli et al., 2014).

The potential role of phenolic acids in combination with other factors in the resistance to the brown rot caused by *Monilinia spp.* thought fungal inhibition have been discussed by several authors (Oliveira-Lino et al., 2016 and references therein). In particular, it has been described that chlorogenic and caffeic acids markedly inhibited the production of the cell wall degrading enzymes polygalacturonase and cutinase in *M. fructicola* cultures (Lee and Bostock, 2007) but had no effect on *M fructicola* growth (Bostock et al., 1999). Another study also pointed out that contents on either neochlorogenic (NCG) or chlorogenic (CG) acids in immature fruits interfere with fungal melanin production to *M. laxa* penetration (Villarino et al*.,* 2011). However, most of the previous studies have been conducted *in vitro* or in immature peach fruits and no conclusion can be derived concerning the preformed (genetic) traits conferring tolerance to brown rot.

From a breeder point of view, it is worthy to know that although greater number of commercial stone fruit cultivars are susceptible to *Monilinia spp*. (Holb, 2008; Martínez-Gómez et al., 2005; Rungjindamai et al., 2014), there exist genetic disease control (Martínez-García et al., 2013; Pacheco et al., 2014) to be introgressed in high fruit quality genetic background (Oliveira-Lino et al., 2016). The objective of this study was to screen eight commercial peach/nectarine cultivars for susceptibility to *M. laxa* and exploring the genetic approach rating cultivars' tolerance for breeding purposes. In addition, we have explored if the fruit antioxidant composition influence the development of brown rot damage after artificial inoculation. Here we discuss if the antioxidant composition such as endogenous ascorbic acid or NCG and CG phenolic acids in peach tissue may be involved in the host brown rot tolerance.

III-2. Materials and methods

III-2.1. Peach cultivars

The studied peach cultivars were established in an experimental orchard at the Experimental Station of Aula Dei-CSIC, Zaragoza (northern Spain). Three trees per genotype were trained to the standard open vase system and planted at a spacing of 4 m x 2.5 m and grown under standard conditions of irrigation, fertilization and pest and disease control. In the two years study (2014-2015), any fungicide treatment was applied in the field before harvest with adequate consideration to the free entry period for evaluation. The preventive fungicide Teldor® 500 SC (fenhexamida) was sprayed

at the EEAD on 29th July, 2014 and 15th September 2015, respectively. All cultivars are non-melting-flesh peach, except the melting-flesh nectarine 'Big Top'. All fruits are yellow-flesh and the origin harvest dates and fruit characteristics are shown in Table 1.

At commercial maturity, 20 fruits were harvested to determine physicochemical traits and biochemical analysis. For inoculation purposes, 25 mature fruits were harvested and disinfected by immersion in aqueous solution of 1.6 % sodium hypochlorite (commercial), 0.005 % Tween® 80 (polysorbate surfactant) and 1.6 % ethyl alcohol for 4 minutes, rinsed in sterile distilled water, and spread out on sterile holding stone-fruit cardboard boxes for 20 minutes ambient air drying in blossom-stem (upside down) position to avoid any possible percolation at the stem position. After incubation, fruit firmness and SSC were also recorded in control (FF2, SSC2) and inoculated fruits (FF3, SSC3) to test storage effect.

III-2.2. Physicochemical and biochemical determinations in fruits

At harvest 20 fruits were hand picked out at commercial maturity to determine basic quality parameters [pH, titratable acidity (TA), fruit firmness (FF), and soluble solids content (SSC) (see details in Saidani et al., 2017)]. Fruits were peeled and flesh tissue was cut in small pieces (three replicates of five fruits each). For each replicate five grams of flesh tissue was frozen in liquid nitrogen and store at -20 or -80 ºC for further biochemical analysis (Saidani et al., 2017).

Contents of ascorbic acid, antioxidant capacity and phenolic compounds were determined according to Saidani et al. (2017). Ascorbic acid and antioxidant capacity were measured by spectrophotometry and polyphenols, including the major hydroxycinnamic acids neochlorogenic (NCGA) and chlorogenic (CGA), were identified by UPLC-MS and quantified by UPLC-DAD. Briefly, the ascorbic acid was extracted with 10 ml of 5% of HPO_3. To determine the antioxidant capacity and total polyphenols, samples were extracted with 10 ml of a mixture of MeOH/H_2O/formic acid (60:38:2 v/v/v). Ascorbic acid determination was based on the reduction of Fe (III) to Fe (II) by L-ascorbic acid followed by the formation of Fe (II) -2, 2'-Bipyridil complex (Okamura, 1980). Absorbance was measured at 525 nm, and the results were expressed as mg of ascorbic acid (AsA) per 100 of FW following the calibration curve daily prepared. The antioxidant capacity was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Brand-Williams et al., 1995). The extract was mixed with DPPH for 60 min in darkness at room temperature. Absorbance was measured at 535 nm, and the results were expressed as relative antioxidant capacity (RAC) in mg of Trolox equivalents (TE) per 100 g of FW following the calibration curve daily prepared.

For analysis of phenolics, one hundred micrograms of internal standard (methyl 4-hydroxybenzoate) was added to an aliquot of the polyphenol extract, which was concentrated to dryness with speed vac at 45 °C. After the addition of 1 ml of the mixture of MeOH/formic acid (95:5 v/v), 1 µl of the solubilized polyphenolic extract was directly injected into UPLC with a photodiode array detector (280, 330, and 520 nm) and UPLC-MS coupling with an ion trap mass spectrometer Bruker Daltonics HCT ultra equipped with an electrospray ionization source (see details in Aubert et al., 2014).

Table III-1: Characteristics of the eight peach cultivars: Cultivar name, origin, harvest dates and fruit physicochemical traits. Data are mean of two years (2014-2015).

Cultivars	Origin	Harvest date	pH	TA	FF1 (N)	SSC1 (^{o}Brix)	AsA*	RAC*	NCGA*	CGA*	TPP*
Crown Princess	USA	19-june a	3.82 b	0.56 b	26.17 a	10.85 a	5.18 a	54.60 b	1.63 ab	5.08 c	7.37 abc
Big Top	USA	03-july b	4.15 c	0.42 a	31.95 b	12.25 ab	4.09 a	20.01 a	1.25 a	3.21 a	4.77 a
Tebana	Italy	03-july b	4.12 c	0.43 a	33.04 bc	10.58 a	9.11 bc	38.43 ab	1.24 a	3.70 a	5.37 ab
Andross	USA	02-aug c	4.31 c	0.35 a	31.56 b	14.15 bc	14.91 d	88.08 d	2.66 bc	5.36 cd	8.91 c
Baby Gold 9	USA	21-aug d	4.17 c	0.41 a	36.41 b	13.68 bc	8.75 b	75.36 cd	2.17 abc	3.77 b	6.99 abc
Miraflores	Spain	10-sep e	3.79 b	0.61 b	35.49 bc	13.43 bc	10.88 c	89.82 d	3.31 c	3.80 b	8.28 bc
Calanda Tardío	Spain	07-oct f	3.68 ab	0.75 c	59.84 e	15.53 c	8.89 b	186.45 e	10.16 e	6.52 d	18.67 e
Calante	Spain	07-oct f	3.65 ab	0.72 c	46.18 d	14.55 bc	10.72 bc	168.63 de	6.37 d	5.88 cd	13.92 d

Abbreviations and units: Titratable acidity (TA) = g / 100 g; Fruit firmness at harvest (FF1) = Newton (N); Soluble solids content at harvest (SSC1); Ascorbic acid (AsA) = mg AsA/100 g FW, Relative antioxidant capacity (RAC) = mg Trolox equivalent / 100 g FW; Neochlorogenic acid (NCGA) = mg /100 g FW, Chlorogenic acid (CGA) = mg /100 g FW; Total polyphenols (TPP) = mg / 100 g FW. * Data are mean of three replications for two years (N=6). For each column, mean values with the same letter are not significantly different at $p < 0.05$ (Duncan test).

III-2.3. *In-vivo* assay: Inoculum, Inoculation and Brown rot evaluation

The culture of the *M. laxa* (Aderhold and Ruhland) Honey, the inoculation and the brown rot evaluation was carried out according to the protocol described in Obi et al., (2017). The isolate (CPML02) used in this study was supplied by the *Collection of Postharvest Pathology Group* of IRTA (Lleida, Catalonia). In brief the inoculation was conducted with 25 µl of a suspension of 25 conidia / µl of *M. laxa* on 20 fruits per cultivar, without skin injury. Five fruits per cultivar were used as control and inoculated with 25 µl sterile water to discard unspecific infections. All fruits were incubated for five days at 23 ^{o}C and 45 - 60% relative humidity. Brown rot incidence (% BRI), colonization (% C), lesion diameter (LD in mm) and colonization extent (CE in mm) were the pathogenic parameters measured. Lesion and colonization severities (LS and CS) in each cultivar were calculated as lesion diameter x percentage of infected fruits and colonization extent x percentage of colonization, respectively (Martínez-García et al., 2013; Obi et al., 2017; RosBreed web-page: https://www.rosbreed.org/node/424). Colonization (% C), colonization extent (CE mm) and colonization severity were recorded to test the correlations with lesion damage.

III-2.4. Statistical analysis

All statistical analysis was performed using SPSS 23.0 (SPSS Inc.**;** Chicago, IL, USA). For biochemical determinations, three biological replicates were considered for year of analysis (2014-2015); mean and standard error (SE) were calculated for each parameter. The pathological traits were recorded for each cultivar in 5 individual fruits inoculated with water for control and 20 individual fruits inoculated with *M. laxa* spores, for year of analysis (2014-2015); mean and standard error (SE) were calculated for each parameter. ANOVA was performed and significance was judged at the level $p \leq 0.05$, and the Duncan test used for mean comparison. Pearson's correlation and regression analysis (Microsoft excel 10.0) were conducted to reveal possible association between pair of traits.

III-3. Results

Peach fruits were harvested between June and October as are ordered in Table V-1. The earliest cultivars were 'Crown Princess', 'Big Top' and 'Tebana' (mid-June to July). 'Andross' and 'Baby Gold 9' were harvested on August. In contrast the Spanish non-melting flesh cultivars 'Miraflores', 'Calanda Tardio' and 'Calante', were harvested the latest from September to October.

III-3.1. Physico-chemical traits and biochemical composition.

All studied physico-chemical traits and biochemical composition of the cultivars at harvest are presented in Table V-1. For all traits, analysis of variance at 5% probability level showed significant differences among the cultivars. The pH values ranged from 3.65 in 'Calante' to 4.31 in 'Andross' (Table V-1). The cultivars with pH>4, showed TA values below 0.5, and when pH<4, TA values were over 0.5. The firmness ranged from 26.17 N in 'Crown Princess' to 59.84 N in 'Calanda Tardio'. Soluble solids content (SSC) ranged from 10.58 to 15.53 °Brix, in 'Tebana' and 'Calanda Tardio', respectively.

In the eight peach cultivars, we have performed the antioxidant composition in flesh fruit and determined ascorbic acid content, Relative Antioxidant Capacity (RAC) and polyphenol profile. Contents for ascorbic, RAC, the major hydroxycinamic acids (neochlorogenic, NCG and chlorogenic, CG) and total polyphenols (TPP) are presented in Table V-1, Supplementary Tables V-1 and V-2.

Ascorbic acid content varied, ranging from 4.09 in 'Big Top' to 14.91 mg/100 g FW in 'Andross'. The antioxidant levels ranged widely from 'Big top' to 'Calanda Tardio' (RAC 20.01-186.45 mg TE/100 g FW and TPP 4.77-18.67 mg /100 g FW). The nectarine 'Big top' showed the lowest contents of AsA, RAC

and TPP although not significantly different from 'Tebana' for RAC and TPP, or 'Crown Princess' and 'Baby Gold 9' for TPP. In contrast the cultivars, 'Calanda Tardio' followed by 'Calante' showed the highest relative antioxidant capacity and amounts of TPP.

Regarding polyphenols, significant variation was found among cultivars for contents of NCG and CG hydroxycinnamic acids and TPP. NCGA content ranged from 1.24-1.25 mg/100 g FW in 'Tebana' and 'Big Top' to 10.16 mg/100 g FW in 'Calanda Tardio'. CGA from 3.21-3.30 mg/100 g FW in 'Big Top' and 'Tebana' to 6.52 mg/100 g FW in 'Calanda Tardio'. Neochlorogenic and chlorogenic acids were the main hydroxycinnamic acids and the major polyphenols found in these peach cultivars (See Supplementary Tables V-1 and V-2 and Supplementary Figures V-1 and V-2, submitted as Data in Brief). The nectarine 'Big Top' and 'Tebana' showed the lowest levels of NCGA, CGA and TPP. On the

The lesion severity (LS) ranged from 30.29 to 50.07 mm, being 'Andross' the more tolerant cultivar and 'Calante' the more susceptible. 'Andross' exhibited the lowest LS significantly different from 'Crown Princess', 'Big Top', 'Calanda Tardío', 'and 'Calante'.contrary, the late-maturing cultivars, 'Calanda Tardio' and 'Calante' showed the highest levels of, RAC, both NCG and CG acids, and TPP. These two cultivars also showed the highest total polyphenols (TPP), total hydroxycinnamic acids (HA) and total flavanols (FA) contents (Supplementary Table V-2 and Supplementary Figure V-2).

III-3.2. Fruit susceptibility.

No visible symptoms of brown rot infection were observed on control fruits that had been inoculated with sterile distilled water. In mature fruits, symptoms of brown rot lesions appeared after three days of fruit inoculation and storage. Brown rot incidence, lesion diameter and colonization extent were recorded as pathological traits after fruit inoculation and storage. Brown rot incidence ranged from 70 to 90 % among the eight studied cultivars (Figure V-1).

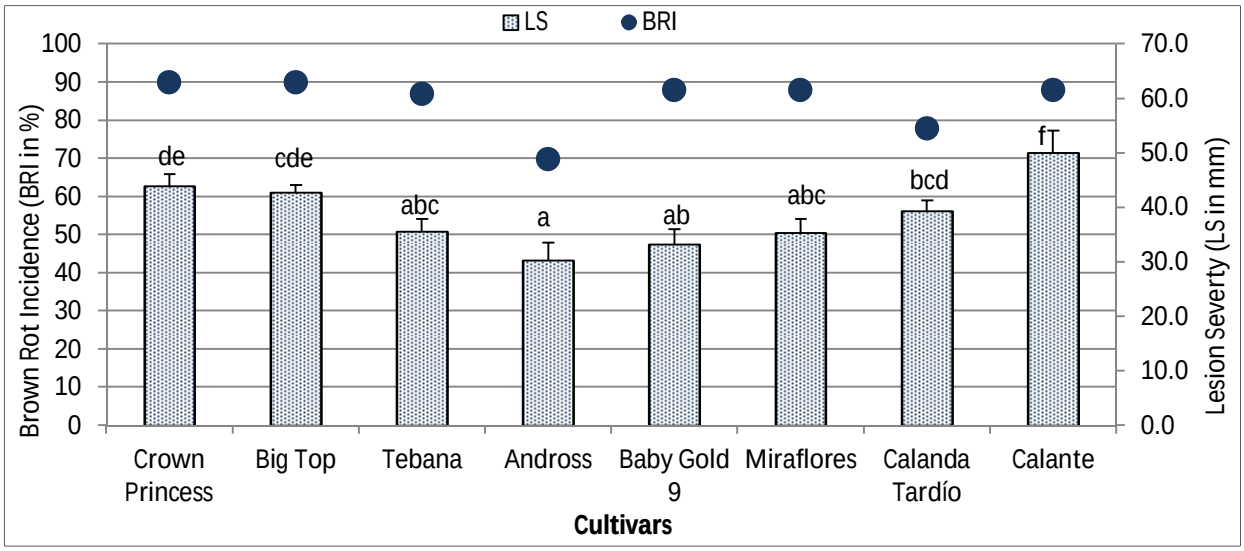

Figure III-1: Brown rot incidence (%) and Lesion severity (mm) on eight peach cultivars evaluated during two consecutive years (2014-2015). Data are mean ± SE (N=20-35 fruits). Different letters show significant differences on lesion severity among cultivars (Duncan test, $p < 0.05$).

After incubation fruit firmness and SSC were also recorded (Supplementary Table 3). Fruit firmness after five days of incubation (mean FF2: 33.5 N) decrease significantly with *M. laxa* inoculation (mean FF3: 29.2 N), however, no significant differences were found in soluble solids content between non-inoculated (mean SSC2 =14.0 ºBrix) and inoculated fruits (mean SSC3 = 13.5 ºBrix).

III-3.3. Pearson's Correlation.

Pearson's correlation coefficients (r) between pairs of pathological and antioxidant traits (Table 2) have been calculated with purpose of highlighting its involvement in the host tolerance.

Table III-2: Pearson`s correlation coefficients between pathological and antioxidant traits in eight peach cultivars harvested during two years (2014-2015). N= 37-319

	LS	%C	CS	AsA	RAC	NCGA	CGA	TPP
%BRI	0.471**	0.832**	0.682**	-0.537**	ns	ns	ns	ns
LS		0.597**	0.749**	-0.579**	ns	ns	ns	ns
%C			0.830**	-0.652**	ns	ns	ns	ns
CS				-0.590**	ns	ns	ns	ns
AsA					ns	ns	ns	ns
RAC						0.876**	0.704**	0.967**
NCGA							0.707**	0.970**
CGA								0.861**

Significance: **$p \leq 0.01$. Units and abbreviations: %BRI: percentage brown rot incidence; LS: lesion severity (mm); %C: percentage colonization; CS: colonization severity (mm); Ascorbic acid (AsA) = mg AsA/100 g FW, Relative Antioxidant Capacity (RAC) = mg Trolox Equivalent / 100 g FW; Neochlorogenic acid (NCGA) = mg /100 g FW, Chlorogenic acid (CGA) = mg /100 g FW; FW: fresh weight, Total Polyphenols (TPP) = mg / 100 g FW.

All pathological traits (brown rot incidence, percentage of colonization, and lesion and colonization severities) were highly correlated among them. Figure 2 shows the correlation between the lesion severity with colonization severity (R^2= 0.562; r = 0.749, $p \leq 0.01$).

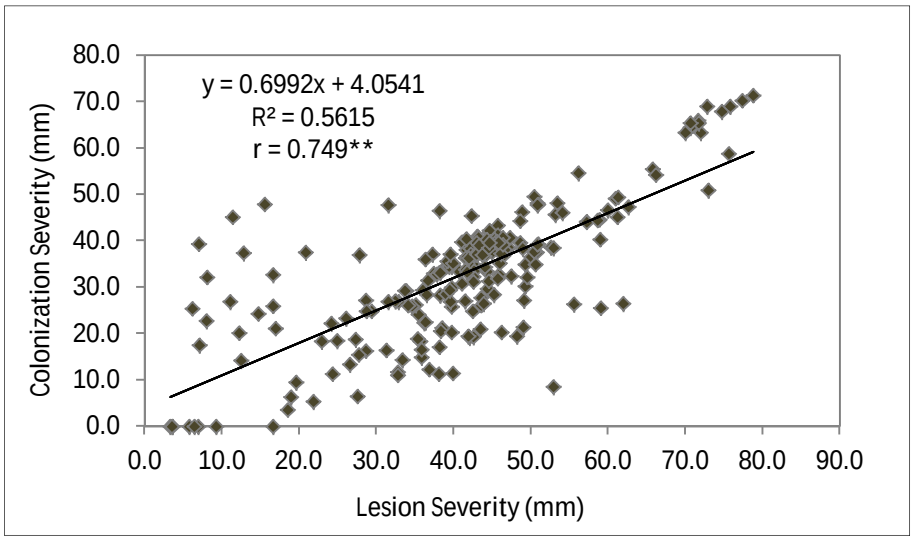

Figure III-2: Linear regression between the lesion severity with colonization severity on eight peach cultivars evaluated during two consecutive years (2014-2015) (N=266 fruits). Significance of Pearson correlation is shown (** $p < 0.01$).

Furthermore, the correlation analysis was carried out to clarify the contribution of the antioxidant content to *Monilinia* tolerance. The analysis between pathological and antioxidant traits surprisingly revealed significant inverse correlation only with ascorbic acid content. Table 2 showed the inverse correlation between the lesion severity with AsA content (r = -0.579, $p \leq 0.01$). On the contrary, no significant correlation was found between pathological traits and RAC or the polyphenolic compounds; neither the neochlorogenic, nor the chlorogenic hydroxycinamic acids or TPP, were significantly correlated.

III-4. Discussion

We have studied eight commercial peach cultivars with different fruit characteristics and tested its tolerance to brown rot caused by *Monilinia laxa* after artificial inoculation. As it was reported in our previous study, symptoms of infection in peach fruits were only developed after inoculation on commercial maturity (Obi et al., 2017, 2018). A similar approach and protocols are routinely used to phenotype tolerance to brown rot caused by *M. fructicola* in peach germplasm (Bostock R, https://www.rosbreed.org/node/679). These cultivars based on the pH can be classified as acid (pH<4) or non-acid fruits (pH>4) (Dirlewanger et al., 1999). However, the range of pH (3.7-4.3) found among cultivars has no effect on the *in vivo Monilinia* growth. Obi et al. (2018) demonstrated a similar *in vitro* mycelial growth at these pH ranges. Values found for pH, TA, FF1 and SSC1 were within the range reported in other studies in peach germplasm (Abidi et al., 2015; Font i Forcada et al., 2013; Saidani et al., 2017). The differences found among cultivars in firmness (FF1) are mainly related to factors, such as harvest date or fruit type. Firmness of ripe peaches tended to be higher for the late cultivars as we have found in 'Calanda Tardio' and 'Calante' (Iglesias and Echeverria, 2009; Montevecchi et al., 2012).

Similarly, the content for Ascorbic acid varied widely (from 4.09 in 'Big Top' to 14.91 mg/100 g FW in 'Andross') and fell within the ranges previously reported for ascorbic acid in peach pulp (Abidi et al., 2015; Font i Forcada et al., 2013, Saidani et al., 2017). Regarding NCG and CG acids content, 'Calanda Tardio' and 'Calante' showed the highest levels for both hydroxycinnamic acids. These cultivars also showed the highest relative antioxidant capacity (RAC), total polyphenols (TPP), total hydroxycinnamic acids (HA) and total flavanols (FA) contents (Table V-1, Supplementary Table V-2 and Supplementary Figure V-2), as reported Saidani et al. (2017) in peel and flesh tissue in a parallel study. The wide variation found among these cultivars has been attributed specially to seasonal influences. The cultivars late harvested contain higher contents on relative antioxidant capacity, flavonoid and TPP than cultivars harvested in earlier season (Saidani et al., 2017). The levels of antioxidants were gradually increases as the harvest date progresses throughout the year. These is consistent with the high and positive correlation ($p \leq 0.01$) found between harvest date and relative antioxidant capacity (r = 0.836), total hydroxycinnamic acids (r = 0.742), total flavanols (r = 0.874) and total polyphenols (r = 0.761) (Saidani 2016).

Based on the results found here, the degree of susceptibility to brown rot on these peach cultivars was not associated to the large differences in harvest dates. The cultivars were harvested from mid - June to early October but no correlation was found between harvest date and brown rot tolerance as was reported before in other peach germplasm (Obi et al., 2017, Obi et al., unpublished results). All pathological traits (brown rot incidence, colonization and lesion and colonization severities) were highly correlated among them (Obi et al., 2017). After 5 days of incubation no significant differences were found between the soluble solid contents (SSC) of control fruits (SSC2=14.0 ºBrix) with inoculated fruits (SSC3 =13.5 ºBrix). Therefore, there is no credible evidence that the activities of *M. laxa* depleted soluble solid contents in the peach as it was found previously in other progenies (Obi et al., unpublished results).

In this work we have showed that only AsA content presented an inverse correlation with lesion severity (r = -0.555, $p \leq 0.01$). On the contrary, any disease parameter correlated with none of the other bioactive compounds neither the relative antioxidant capacity nor NCGA, CGA or TPP (Table V-2). In agreement with these findings, no relation was detected between brown rot resistance to *M. fructicola* and concentration of phenolic compounds in Californian peach germplasm (Gradziel and Wang, 1993). Apparently, phenolic compounds were not specifically involved in the cultivar tolerance to brown rot caused by *Monilia fructicola* or *M. laxa*. Nevertheless, we could suggest that a

combination of different antioxidant compounds may confer partial immunity as was found in 'Andross' (this study, Gradziel and Wang, 1993). This cultivar showed the highest level of ascorbic acid, high CG acid and moderate levels of relative antioxidant capacity that may contribute to its tolerance. 'Andross' also presented moderate levels of flavonoids and total phenolic content in peel and pulp tissues (Saidani et al., 2017). On the contrary, the levels of ascorbic, NCG and CG acids found in 'Calante', and the highest contents in RAC, NCGA and CGA and TPP found in 'Calanda Tardio' cannot explain its susceptibility to brown rot.

As it was mentioned above, the role of plant phenolic acids in fungal inhibition has been widely discussed by several authors (Oliveira-Lino et al*.,* 2016 and references therein). CG and caffeic acids at levels similar to or in excess of those in the exocarp of immature resistant fruits did not affected *M. fructicola* growth (Bostock et al., 1999), however, these acids down regulate cutinase production in *M. fructicola* cultures (Wang et al., 2002) and markedly inhibited the production of the cell wall polygalacturonase and cutinase (Lee and Bostock, 2007). In the same direction, Villarino and coworkers (2011) found that NCGA and CGA contents in immature fruits were negatively correlated to brown rot incidence and *in vitro* demonstrated that high CGA concentration modified fungal melanin production that might interfere to *Monilia laxa* penetration. These studies suggested that phenolic acids may suppress the cellular activities in the fungal pathogens that may be crucial for its growth and colonization on a host. However, in this study the correlation analysis revealed that NCGA and CGA contents or total polyphenols in fruits had not effect on fungal lesion after artificial inoculation. In other words, contents of NCG and CG acids at harvest probably are independent and do not indicate cultivar tolerance.

On the contrary, the ascorbic acid that is considered one of the most important antioxidants in plant tissues was negatively correlated with fungal growth after artificial inoculation. Our results are in agreement with Wang et al. (2002) who demonstrated that other antioxidants such as glutathione and lipoic acid significantly attenuate cutinase production in *M fructicola* and discuss that the effect of phenolics is due to a general antioxidant effect rather than a specific chemical interaction. Lee and Bostock (2007) also mentioned that phenolics in plant tissue may influence the antioxidant level in the pathogen and, as a consequence, the expression of genes associated with infection. The ascorbic acid, which concentration has been considered as an important nutritional quality indicator for peach, can be considered relevant in plant breeding for its antioxidant role conferring *in vivo* brown rot tolerance. These findings will open new research to test the effect of ascorbic either *in-vivo* on brown rot tolerance or *in-vitro* on microbial growth.

III-5. Conclusions

In conclusion the result of evaluation of tolerance in the eight commercial cultivars to brown rot disease demonstrates variability in the genetic susceptibility *to Monilinia laxa*, with 'Andross' and 'Baby Gold 9' the more tolerant but not significantly different from 'Tebana' and 'Miraflores'. 'Crown Princess', 'Big Top' 'Calante' and 'Calanda Tardío' were the less tolerant peach cultivars. The content of ascorbic acid can only partially explain the tolerance-susceptibility observed, indicating that other factors are probably involved in the response. Identification of these factors will be fundamental for breeding programs focused on improving resistance.

In addition, neither neochlorogenic nor chlorogenic or TPP contents at harvest can explain the differences in tolerance to brown rot found in these peach cultivars. We suggest that together with the ascorbic acid, other physico-chemical factors may confer the tolerance to 'Andross', 'Tebana', 'Baby Gold 9' and 'Miraflores'. To our knowledge this is the first study that correlates the ascorbic acid

contents in mature peach fruit with *Monilinia laxa* brown rot tolerance. From a practical point of view application of ascorbate based formulae may be promising. Furthermore, we may speculate that the complex interaction *Monilinia-Prunus* establish the redox environment that modulate or restrict the fungal infection. Nonetheless, further studies need to be done in order to know the effect of AsA as a curative or preventive treatment to control *M. laxa* infections and/or to disentangle the role of each specific antioxidant compound in the genetic brown rot tolerance in peach.

III-6. Acknowledgments

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Chapter IV. Breeding strategies for identifying superior peach genotypes tolerant to brown rot

Manuscript. Breeding strategies for identifying superior peach genotypes tolerant to brown rot

Breeding strategies for identifying superior peach genotypes tolerant to brown rot

Abstract

A sustainable approach to control brown rot incidence in pre- and postharvest management is to select genotypes with high contents in antioxidant compounds in combination with tolerance to *Monilinia laxa* [(Aderhold and Ruhland) Honey]. In this research sixty eight progenies from the 'Babygold 9' × 'Crown Princess' population of the breeding program of EEAD-CSIC were screened (under controlled conditions in post inoculation test) for a period of 3 years (2013-2015). To evaluate the susceptibility to brown rot, twenty healthy fruits per genotype were surface inoculated with 625 spore suspension of the *M. laxa*. After incubation at 23 ^{o}C for 5 days, the brown rot incidence, lesion diameter, colonization extent and their severities were calculated. Physico-chemical traits, such as fruit firmness and soluble solids content were also recorded before and after storage. Titratable acidity, pH, and antioxidant composition were measured at harvest. Significant differences were found for pathogenic traits, vitamin C, total phenolic, flavonoids and anthocyanins within genotypes in this population. Also inverse correlations have been found between the content of phytochemical compounds (anthocyanin, and total phenolic) and disease incidence and severity. Differences in susceptibility to brown rot confirm the genetic variability available in this progeny and therefore, the selection of six of them found highly tolerant to brown rot of *M. laxa* with high organoleptic properties, rich in phenols to be introduced in our peach breeding program.

Key words: Genetic tolerance, bioactive, susceptibility, screening, brown rot, plant breeding**.**

IV-1. Introduction

The length of conservation and commercial shelf life of peach [*Prunus persica* (L.) Batsch] are negatively influenced due to pre and post-harvest diseases principally associated with the brown rot (Sisquella et al., 2014). Brown rot of stone fruits is a disease primarily caused by *Monilinia* species which includes: *M. laxa* (Aderhold and Ruhland) Honey; *M. fructigena* (Honey); *M. fructicola* (G. Winter) and an anamorph of *Monilinia*, *M. polystroma* (G. Leeuwen) (Jansch et al., 2012). In this crop the pathogen initiates and encourages flower blights, spurs, twig/branch death and fruit rot in the field (Gell et al., 2007). Hence the pathogen`s activity on peach is highly destructive from flowering stage via peach formation to storage (Thomidis and Exadaktylou, 2010).

In Spain *M. laxa and M. fructicola* have been the most recurrent, subsequent to the dislodgment of *M. fructigena* after the year 2010 (Villarino et al., 2016), causing over sixty per cent fruit losses after harvest (Villarino et al., 2012; Egüen et al., 2015), mostly under favorable environmental conditions for the commencement and growth of the diseases in the orchard.

Host tolerance to plant pathogens is important to cost effective and environmentally safe strategy for disease management (Gradziel, 1994). In the same idea according to Gell et al., (2007), the use of tolerant cultivars in crop improvement is the topmost principle of crop protection as plants and plant products are usually protected (prophylactic) (Mooney et al., 2012) and, often, not cured of diseases (chemotherapeutic).

Cultivar significantly influence rot incidence and severity among other potential factors in stone fruits (Tarbath et al., 2014) and, therefore, fits an ideal component in the measures for disease control (Kreidl et al., 2015). Lasting prophylactic treatment of peach, using *M. laxa* tolerant cultivars, means prevention of the pathogenic problems first in the orchard. Tolerant genotypes will allow sustainable control with zero residues in fruits safety harvesting and at least decreasing disease problems in commodities in storage leading to better economic benefit. The total absence of residue in

prophylactic tolerant peach cultivar is friendly to enhanced environment compatibility (Usall et al., 2016). However, disease resistant varieties are not readily available in many fruit crops (Spiers et al., 2005) including commercial peach cultivars.

Developing peach cultivars tolerant to *M. laxa* pathogen requires, in the first instance, identification of existing tolerant/susceptible genotypes by screening from a germplasm (Rubos et al., 2008). Although most commercial cultivars are susceptible to *Monilinia* spp., few tolerant cultivars have been identified in peaches (Gradziel and Wang, 1993; Martínez-García et al., 2013; Oliveira-Lino et al., 2016; Obi et al., 2017). Hence relative tolerance/susceptibility of fruit to disease has often been used for selecting disease resistant genotype for peach breeding purpose (Gradziel, 1994).

Selection within breeding descendant population have been carried out in peach and nectarine (Bassi et al., 1998; Pacheco et al., 2014; see Oliveira-Lino et al., 2016, for details) and other fruits germplasm such as apricot (Walter et al., 2004); plum (Pascal et al., 1994) and apple (Biggs and Miller, 2004). Evidence exists showing that powerful antioxidants such as phenolic acids, flavonoids and anthocyanins can be found in the phytochemical compounds of some peach cultivars (Abidi et al., 2011; Giménez, 2013; Ágreda, 2016; Saidani et al., 2017), and that these bioactive compounds, especially the chlorogenic and neochlorogenic acids, major of the phenolics maintain important preservative functions in postharvest handling in peach commodity (Villarino et al., 2011; Pacheco et al., 2014; see Oliveira-Lino et al., 2016, in details). In addition, considering the recent drive for alternative technologies effective to control postharvest diseases of stone fruits (Mari et al., 2015; Usall et al., 2015, 2016), any documentation of composites inhibitory to brown rot susceptibility would have influence in breeding schemes and useful in the postharvest peach commerce.

In this context, information on the global evaluation of fruit pathogenic tolerance to brown rot of *M. laxa* in breeding descendants and their relationships with quality and phytochemical traits in peach postharvest handling appears limited. We aim to search for the Spanish industry superior peach cultivars with high tolerance to brown rot of *M. laxa* and significant antioxidant outline. The specific objectives of this work, therefore, were to evaluate for tolerance to brown rot of *Monilinia laxa* within the breeding descendant population of 'Babygold 9' × 'Crown Princess', and to examine fruit quality and phytochemical composition correlated to the tolerance. Finally, the identification of any biochemical compound associated to brown rot tolerance would have impact in breeding strategies, relevant to go further in the postharvest industry and ample environmental sustainability.

IV-2. Materials and Methods

IV-2.1. Plant material

The plant materials are progenies from a controlled biparental cross of two commercial cultivars, 'Babygold 9' × 'Crown Princess' (B9 × CP). These genotypes were propagated during 2000 and 2001 in collaboration with Agromillora Catalana S.L. (Barcelona, Spain). Both progenitors and the complete progeny are yellow fleshed, clingstone peaches. The resulting seedlings were budded on the GF677 rootstock and established in 2002 at the Experimental Station of AulaDei - CSIC Zaragoza Spain. Trees were trained to the standard open vase system, hand thinned and subsequently grown under standard conditions of irrigation, fertilization and chemical spray programs for pest and disease control (Giménez, 2013).

For the three years study (2013-2015), any fungicide treatment was applied in the field before harvest with adequate consideration to the free entry period and harvest for evaluation. A total of 68 genotypes were harvested in 2013 and 2014 seasons (Supplementary table III-1). Then, seventeen

genotypes were pre-selected with lesion severity (LS) < 40 mm, either in 2013, or 2014, or when the mean value for both years was below 40 mm (Obi et al., 2017), and harvested in 2015 to validate results concerning *M. laxa* tolerance. The pathogenic traits [percentage of brown rot incidence (%BRI), lesion diameter (LD) and colonization extent (CE)] were measured for each seedling tree separately over the 3-year period and means on the 17 selected genotypes were calculated. Fruits were subjectively selected and harvested based on optimum maturity [(Cantín et al., 2009) (expressed on visual color change and manual evaluation of firmness, apparently healthy with uniform ripeness and size)]. Fruits were disinfected as described by Obi et al., (2017).

IV-2.2. Pathogen culture, conidia production and inoculation

The procedure adopted is as described by Obi et al., (2017). Briefly, the culture of the *Monilinia laxa* (Aderh. & Ruhl.) Honey, isolate number: CPML02, used in this study was supplied by the *Collection of Postharvest Pathology Group* of IRTA (Lleida, Catalonia). Conidia from wounded fruits were efficiently harvested into a solution of sterile distilled water and Tween® 80 (0.0005%) surfactant. Quantification of conidia suspension was determined as in Obi et al., (2017) and adjusted to 25 x 10^3 mL^{-1} spore for fruit inoculation. To evaluate tolerance to brown rot, twenty disinfected fruits were inoculated with 25 μL of spore load of the virulent pathogen. Five fruits used as control were loaded with 25 μL sterile water and incubated for 5 days in darkness at 23 ^{o}C.

IV-2.3. Brown rot disease evaluation

Pathogenic traits were evaluated according to Obi et al., (2017). In brief inoculated fruits were daily observed during 5 days of incubation. Percentage brown rot incidence (%BRI) was assessed using the percentage fraction infected over total number of inoculated fruits. Percentage of colonization (%C) was assessed using the percentage colonized over total number of fruits. Lesion diameter (LD) and colonization extent (CE) were also measured. These parameters were used in the determination of brown rot disease severity for genotype tolerance rating as it was reported previously (Martínez-García et al., 2013; Obi et al., 2017). Lesion severity (LS) was calculated by the percentage of brown rot incidence (%BRI) x lesion diameter (LD)/100 and colonization severity (CS) by the percentage of colonization (%C) x colonization extent (CE)/100.

IV-2.4. Fruit quality trait evaluation

During the 2014 and 2015 seasons, twenty fruits were harvested to evaluate fruit quality individually in each seedling tree. Harvesting date (Julian days) ranged from late-May to mid-September, depending on each genotype of the population. Fruit weight and physicochemical traits were determined for each genotype. Titratable acidity and pH were determined at harvest as explained in previous studies (Abidi et al., 2015; Zeballos et al., 2016).

Fruits were evaluated for firmness (FF) and soluble solids content (SSC) at three different levels: at harvest and after 5 days of storage (inoculated and no inoculated). At harvest, firmness was determined on 5 fruits/genotype on opposite sides of the equator of each fruit, after a slash of the peel (about 2 cm^2) was removed, with a penetrometer fitted with an 8-mm diameter probe (Effegi, Milan Italy). Both measures were averaged for each fruit, and data are given in Newton (N). Firmness on no-inoculated and inoculated fruits was determined on 5 and 20 fruits / genotype, respectively, in the undamaged fruit part after 5 days of incubation. The soluble solids content (SSC) of the juice was also measured at harvest and after incubation with a temperature compensated refractometer (model ATC-1, Atago Co., Tokyo, Japan); and data are given as ^{o}Brix.

IV-2.5. Antioxidant compounds analysis

In sampling for biochemical analysis on fruit pulp and peel, ten fruits out of the twenty were randomly selected from the harvest, peeled using a mechanical peeler, and later cut into smaller pieces for relative homogeneity, and 3 g of peel and 5 g of fresh fruit weighed into 50 mL transparent polypropylene (PP) jars, frozen in liquid nitrogen and conserved under - 20°C for later use in determining (total phenolics, flavonoids, anthocyanins assays). For vitamin C determination samples were stored with metaphosphoric acid (HPO_3) and subsequently conserved at - 20°C until analysis. Biochemical extractions were done as it was reported in Cantín et al. (2009).

Vitamin C, total phenolic, flavonoid, and anthocyanin contents were determined with colorimetric methods (Cantín et al., 2009) and measured using a spectrophotometer [BIOCHROM ASYS UVM 340 microplate reader, (see details in Ágreda, 2016)]. Standard calibration curves were daily prepared for all determinations. For vitamin C, absorbance was measured at 525 nm and the amount of vitamin C was expressed as milligrams (mg) of ascorbic acid (AsA) per 100 g fresh weight (FW). For total phenolic content, the colorimetric method based on the chemical reduction of the Folin-Ciocalteau reagent was used. Absorbance was measured at 725 nm and the content was expressed in mg of gallic acid (3, 4, 5-Trihydroxy-benzoic acid) equivalents (GAE) per 100 g of FW.

Total flavonoids content was determined measuring absorbance at 510 nm and the results were expressed as milligrams of catechin equivalents (CE) per 100 g of FW. The total anthocyanin content was evaluated measuring in the hydroalcoholic extract the absorbance at 520 and 700 nm. The anthocyanin concentration was calculated using the molar extinction absorptivity coefficient ε = 26,900 / cm and was expressed in milligrams of cyaniding-3-glucoside equivalents (C3GE) per 100 g of FW (Liu et al., 2015; Saidani et al., 2017).

IV-2.6. Statistical analysis

Means and standard errors (SE) and Pearson's correlation were carried out with SPSS-23 (Statistical Product and Service Solutions Inc., Chicago USA) statistical software. The incidence and severity of brown rot including influence of quality parameters were also analyzed using analysis of variance (ANOVA) with SPSS-23 statistical software. Statistical significance was judged at the level $P < 0.05$, and the Duncan test was used for mean comparison.

IV-3. Results

We have studied a total of 68 descendants from the population 'Babygold 9' × 'Crown Princess' during three years (2013, 2014, and 2015) for tolerance against brown rot of *Monilinia laxa* (Supplementary table III-1). The disease parameters include: percentage brown rot incidence (%BRI), lesion diameter (LD), lesion severity (LS), percentage of colonization (%C), colonization extent (CE) and colonization severity (CS). As we have previously mentioned, we have selected seventeen genotypes that showed *M. laxa* lesion severity (LS) < 40 mm, either in 2013, or 2014, or with the mean value for both years (Supplementary Table III-2) to later evaluate and validate in 2015 the *M. laxa* tolerance in these genotypes.

Furtherance in these seventeen genotypes, harvest date (HD) was recorded and the physicochemical traits [fruit weight (FW), fruit firmness (FF), soluble solids content (SSC), pH and titarable acidity (TA)] were evaluated for three years [(2013-2015) (Table III-1)] and parametric test of Pearson correlation conducted within pairs of fruit quality traits (Table III-2). We have, also determined

phytochemical traits compounds as: Vitamin C (Vit. C), total phenolic (TPC), flavonoid and anthocyanin contents in flesh (2014-2015, Table III-3) and in peel only for 2015 (Table III-4).

IV-3.1. Effect of phytopathogen activities

The evaluation of 68 genotypes of 'Babygold 9' × 'Crown Princess' for brown rot tolerance in 2013 and 2014 are shown in Supplementary table 1. The incidence of brown rot (%BRI) in both years was between 50 to 100 %. Among years differences exist, although similar average percentage brown rot incidence was found for 2013 (91.9%) and 2014 (91.6%). The average percentage of colonization (%C) in 2013 was 84.8 and lower in 2014 80.2 %.

The average lesion diameter (LD) 2013 was 56.5 mm, while the average LD 2014 was 48.9 mm. In 2013 mean lesion severity (LS) was 52.5 mm and 45.3 mm in 2014. A corresponding pattern was repeated in both years in the range of colonization severity (CS) with average CS (44.0 mm in 2013 and 36.6 mm in 2014). Almost all the associated pathologic parameters indicate that the progeny in 2014 showed fewer symptoms of *M. laxa* than in 2013. Concisely in 2015, the seventeen genotypes, average BRI (92.9 %) and % of colonization (89.4%) had been higher than in the previous years. On the contrary, %LD and LS with 48.1 and 44.7 mm of average were lower.

From the mean of these seventeen genotypes evaluated in 2013, 2014 and 2015, only six genotypes (BC1, BC48, BC58, BC63, BC67, and BC68) showed lesion severity < 40 mm and colonization severity below 32 mm (Supplementary table III-2). The Pearson correlation between pairs of traits for pathological traits showed positive and significant correlation coefficients between 0.406 and 0.959 at $P < 0.01$. Among the strongest ones are brown rot incidence (%BRI) with percentage colonization (%C) ($r = 0.814$, $P \leq 0.01$); lesion diameter (LD) with colonization extent (CE) ($r = 0.859$, $P \leq 0.01$) and lesion severity with colonization severity ($r = 0.959$, $P \leq 0.01$) (Figure III-1).

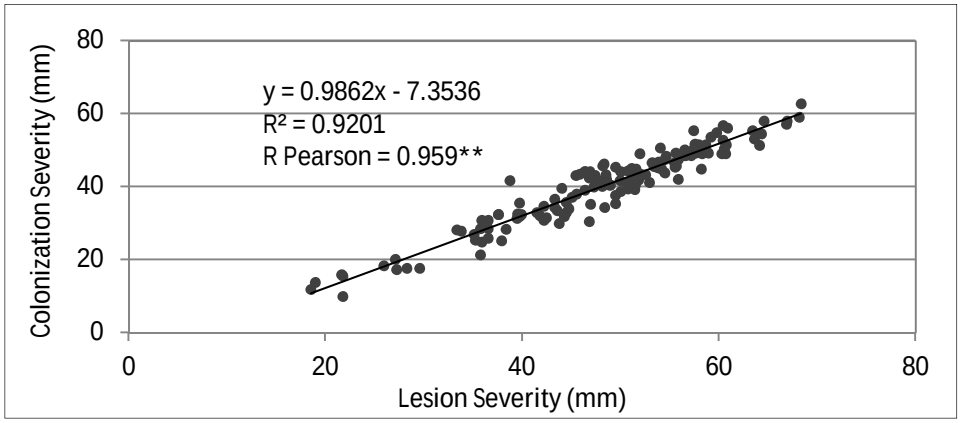

Figure IV-1: Correlation between lesion and colonization severities in all the 'B9' × 'CP' genotypes evaluated for three years (2013 - 2015). N = 138.

Table IV-1: Effect of storage and inoculation in FF and SSC and physicochemical traits in the 17 descendants of 'B9' × 'CP' population. Data are mean ± SE of three years (2013 – 2015). In bold tolerant genotypes.

Genotype	HD (JDs)	FW (g)	FF at harvest (N) [1]	FF no-inoculated (N) [1]	FF inoculated (N) [1]	SSC at harvest [a] (^{o}Brix)	SSC after storage no-inoculated (^{o}Brix) [1]	SSC after storage inoculated (^{o}Brix) [1]	pH [a]	TA (%) [a]	RI [a]
BC1	**175 ± 5**	**175 ± 21.1**	**26.50 ± 1.9 ab**	**33.40 ± 1 bcde**	**28.10 ± 0.8 b**	**8.2 ± 0.4**	**8.0 ± 0.5ab**	**8.0 ± 0.3 b**	**3.62 ± 0.0**	**0.6 ± 0.0**	**13.29 ± 0.8**
BC11	227 ± 6	209 ± 27.1	51.16 ± 3.8 d	48.13 ± 4.6 f	41.55 ± 3.0 d	9.8 ± 0.7	10.0 ± 0.4 cd	9.2 ± 0.2 c	3.96 ± 0.1	0.5 ± 0.1	17.70 ± 1.3
BC19	175 ± 5	186 ± 21.0	18.58 ± 2.3 a	24.05 ± 1.2 a	22.31 ± 0.8 ab	9.7 ± 0.1	8.0 ± 0.6 ab	7.5 ± 0.3 ab	3.82 ± 0.1	0.4 ± 0.0	20.05 ± 0.5
BC24	226 ± 7	208 ± 32.6	39.31 ± 3.3 c	34.18 ± 2.1 cde	34.35 ± 1.9 c	10.4 ± 0.4	9.2 ± 0.3 bc	9.2 ± 0.3 c	3.96 ± 0.1	0.6 ± 0.1	17.31 ± 1.6
BC44	175 ± 5	171 ± 21.5	20.35 ± 2.1 ab	19.07 ± 1.5 a	17.65 ± 0.6 a	8.2 ± 0.2	7.9 ± 0.3 ab	7.5 ± 0.2 ab	3.84 ± 0.3	0.5 ± 0.1	15.88 ± 3.1
BC48	**224 ± 4**	**208 ± 15.7**	**47.51 ± 2.9 cd**	**36.23 ± 1.4 e**	**35.11 ± 0.9 c**	**11.1 ± 0.7**	**11.1 ± 0.4 d**	**9.3 ± 0.2 c**	**3.88 ± 0.1**	**0.6 ± 0.1**	**16.56 ± 0.9**
BC51	175 ± 5	181 ± 21.0	24.17 ± 4.4 ab	25.15 ± 2.8 ab	25.50 ± 1.2 b	8.9 ± 0.9	8.0 ± 0.5 ab	7.9 ± 0.2 b	3.71 ± 0.1	0.5 ± 0.1	16.17 ± 2.9
BC53	178 ± 3	143 ± 16.2	19.22 ± 0.9 a	24.50 ± 0.8 a	23.73 ± 0.7 b	8.5 ± 0.3	7.1 ± 0.4 a	7.6 ± 0.3 ab	3.68 ± 0.0	0.5 ± 0.1	14.63 ± 1.2
BC57	227 ± 6	241 ± 18.0	48.19 ± 4.9 cd	46.76 ± 3.9 f	49.82 ± 3.1 e	9.3 ± 0.5	9.6 ± 0.5 c	7.9 ± 0.3 b	3.95 ± NE	0.5 ± NE	17.44 ± NE
BC58	**180 ± 6**	**187 ± 15.5**	**23.79 ± 2.1 ab**	**27.20 ± 1.1 abcd**	**24.27 ± 0.7 b**	**7.7 ± 0.9**	**7.6 ± 0.5 a**	**7.1 ± 0.3 a**	**3.62 ± 0.0**	**0.6 ± 0.1**	**12.98 ± 1.3**
BC59	176 ± 8	163 ± 24.3	29.08 ± 1.5 b	34.06 ± 1.7 ced	28.07 ± 0.8 b	9.7 ± 1.8	8.0 ± 0.3 ab	8.0 ± 0.3 b	3.76 ± 0.1	0.5 ± 0.0	17.16 ± 3.0
BC60	224 ± 7	187 ± 13.2	51.09 ± 3.1 d	52.29 ± 3.6 f	42.00 ± 1.6 d	11.0 ± 0.6	10.3 ± 0.3 cd	9.0 ± 0.2 c	3.92 ± 0.2	0.7 ± 0.2	14.40 ± 2.7
BC61	227 ± 6	220 ± 22.4	41.27 ± 2.6 c	36.26 ± 3.2 e	33.56 ± 1.5 c	9.2 ± 0.7	10.0 ± 0.3 cd	9.6 ± 0.2 cd	4.17 ± 0.0	0.4 ± 0.1	21.20 ± 2.1
BC63	**222 ± 2**	**235 ± 18.0**	**43.28 ± 1.9 cd**	**35.91 ± 1.6 de**	**36.37 ± 1.0 c**	**9.4 ± 1.0**	**9.5 ± 0.4 c**	**9.1 ± 0.2 c**	**3.89 ± 0.1**	**0.6 ± 0.1**	**14.28 ± 0.6**
BC66	216 ± NE	151 ± NE	29.29 ± 2.1 b	33.87 ± 1.6 bcde	27.22 ± 1.3 b	8.8 ± NE	9.5 ± 0.5 c	10.1 ± 0.3 d	3.87 ± NE	0.6 ± NE	12.68 ± NE
BC67	**180 ± 6**	**169 ± 10.6**	**21.70 ± 1.1 ab**	**25.60 ± 1.2 abc**	**24.73 ± 0.5 b**	**8.5 ± 1.2**	**7.6 ± 0.5 a**	**6.9 ± 0.2 a**	**3.67 ± 0.0**	**0.5 ± 0.1**	**17.01 ± 0.3**
BC68	**180 ± 6**	**186 ± 4.2**	**17.51 ± 1.0 a**	**25.32 ± 0.7 abc**	**24.05 ± 0.5 b**	**9.4 ± 1.0**	**7.2 ± 0.5 a**	**7.1 ± 0.2 a**	**3.76 ± 0.0**	**0.4 ± 0.1**	**18.01 ± NE**

a: No replication (data from pooled fruits of 5). Abbreviations: HD, harvest date; JDs, Julian days; FW, fruit weight; FF, fruit firmness; SSC, soluble solids content; TA, titratable acidity; RI, ripening index (SSC/TA); SE, standard error; NE, not available, because replications were less than 3 or harvested one year. [1] Different letters show differences among genotypes.

titratable acidity; RI, ripening index (SSC/TA); SE, standard error; NE, not available, because replications were less than 3 or harvested one year. [1] Different letters show differences among genotypes.

IV-3.2. Effect of storage, inoculation and physicochemical traits on fruits

Table 1 showed the effect of storage and inoculation on fruit firmness (FF) and soluble solid contents (SSC) and physicochemical traits in 17 selected fruits evaluated during three years (2013-2015). Genotypes from this progeny were harvested between 175 and 227 Julian days (JDs), which is late June and middle of August, respectively. Incidentally the six tolerant genotypes (in bold in Table 1) mature between 175 and 224 JDs. The fruit weight (FW) ranged between 143 g and 241 g. Marked variability was encountered in the state of firmness (FF) at harvest and after storage. In the seventeen genotypes the mean FF at harvest was 32.47 N. Specifically the least FF at harvest (17.51 N) was recorded for genotype BC68, while the most FF (51.16 N) was documented on behalf of genotype BC11. Mean FF at harvest (32.47 N) was lower than mean FF at storage (33.06 N) in the seventeen genotypes. Mean SSC at harvest was 9.3 ^{o}Brix (7.7 in BC 58 to 11.1 ^{o}Brix in BC 48). Within the stored peaches, mean SSC in no inoculated was 8.7 ^{o}Brix, while in inoculated it was 8.3 ^{o}Brix. After storage significant differences were found in SSC among the 17 selected genotypes.

There were also marked variability in pH (3.62 – 4.17), TA (0.40 – 0.60 %) and RI (12.68 – 21.20). As shown in Table 2, most of the physicochemical traits indicated positive and significant correlations among them. Harvest date (HD) correlated positively and significantly with FW, FF, SSC pH and RI. The FW correlated positively and significantly with FF and SSC (at harvest, inoculated and at storage), and pH and RI. The fruit firmness (FF) and SSC, at harvest, highly correlated with both parameters at storage.

Table IV-2: Pearson's correlations (parametric test) within pairs of fruit quality traits in 'B9' × 'CP' population studied during three years (2013-2015).

	FW	FF at harvest	FF no inoculated	FF inoculated	SSC at harvest	SSC no inoculated	SSC inoculated	pH	TA	RI
HD (JDs)	0.554**	0.602**	0.385*	0.552**	0.677**	0.630**	0.687**	0.759**	0.092	0.497**
FW		0.220*	0.200*	0.319**	0.334**	0.463**	0.445**	0.464**	0.167	0.421**
FF at harvest			0.833**	0.800**	0.418**	0.514**	0.363**	0.316**	0.261*	0.115
FF no-inoculated				0.837**	0.367**	0.547**	0.386**	0.369**	0.245*	0.175
FF inoculated					0.391**	0.562**	0.407**	0.415**	0.260*	0.173
SSC at harvest						0.786**	0.829**	0.667**	0.174	0.586**
SSC no-inoculated							0.810**	0.667**	0.133	0.518**
SSC inoculated								0.696**	0.199	0.514**

**Correlations significant at $P \leq 0.01$, N = 138

Abbreviations: HD, harvest date; JDs, Julian days; FW, fruit weight; FF, fruit firmness; SSC, soluble solids content; TA, titratable acidity; RI, ripening index (SSC/TA)

IV-3.3. Effect of antioxidant compound contents

Table III-3 shows contents for all antioxidant compounds (ascorbic acid, total phenolic, flavonoid, and anthocyanin) in the flesh of 17 genotypes evaluated in 2014 and 2015. In addition, we have included as preliminary results the content of these compounds in the peel measured in 2015 to find any compound associated with the tolerance to *M. laxa* (Table III-4). Significant differences among genotypes were found for all antioxidant contents either in flesh or peel tissues.

Table IV-3: Antioxidant compound contents in the flesh of the 17 genotypes of 'B9' × 'CP' population evaluated for two years (2014 - 2015). Data are mean ± SE. In bold are tolerant genotypes

Genotype	Ascorbic acid (mg of AsA/100 g of FW)	Total phenolics (mg of GAE/100 g of FW)[1]	Flavonoids (mg of CE/100 g of FW)	Anthocyanins (mg of C3GE/100 g of FW)
BC1	**9.19 ± 3.3 d**	**49.78 ± 2.3 bcde**	**17.99 ± 1.8 abc**	**0.13 ± 0.0 a**
BC11	4.41 ± 0.3 abc	61.71 ± 3.3 e	33.49 ± 7.6 d	0.16 ± 0.0 ab
BC19	7.89 ± 0.5 cd	48.91 ± 6.4 abcde	24.46 ± 1.1 abcd	0.14 ± 0.0 a
BC24	7.74 ± 1.4 cd	56.06 ± 4.8 cde	35.09 ± 3.5 d	0.17 ± 0.0 ab
BC44	6.12 ± 0.7 abcd	34.04 ± 2.4 abc	12.08 ± 1.2 ab	0.09 ± 0.0 a
BC48	**5.22 ± 0.5 abc**	**47.82 ± 2.0 abcde**	**17.69 ± 1.5 abc**	**0.09 ± 0.0 a**
BC51	3.64 ± 0.9 ab	60.07 ± 3.7 de	35.45 ± 6.9 d	0.15 ± 0.0 ab
BC53	6.47 ± 0.9 bcd	28.30 ± 2.4 ab	10.02 ± 1.9 a	0.17 ± 0.0 ab
BC57	2.76 ± 0.3 a	37.31 ±1.2 abcd	10.96 ± 0.6 ab	0.16 ± 0.0 ab
BC58	**3.16 ± 0.2 ab**	**42.84 ± 3.1 abcde**	**17.48 ± 2.9 abc**	**0.22 ± 0.0 ab**
BC59	5.69 ± 1.3 abc	46.57 ± 7.2 abcde	25.86 ± 9.9 bcd	0.10 ± 0.0 a
BC60	5.25 ± 1.0 abc	27.49 ± 3.3 a	09.95 ± 3.0 a	0.29 ± 0.1 bc
BC61	6.36 ± 0.4 bcd	42.35 ± 5.1 abcde	13.44 ± 2.1 ab	0.20 ± 0.0 ab
BC63	**2.55 ± 0.4 a**	**50.09 ± 6.9 abcde**	**29.04 ± 8.7 cd**	**0.16 ± 0.0 ab**
BC66	3.86 ± 0.6 ab	41.32 ± 4.0 abcde	19.04 ± 1.4 abc	0.12 ± 0.0 a
BC67	**4.88 ± 1.9 abc**	**49.40 ± 2.1 bcde**	**19.09 ± 2.2 abc**	**0.16 ± 0.0 ab**
BC68	**4.66 ± 0.3 abc**	**40.10 ± 3.5 abcde**	**09.57 ± 0.7 a**	**0.40 ± 0.1 c**

Abbreviations: AsA, ascorbic acid; GAE, gallic acid equivalents; CE, catechin equivalents; C3GE

The Ascorbic acid (AsA) content in flesh ranged from 2.55 to 9.19 mg of AsA/100 g of FW TPC ranged from 27.49 to 61.71 mg of GAE/100 g of FW) among the selected 17 genotypes Flavonoids contents varied from 9.57 to 35.45 mg of CE/100 g of FW. Regarding anthocyanins, particularly in fruit flesh, the variation was from 0.09 to 0.40 mg of C3GE/100 g of FW. Furthermore, in peel a wide range of antioxidant contents was found in the seventeen studied genotypes.

In general, vitamin C, TPC and flavonoid contents were higher in peel than in the flesh. Contents for TPC in BC67, ascorbic and anthocyanins in BC1 and BC67 contained significantly higher values comparing with the other genotypes. Flavonoid contents were not significantly different in the tolerant compared to non-tolerant genotypes. As shown in Table 4, the ascorbic acid (AsA) content in peel of the 17 genotypes ranged from 5.89 to 16.29 mg of AsA/100 g of FW.

Table IV-4: Antioxidant compound contents in the peel of the 17 genotypes of 'B9' × 'CP' evaluated in 2015. Data are mean ± SE of 10 fruits per genotype

Genotype	Ascorbic acid (mg AsA/100 g FW)	Total phenolics (mg GAE/100 g FW)	Flavonoids (mg CE/100 g FW)	Anthocyanins (mg C3GE/100 g FW)
BC1	**15.48 ± 0.7 e**	**153.54 ± 1.1 hi**	**96.42 ± 2.7 fg**	**9.66 ± 0.1 i**
BC11	9.01 ± 0.7 abcd	158.92 ± 5.0 ij	106.18 ± 3.4 g	4.17 ± 0.0 e
BC19	9.24 ± 0.9 abcd	112.17 ± 1.2 bcd	75.70 ± 4.5 de	6.00 ± 0.2 f
BC24	8.45 ± 0.5 abcd	168.24 ± 2.8 j	128.13 ± 2.4 h	0.62 ± 0.0 a
BC44	10.58 ± 0.4 bcd	116.81 ± 1.1 cde	67.74 ± 0.4 cd	2.42 ± 0.0 c
BC48	**9.87 ± 0.6 bcd**	**141.01 ± 7.5 gh**	**74.25 ± 6.7 de**	**5.99 ± 0.1 f**
BC51	8.12 ± 0.4 abcd	150.15 ± 6.7 hi	142.04 ± 5.4 hi	2.81 ± 0.0 d
BC53	11.10 ± 0.9 cd	89.98 ± 3.2 a	50.00 ± 0.8 b	4.36 ± 0.1 e
BC57	7.37 ± 0.7 abc	123.00 ± 2.4 de	61.69 ± 7.6 bcd	1.69 ± 0.0 b
BC58	**10.89** ± 0.1 **cd**	**128.13 ± 5.2** ef	**86.47 ± 6.6 ef**	**8.26 ± 0.2 h**
BC59	9.48 ± 0.1 abcd	144.07 ± 3.0 gh	132.70 ± 1.3 h	2.17 ± 0.0 c
BC60	11.31 ± 0.2 d	106.19 ± 4.5 ab	56.30 ± 7.5 bc	6.94 ± 0.2 g
BC61	8.45 ± 0.3 abcd	135.70 ± 3.1 fg	105.45 ± 5.5 g	2.94 ± 0.0 d
BC63	**5.89** ± 0.3 **a**	**115.61 ± 3.8 bcde**	**88.72 ± 1.4 ef**	**4.28 ± 0.1 e**
BC66	08.42 ± 0.3 abcd	103.73 ± 2.7 b	74.16 ± 1.0 de	0.68 ± 0.0 a
BC67	**16.29** ± 1.1 **e**	**189.43 ± 5.7 k**	**148.61 ± 3.4 i**	**12.94 ± 0.2 j**
BC68	**6.77** ± 0.1 **ab**	**148.59 ± 2.5 hi**	**36.54 ± 6.9 a**	**2.18 ± 0.0 c**

Abbreviations: AsA, ascorbic acid; GAE, gallic acid equivalents; CE, catechin equivalents; C3GE,

IV-4. Discussion

Within the phytopathogenic activities in fruits, we have found that genotypes with smaller diameter of the fungus injury correspond to the smaller diameter of colonization. In addition, these genotypes are also associated with a lower incidence, that is, a lower percentage of damaged fruits (susceptibility).
The annual disparity found in the genotypes response to brown rot after inoculation may be due to different levels of cuticular cracking or fractures as other authors have reported for stone fruits (Gradziel et al., 2003; Kappel and Sholberg 2008).

Cuticular cracks are considered as preferential entry spots for fungi pathogens of the *Monilinia* species (Gibert et al., 2007) and incidence of fruit infection increased with increasing surface area of fruit cuticular cracks (Borve et al.,2000; Gibert et al., 2009).
In this experiment fruits were not wounded before they were inoculated; therefore, the brown rot pathogen would require naturally occurring wounds or micro-cracks in the cuticle to gain entry into the fruit (Oliveira-Lino et al., 2016). Secondly since we used a uniform quantity of artificial inoculum density throughout our experiment the yearly variation would be attributed to the natural differences in surface cuticular cracks. Gradziel et al., (2003) reported that cuticular and epicuticular waxes of peaches were influenced by the environment during the growing season. Ágreda, (2016) has also reported yearly variation in the *in-vitro* infection of brown rot in another peach population evaluated under the same conditions.

In the scrutiny of the brown rot tolerance along three years, the seventeen genotypes showed a wide range of variability in most of the pathogenic parameters studied (Supplementary Table III-1). The lowest BRI (73.3 %) and colonization (51.7%) occurred in genotype BC67, while the lowest LD (41.98 mm), LS (31.75 mm), CE (39.05 mm) and CS (21.75 mm) were observed in genotype BC58. The highest values for brown rot incidence (BRI, 100%) occurred in four different genotypes (BCs:11, 24, 61 and 66), for LD (52.34 mm), LS (50.27 mm) and CS (42.51 mm) highest values were recorded in BC11, while for colonization (91.7%) and CE (49.47 mm) were observed in BC61 and BC60 genotypes, respectively. Consequently, the level of susceptibility to brown rot depends largely on peach genotype (Gradziel, 1994).

The positive and significant correlation between pairs of pathological traits in our study is rather a typical trend. This undoubtedly inclines to indicate that the level of infection significantly influenced the lesion diameter and colonization extent including severities in the diseased situation (Michailides et al., 2000). Thus, lesion and colonization are usually two associated brown rot parameters evaluating brown rot tolerance in peach. Susceptibility information from the two traits is important in the evaluation for tolerance considering their genetic and pathogenic points of view, respectively (Xu et al., 2008; Burnett et al., 2010).

Considering the physicochemical variables, the observations in harvest date (HD) of our work are in agreement with the report of Giménez, (2013) within the entire population which was harvested during 2009, 2010 and 2011 between 163 to 248 JDs. And considering the fact that we obtained infection in all the sixty eight genotypes spread between late June and middle of August it tends to suggest, therefore, that peach susceptibility to brown rot of *M. laxa* occur even in early or late harvest season (Obi et al., 2017). Though when fruits are harvested late they are sweeter, larger, and have higher total phenolic, flavonoids and total sugar concentration (Font i Forcada et al., 2013), both very early-maturing, as well as very late-maturing peach genotypes are of significant interest for the peach industry, particularly, in the Mediterranean area (Cantín et al., 2010). The fruit weight (FW) range of 143 g and 241 g obtained in the 17 genotypes is similar to the range reported, in the same population, by Giménez (2013).

Though mean FF at harvest (32.47 N) was lower than mean FF at storage (33.06 N) in the 17 genotypes, there was no significant differences, indicating that the incubation conditions did not affect fruit firmness (data not showed). On the contrary, there was a significant decrease ($P \leq 0.05$) at storage in FF no-inoculated (33.06 N) vs FF inoculated (30.49 N), indicating that fruit firmness in inoculated fruit is due to the activity of *M. laxa* and that may have affected the surrounding tissues. Our findings may explain the observation of Yaghmour et al., (2011) that found more severity as the state of FF decreases. Our analysis revealed a broad range of FF of 17.51 to 47.51 N within the genotypes with LS below 40 mm, indicating that brown rot is not dependent on fruit firmness.

Our study also revealed that there is loss in SSC as fruit is stored, inoculated or no inoculated, from harvest. In the seventeen peach genotypes mean SSC at harvest was 9.3 ^{o}Brix which ranged from 7.7 in BC 58 to 11.1 ^{o}Brix in BC 48. Within the stored peaches, mean SSC in fruits no inoculated was 8.7 ^{o}Brix, while in inoculated was 8.3 ^{o}Brix. After storage significant differences were found in SSC among the 17 selected genotypes. The fact that we have found significant differences in SSC at harvest (9.3 ^{o}Brix) vs SSC after storage (8.5 ^{o}Brix) ($P \leq 0.001$) indicates that the decrease in SSC might be due to the effect of storage including the pathogenic activities of the fungus on the inoculated host. We can speculate with our results that in inoculated fruit that SSC influences pathogenic activities of *M. laxa* in peach inferring that as the pathogen preys on the host, the interaction leads to the depletion of SSC as sugars are used for its mycelia biosynthesis, growth and development. There were significant differences ($P \leq 0.001$) in SSC mean of the seventeen genotypes in 2014 (8.2 ^{o}Brix) comparing to 2013 (8.9 ^{o}Brix) and 2015 (8.8 ^{o}Brix), but any difference between 2013 and 2015. This indicates that SSC were significantly lower in fruits with less pathogenic damage in the population.

Concisely, our findings in the effect of storage and inoculation on fruit properties are in agreement with the reports of Biggs and Miller (2004) that showed positive correlations between *Botryosphaeria dothidea* pathogen activity and sugar content. However, under storage, SSC in inoculated peach presented an inverse significant correlation with CE, LD and LS ($r = -0.273$, $P \leq 0.01$ and $r = -0.236$, $P \leq 0.01$; $r = -0.178$, $P \leq 0.05$, respectively), which is in concordance with Gradziel (1994) that associated less susceptibility to brown rot with high SSC content as the peach fruit ripens.

In relation to disease parameters with FF within the seventeen genotypes, BC58 at harvest recorded one of the lowest FF (23.79 N) was associated with the lowest disease parameters in LD (41.98 mm), LS (31.75 mm), CE (39.05 mm) and CS (21.75 mm), while genotype BC11 recorded the highest FF at harvest (51.16 N) and the highest disease parameters in LS (50.27 mm) and CS (42.51 mm). However, genotype BC44 which demonstrated FF of 20.35 N) did not correspond to tolerant or susceptible genotype (LS = 43.64 mm and CS = 34.96 mm).

Hence, state of FF, especially at harvest, does not seem to influence brown rot development significantly. In the same manner the genotype BC58 which registered the least SSC at harvest (7.7 ^{o}Brix) was associated with the lowest disease parameters as well as the other genotype BC67 (6.9 ^{o}Brix), however, the genotype BC48 with the highest SSC (11.1 ^{o}Brix) reflected low damage too. Other genotypes with intermediate SSC content at harvest as BC 44 (9.8 ^{o}Brix) showed the highest brown rot severities.

In furtherance, the positive and significant correlation of HD with FW, FF, SSC and pH in our test is compatible with the report by other authors (Cantín et al., 2010; Giménez, 2013; Font et al., 2014; Ágreda, 2016). The correlation found in FF and SSC at harvest with same parameters after storage ($r = 0.418$, $P \leq 0.01$) is similar to the report of Giménez (2013) ($r = 0.226$, $P \leq 0.01$) studying one hundred progenies of the same population. The positive association between FF and SSC in the tolerant genotypes is important since the selection of genotypes with high SSC will aim first at higher firmness

and second lower susceptibility to pathogen predisposing mechanical damage during handling and package (Crisosto et al., 2001).

The variation of pH from 3.62 to 3.89, in our six tolerant genotypes, indicates values of normal acidity fruits since pH lower than 4.0 at maturity are considered as acidic (Abidi et al., 2015). The inverse and significant correlations found between pH and TA ($r = - 0.327$, $P \leq 0.01$) and TA vs RI ($r = - 0.665$, $P \leq 0.01$), are similar to the report by other authors (Giménez, 2013; Abidi et al., 2015). We have observed in previous experiments that the pH of the fruit increased with progress in fruit maturity while the titarable acidity (TA) decreased (Obi et al., 2018). These parameters can be important since it has been reported that acidity preserve fruits from pathologic damage (Hajilou and Fakhimrezaei 2011; Cropotova et al., 2013; Tarabih and El-Metwally, 2014).

Regarding the bioactive compounds, ascorbic acid (AsA) content in flesh ranged from 2.55 to 9.19 mg of AsA/100 g of FW as reported by Giménez, (2013) in the same population. However, the total phenolic content (27.49 to 61.71 mg of GAE/100 g of FW) among the selected 17 genotypes was over the range found by Giménez, [(2013) (11.22 to 37.42 mg of GAE/100 g of FW)] in the same progeny studied during three years (2009-2011). The differences found here can be due to the screening of genotypes with LS lower than 40 mm. Concerning flavonoids contents varied from 9.57 to 35.45 mg of CE/100 g of FW, being also higher than those obtained in previous studies in different peach progenies by Giménez, 2013, (1.6 to 13.7 mg of CE/100 g of FW); Abidi et al., 2015, (2.3 to 18.0 mg of CE/100 g of FW) and Ágreda (2015) (3.79 to 27.63 mg of CE/100 g of FW). Regarding anthocyanins, particularly in fruit flesh, the variation was from 0.09 to 0.40 mg of C3GE/100 g of. These values were below those reported by other authors [(0.7 to 12 mg of C3GE/100 g of FW) in a broad germplasm collection (Font i Forcada et al., 2013); (0.23 - 11.83 mg of C3GE/100 g of FW), in the same progeny (Giménez, 2013)]. The differences can be due to the flesh-colorless of these seventeen 'B9' × 'CP' genotypes and / or for the different method used for the quantification.

Furthermore, in peel a wide range of antioxidant contents was found in the seventeen studied genotypes. In general vitamin C, total phenolic and flavonoid contents were higher in peel than contents found in the flesh in agreement with previous reports (Ágreda, 2016; Saidani et al., 2017). We have found around 65% of vitamin C, 75% of total phenolics, 81% of flavonoid and 96% of anthocyanin contents are concentrated in peel in our progeny. Contents for total phenolics in BC67, ascorbic and anthocyanins in BC1 and BC67 contained significantly higher values comparing with the other genotypes. However, flavonoid contents were not significantly different in the tolerant compared to non-tolerant genotypes.

As shown in Table III-4, the ascorbic acid (AsA) content in peel of the 17 genotypes ranged from 5.89 to 16.29 mg of AsA/100 g of FW, as other investigators have recently reported (Ágreda, 2016; Saidani et al., 2017). The content of anthocyanins from 0.62 to 12.94 mg of C3GE/100 g of FW in peel tissue of the 17 selected genotypes reveals that most of the tolerant genotypes had 27-81 times higher contents of anthocyanins in peel compared to the flesh. These values are in agreement with previous reports (Prior et al., 1998; Gil et al., 2002; Saidani et al., 2017) inferring and supporting that anthocyanins are concentrated more in fruit peel than in the flesh.

Unequal distribution of vitamin C, total phenolic in flesh (≈ 25-30 %) and peel (≈ 65-70%) of peach has also been documented (Ágreda, 2016; Saidani et al., 2017). Of great significance, therefore, is that high content of these bioactive compounds in peel help the fruit to resist the forces of abiotic stress (Cantín et al., 2009), whose activities often predispose peach fruits to biotic inversion. And even directly fruit peel has been extensively indicated as an important broad range resistance source against opportunistic pathogens such as *Monilinia* spp. (Pacheco et al., 2014).

Interestingly, pathologic variables, brown rot incidence and colonization (%BRI, %C) and lesion and colonization severities (LS, CS) correlated inversely with peel anthocyanin contents (r = - 0.551, r = - 0.552, r = - 0.481, r = - 0.491, $P \leq 0.05$ respectively (Figure III-2). However, only %BRI negatively correlated with anthocyanin content from fruit flesh (r = - 0.219, $P \leq 0.05$).

Anthocyanin is the most common pigment in nature (Khoddami et al., 2013), a phytochemical that gives plants their colour and protects tissues from oxidative abiotic stress, which invariably extends the life span of the plant organ. Hence it is found more concentrated in the skin portion of fruit, particularly near maturity (Prior et al., 1998) for protective barrier against potential phytopathogenic invaders and that, in our genotypes, could be an advantage to tolerance.

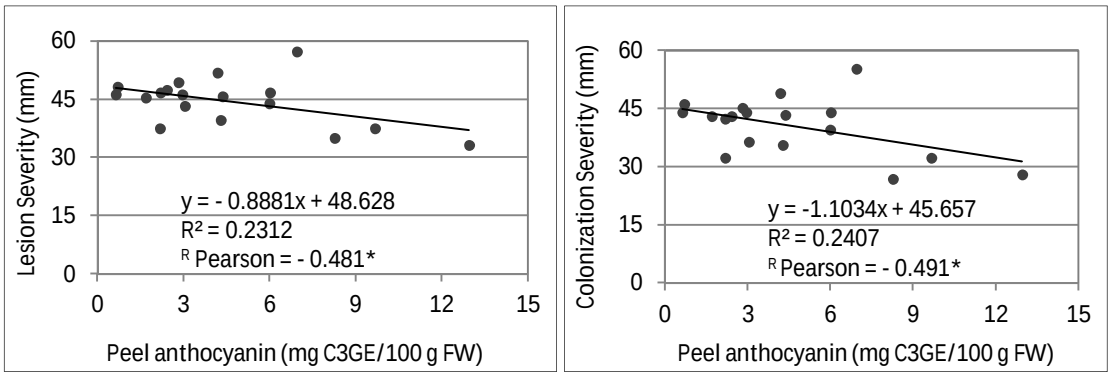

Figure IV-2: Correlation between lesion and colonization severities and peel anthocyanin-content in the 17 (B9 × CP) genotypes evaluated for 2015. N = 17.

Nevertheless, it was only TPC from flesh that correlated inversely and significantly in this progeny with LD, LS and CE (r = - 0.282, r = - 0.279, and r = - 0.225, all at $P \leq 0.05$, respectively) as found by Ágreda (2016). Other authors also have reported significant inverse correlation between phenolic acids and BRI in peach and nectarine cultivars (Villarino et al., 2011). Apparently high contents of antioxidants tend to influence brown rot negatively by reducing pathogenic activities; however, in this work the genotypes with LS < 40 mm were not those with the highest TPC content and vice-versa. In particular, major phenolic acids, such as chlorogenic and neochlorogenic acids (Villarino et al., 2011) of high and potent antioxidant properties (Dai and Mumper, 2010; Khoddami et al., 2013), may protect the plant and plant materials against fungi and other phytopathogenic organisms (Prasad et al., 2014; Spadoni et al., 2014).

However, at harvest fruit phenolic contents has decreased and their effectiveness to control brown rot infection can vary with peach cultivar (Obi et al., 2018; Cindi et al., 2016).

Pearson's correlation coefficients among bioactive compounds were between 0.790 and 0.506. TPC flesh correlated positively and significantly with flesh and peel flavonoids (r = 0.790, r = 0.718, respectively) all at $P \leq 0.01$. Moreover, TPC and flavonoid contents in peel were also highly correlated (r = 0.722, $P \leq 0.01$). The results found in this progeny were in agreement with studies carried out previously in this o other progenies or peach germplasm (Giménez 2013; Font et al., 2014; Abidi et al., 2015). Furthermore, Ágreda, (2016), Saidani et al., (2017) found flesh ascorbic positively and significantly correlated with peel anthocyanin (r = 0.797, $P \leq 0.01$), and Obi et al., (2017) found flesh ascorbic negatively and significantly correlated with disease incidence. This sort of high association found in this study within the biochemical compound implies that they are important antioxidant phytochemicals acting in coordination inducing tolerance to brown rot in peach but further studies need to be done in order to determine this.

Infection or incidence, sporulation and dissemination make up the three major components of a fungal pathogen life cycle in a disease situation (Agrios, 2005) From the genetic point of view, lesion severity is a good parameter to consider in selection for breeding because though there is damage from the fungi, dispersion of pathogen is limited due to lack of sporulation in the incidence. But from the pathogen point of view colonization severity is a better option to be considered because there is the possibility of sporulation due to colonization which leads to spore dispersion within the environment for further damage.

IV-5. Conclusions

The selection of genotypes with bioactive contents in peach breeding in combination with brown rot tolerance may avoid dramatic consequences in commodities safe alternative to control measures. Based on our three years screening protocol, we have found phenotypic differences in susceptibility to brown rot caused by *Monilinia laxa* in the population 'Babygold 9' × 'Crown Princess'. Furthermore, we have found fruit firmness decreases due to 5 days of storage and to the activity of *M. laxa* in the surrounding tissues.

It was possible to identify and select six genotypes (BC1, BC48, BC58, BC63, BC67, and BC68) of low brown rot susceptibility and high fruit quality in the germplasm of the Experimental Station of Aula Dei-CSIC. Though we have found genotypes that possess bioactive compounds as total phenolic, ascorbic acids, flavonoids and anthocyanin compounds associated to potential brown rot tolerance, not all the genotypes with lesion severity less than 40 mm contained the highest bioactive contents. BC1 and BC67 had significantly higher contents for ascorbic, total phenolics and anthocyanins. However, flavonoid contents were not significantly different in the tolerant compared to non-tolerant genotypes. The inverse correlations observed between anthocyanin and brown rot severity highlight their potential influence on susceptibility to *M. laxa*. This interaction is of paramount importance to be considered in the current breeding programs to select cultivars with bioactive compound contents, health-enhancing properties and good postharvest performance.

IV-6. Abbreviations used

B9 × **CP** ('Babygold 9' × 'Crown Princess'); **%BRI** (percentage brown rot incidence); **BR** (brown rot); **LD** (lesion diameter); **CE** (colonization extent); **LS** (lesion severity); **CS** (colonization severity); **%C** (percentage colonization); **HD** (harvest date); **FW** (fruit weight); **FF** (fruit firmness); **SSC** (soluble solids content); **TA** (titarable acidity); **RI** (ripening index); **Vit**. **C** (vitamin C); **TPC** (total phenolic content); **JDs** (Julian days; **Vs** (versus); **A** × **C** ('Andross' × 'Calante'); MS (master of science); TFM (trabajo fin de máster).

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General discussion

The use of tolerant cultivars in plant breeding is topmost in the principle of crop protection and improvement (Gell et al., 2007 a), as plants and plant products are usually secured (prophylactic) and, often, not cured of diseases (chemotherapeutic). Consequently, host tolerance to plant pathogens is cost effective and environmentally safe strategy for disease management (Gradziel, 2003 a; Martínez-Gómez et al., 2005). Peach [*Prunus persica* (L.) Batsch] is third most important global tree crops after apples and pears within the economically important Rosaceae family (Obi, et al., 2017). Its production and commercialization is often hindered by the activities of brown rot or moniliosis (Oliveira-Lino et al., 2016) caused by *Monilinia* species which includes: *M. laxa* (Aderhold and Ruhland) Honey, *M. fructigena* (Honey), *M. fructicola* (G. Winter) and *M. polystroma* (G. Leeuwen) (EPPO, 2009; Jansch et al., 2012). In Spain, *M. laxa* is one of the principal species against successful production of peach (Villarino et al., 2012). Damage of 59-100% after harvest is reported (Casal et al., 2010 a; Villarino et al., 2012). And due to its high incidence in Spain, numerous control measures, both in the plot (pre-harvest), and during the shelf-marketing (post-harvest) stages have been employed.

In summary, we have made observations that commercial peach fruits are highly susceptible to brown rot disease in our Mediterranean conditions. We have also observed an enhanced stricter regulation of fungicide use in EU countries, in recent times (Oliveira-Lino, 2016) due to rising concern about the effects of biochemical fungicides on the environment (Thomidis and Exadaktylou, 2010), and strain fungicide resistance (Egüen et al., 2015). These justify new alternatives of host resistance, among the most cost effective and environmentally safe strategies to be pursued for disease control in peach farming. To breed new peach crops more tolerant to brown rot than the present cultivars would involve the possibility of increasing the general level of tolerance to brown rot by crossing tolerant cultivars and followed by recurrent selection in progeny. Lasting prophylactic treatment of peach, using *M. laxa* tolerant cultivars means prevention of the pathogenic problems from the orchard. This will enhance good harvest and zero disease problem in storage leading to better economic benefit. Therefore, in this thesis, we aimed at, and evaluated for phenotypic tolerance to brown rot (*Monilinia laxa*) disease in the peach breeding program of the Experimental Research Station of Aula Dei-CSIC, Zaragoza, Spain, a novelty of being the first of its kind in this station. This thesis was organized in a series of four papers that are discussed below.

Optimizing protocols to evaluate brown rot of *Monilinia laxa* susceptibility in peach fruits

In the **first paper**, we have modified the existing methods in order to screen a large peach germplasm, of the estacion experimental de Aula Dei -CSIC, for tolerance to brown rot of *Monilinia laxa*. Incubation factors as temperature (20-26 °C), relative humidity (40-60%) and 12 h photoperiod enhanced colonization and sporulation for substantial conidia production. We have found that the peculiar lighting source (mixture of two fluorescent lighting systems, Sylvania Gro-Lux, F36W / GRO and Ogram Daylight, F36W / 840DL) was positive to obtain enough inoculum in a short period of time. We have also found that artificial injury ensured adequate and prompt infection of host (Casals et al., 2010 a; de Cal et al., 2013). Fruit injury encouraged short period of incubation making available inoculum source within 3 days post inoculation and avoiding cross-contamination of other fungi such as *Botrytis* and *Rhizopus* species. Moreover, the purity of inoculum load was ensured with this method because potential contaminants such as nematodes were avoided during conidia harvest by mildly rubbing over the infected regions of the fruit using a sterile dental brush. Initially enough *M. laxa* inoculum was produced from PDA culture incubated (Memmert CO_2 incubator INCO, Germany) at least 35 days at 23 ^{o}C in the dark. Nevertheless, we tried to reduce the time to obtain inoculum adapting the methods used by Gell et al., (2007 b) and Jansch et al., (2012). They incubated prepared PDA cultures of *Monilinia* spp. in the dark at 20-25 ^{o}C for 7 to 10 days for adequate sporulation. But our preliminary trials (data not shown) indicated poor inoculum production when incubated in the dark even with more than 6 days of incubation at 23 ^{o}C. Finally, we have assured effective inoculum in 4 to 6 days after incubation of inoculated peaches under at temperature (20-26 ^{o}C), relative humidity (40-60%) and 12 h photoperiod.

Furthermore, the composition of inoculum for susceptibility screening could involve one pathogenic strain (Gradziel and Wang, 1993) or two or more pathogenic isolates pooled (Biggs and Miller, 2005) to enhance pathogen virulence. A monostrain isolate was used in our study because our inoculum source was tested for stable virulence and was stable enough to initiate pathogenesis without need for strain synergism. We established effective inoculum density of 25 x 10^3 mL^{-1} with inoculating load of 625 spores per fruit. Pascal et al., (1994) though employed a monostrain isolate but higher loading of 20,000 spores / fruit that may be less sustainable.

We have evaluated 20 fruits per genotype as enough sample size to assess representative evaluation considering the large population involved in plant breeding. The number of samples used to evaluate disease tolerance against brown rot in stone fruit varies among researchers from 10 peaches per genotype (Gradziel and Wang, 1993) to 30 fruits in apricot, plum and peach (Pascal et al., 1994). Moreover, the preference for the *ex-situ* evaluation is that the methodology enables for effective and efficient handling of large population and sample material. The *ex-situ* system also enables for thorough selection of healthy fruits and disinfection from insects and potential contaminants. Also essential is that direct influence such as variation in temperature, precipitation and relative humidity are avoidable environmental factors when screening, for brown rot tolerance, under a controlled environment. The germination and other pathogenic activities of conidia are markedly influenced by the interaction of the above mentioned factors (Xu et al., 2001; Casals et al., 2010 b).

The 3_(A×C) genotype, and 'Big Top', 'Venus' and 'Calante' cultivars exhibited brown rot severity (BRS) lower than 40 mm. Paunovic and Paunovic, (1996) have reported similar susceptibility values in peach germplasm in Yugoslavia. However, their investigation was done *in-situ* involving five pathogens (*M. laxa, M. fructicola, Sphaerotheca pannosa, Tapharina deformans* and Sharka virus).

Brown rot incidence (% BRI) showed positive and significant correlations with all pathologically associated factors (LS, CE and CS). This indicates that the level or frequency of infection will always and significantly influence the lesion diameter and extent of colonization including severities in a disease situation. Also harvest date showed a positive and significant correlation with % BRI, LS and CS. Inferring that genotypes that mature later generally tended to have bigger lesions and would be less tolerant to fungal pathogen. This is the first time to find that the pathological parameters correlate, positively, with Julian days.

There was a particular interest on the 'Calante' cultivar which, though produced lesion, was devoid of colonization (sporulation), an important factor in disease spread. This feat is biologically and epidemiologically an advantage for plant breeding. Its high level of tolerance may be related to a late harvest or to the agronomical practices performed in Alcañiz. In these orchards, due to the singularity of this late cultivar harvested in October, fruits are protected to pathogen injuries using paper bags. Other authors have found annual variation for brown rot infection in 'Calante' (Obi et al., 2015; Ágreda et al., 2016). It was described that colonization in nectarines and peaches fruit infected by *Monilinia* spp. could be associated with the local acidification of the host tissue (de Cal et al., 2013) and that acidic environment can prevent brown rot colonization on peach (Bonaterra et al., 2003), however, it is not the explanation for our results since the fruit has at maturity the same pH or acidity year by year. Other uncontrolled factors such as environmental instead of genetic may be responsible for non-colonization in 'Calante' harvested in the 2012. Moreover, colonization on host (extensive differentiation of hyphae at the surface of host) is not a genetic trait (Giobbe et al., 2007).

Finally, according to this research, we have not found differences indicating the impact of hairiness on susceptibility although other authors have proposed that fruit hairiness could encourage susceptibility to disease infection in some stone fruits (Wade and Cruickshank, 1992; Xu et al., 2007;

Garcia-Benitez et al., 2016). In our plant material using this protocol we have demonstrated a relatively similar degree of tolerance / susceptibility between peach and nectarine fruits. An example are the low levels of *Monilinia laxa* incidence registered with the 'Venus' nectarine and 3_(A x C) peach genotype or the lower LS found in 'Venus' in comparison with the rest of the cultivars.

Effects of Acidity (pH and TA) on the growth and development of *Monilinia laxa*

In the **second paper**, we have focused on the resultant effect of host pathogen interaction, *in vitro* and *in vivo,* specifically implicating acidity (pH and TA)**.** There is an ample influence of pH and titratable acidity (TA) in the solid PDA and the peach cultivars inhibiting the growth of the *M. laxa*. According to Tutu and Ciornea, (2011) pathogen host interaction involves the process of nutrition which leads to either the growth / reproduction or the inhibition of activity and the inactivation of the pathogen as a result of available acidity factors. In the pH-amended PDA there was no effective pathogen growth / development under a high acidic condition. Similarly, *M. laxa* developed no infection in the fruit until commercial maturity at pH 4.19 ± 0.10. This is relatively a moderate state of non-acidity in the fruit. Previously, peach fruits with a non-acid character have been characterized at maturity by a pH higher than 4.0 (Dirlewanger et al., 1999).

Mycelia growth (colonization) and sporulation are the most accurate variables used in plant pathology to effectively compute the degree of disease development (Douds, 1994; Gigot et al., 2009; Miles et al., 2009; Burnett et al., 2010; Obi et al., 2017). Consequently, determining the influence of pH and TA on the subsistence and development of *M. laxa,* at both *in-vitro* and *in-vivo* levels, is an avenue to understanding the epidemiology of brown rot and subsequent development of disease management strategies to effectively combat the problem (Tian and Bertolini, 1999; Hong et al., 2000).

Sporulation itself is a function of colonization (Douds, 1994). Colonization concerns dimension (size or area) occupied by infection while sporulation deals with population (conidia or spores) involved in an occupied or diseased area. This implies that sporulation is the subsequent effect of colonization due to infection (Xu et al., 2001). However, we have found, according to our results, that high rate of colonization did not equate to high rate of sporulation. Hence the extent of lesion or colonization does not always equate to the degree of sporulation in a host pathogen interaction but depends on available level of acidity as exponent by this experiment.

For example in our assay the mycelia growth or extent of colonization against pH 6.40 and 5.30 were 80.61 mm and 77.50 mm producing mean conidia concentration of 60800 and 110800 spores mL^{-1} respectively. Though the extent of colonization was higher in pH 6.40 than in pH 5.30 but sporulation was found to be higher in pH 5.30 than in pH 6.40 with about 45.13%. This appears to suggest that sporulation of *M. laxa* in a disease situation increases as the pH increase, reaches its maximum still in the acidic region and then begins to descend as the pH approaches neutral or the alkaline precinct.

We are of the view and assertion, therefore, that *M. laxa* could sporulate at a wide range of pH between 3.5 and 9.5 with the optimum between pH 4.5 and 5.5. This is similar to, and in agreement with, the results of Agarwal and Sarbhoy, (1978) that acidic pH favours fungi growth with best performance within a range of pH 3.5-6.5, and Pascual et al., (1997) that observed pH 4-6 range for optimum growth of their working fungal pathogen, *Penicillium oxalicum*. Furthermore, Amiri et al., (2009) found, as most suitable, pH 3.6 to enable selective isolation and enumeration of three *Monilinia* spp. of stone fruits. However, our working pathogen (*M. laxa*) sporulated best under acidic state at pH 5.3 while in the work of Pascual et al., (1997), the fungi sporulated best under a neutral / alkaline range of pH 7-8. Though, on different fungi from *M. laxa*, Gupta et al., (2010) observed maximum growth and sporulation of *F. solani* at pH 5.5. Hence *M. laxa* could be associated with the greater percentage of

fungi that grow well in marginally acidic condition. It is equally significant to note here that *M. laxa* has had less attention, notwithstanding the fact that it is as important as *M. fructicola* and *M. fructigena* especially, in the study of epidemiology and management of brown rot (Rungjindamai et al., 2014).

The evolution of pH and TA in the growth and development of peach fruit is non-sinusoidal waveform, contrasting in beats or waves between them. This is found to corroborate the report of Moing et al., (1998). High pH value gives a corresponding lower content in the TA and vice versa. This is so obvious, for example, at 208 JDs (77 BBCH scale) of 'Babygold 9' where the highest pH peak corresponds to the lowest dip in TA value. Also this tends to validate the works of Dirlewanger et al., (1999) that TA and pH in peach are negatively correlated, and Lobit et al., (2002) that pH and TA, the most common measure of acidity with perceived sourness or sugariness in peach fruit, well correlate inversely.

The non-sinusoidal waveform peak of pH reached by fruits at 208 JDs (77 BBCH scale), which corresponds to 136 and 108 days of complete floration and fruit setting respectively, must have occurred at the cell expansion phase. In peach fruit there is usually reduction in organic accumulation, which results in fruit with lower acidity and higher pH as was determined in our work at 208 JDs (77 BBCH scale). This tends to support *Moing* et al., (1998) that such physicochemical activity occurs far before reaching ripening. Stages of peach development are considered to occur in four phases which includes: fruit set, rapid cell division, cell expansion, and ripening/maturation (Tutu and Ciornea 2011).

Hence the most resistant period to pathogen infection in peach fruit is during the stages covering pit hardening to pre-harvest (Keske et al., 2011). It could, therefore, be inferred that immature peaches are very resistant to brown rot because of high levels of acidic pH found in the epidermal cells. This high level of pH could have inhibited brown rot incidence at the immature stages in'Babygold 9' due to acidification activities.

Gluconic acid has been reported as the main organic acid associated with the enhancement of peach acidification in host-pathogen interaction (de Cal et al., 2013). *M. laxa* colonized the 'Babygold 9' at the commercial stage of maturity (232 JDs) (79 BBCH scale) when the acidic pH has run down and probably aided by local acidification of the host tissue (de Cal et al., 2013).

It is found worthy to mention, however, that in the inoculation of uninjured 'Babygold 9' with the normal conidia from immature to mature fruit state, the effects of inoculating with mycelia on both injured and uninjured immature fruits were also determined simultaneously. The observations indicate that: at 145 JDs (65 BBCH scale) (pH 3.69 ± 0.13) there was no BRI when fruit was inoculated with 25μL of 25 x 10^3 cfu mL^{-1} spores on artificial injury.

There was also no BRI in fruits without artificial injury inoculated with 25μL of 25 x 10^3 cfu mL^{-1} spores. There was, however, BRI in the immature fruit with artificial injury inoculated with a 6.5 mm mycelia PDA. In addition, at 222 JDs (78 BBCH scale) (pH 3.89 ± 0.11), within the fruit colour break, there was no BRI in fruits with intact skin (no artificial injury) when inoculated with a 25μL of 25 x 10^3 cfu mL^{-1} spores. In the contrary, artificially injured fruits showed BRI when inoculated with 25μL of 25 x 10^3 cfu mL^{-1} spores.

The significance of these observations is that the degree of susceptibility to infection by *Monilinia* spp. is variable throughout fruit development (Gradziel, 1994; Guidarelli et al., 2014). Our findings support this assertion because in our experiment at the early stage of growth and development, immature fruit could not exhibit brown rot symptoms when inoculated with conidia, neither through injury site nor on intact epidermis, but could only develop infection when inoculated with mycelium in an injury situation. Furthermore, at colour-break 222 JDs (78 BBCH scale), peach fruit at pH 3.89 ± 0.11 could develop infection with conidia inoculation only through an injury and not possible when there was no injury on

the fruit epidermis. Hence skin injury could be responsible for the incident of brown rot on immature peach fruits observed in orchards (Northover and Biggs, 1990; Holb, 2004).

In furtherance, FW at maturity (194.04 ± 1.99) was significantly different ($P \leq 0.05$, Duncan test) from the rest of the FW at development. Also the pH (4.19 ± 0.10) and TA (0.41 ± 0.01) values at maturity were all significantly different from those of the development values. Pearson´s correlation shows inverse correlation between pH and all the pathologic activities of peach cultivars at harvest. Harvest date (HD) significantly correlated with fruit size (FS) ($r = 0.912$, $P < 0.01$); fruit weight (FW) ($r = 0.889$, $P < 0.01$); and pH ($r = 0.440$, $P < 0.01$). Also FS significantly correlated with FW ($r = 0.980$, $P < 0.01$). There was a fair and significant correlation between pH and FW ($r = 0.356$, $P < 0.05$). Expectedly, however, pH inversely correlated significantly with TA ($r = -0.604$, $p < 0.01$).

Finally while there was a continuous and stable increase in weight of fruit as it develops, the reverse was the case in fruit size. There was fluctuation in the size as the fruit developed. Fruit size increased, later decreased at a time and finally increased as the fruit developed. The obvious dynamics in pH and TA values also occurred when there were clear changes in fruit size and weight evolution. In addition the pH 4.19 ± 0.1 at which *M. laxa* could infect the peach in this work relatively correlate with the range of pH (3.5-9.5) in the solid PDA considered to support sporulation *in vivo*. Hence brown rot infection and expression in peach fruit is dependent on the influence of pH and TA as chemical factors and in extension upon the stage of the fruit growth (Emery et al., 2000; Holb, 2004; Gell et al., 2009). This study encompasses the necessity to know the evolution of fruit maturity in new and old varieties in relation to potential *Monilinia* infection in immature fruits. The knowledge, in addition, could be useful in the determination of the presence of pathogen in latent infection within molecular techniques.

Finally, the study has shown that *M. laxa* exhibited variation in its growth and sporulation capacities on the seven pH amended PDA, preferring relatively moderate acidic conditions for their optimum performance. We found, in the *in-vitro* analysis, that there was mycelia growth in pH from 2.40 to 8.84 while pH 11.52 did not support any mycelia growth. The pH 5.30 supported the highest sporulation while pH 6.40 encouraged the highest colonization extent or mycelia growth. This is in support of the findings of Holb, (2004) that the most favorable initial hydrogen-ion concentration for mycelial growth occurs between pH 3.5 and 5.5. We found that there was a continuous and stable increase in weight of fruit as it develops, the reverse being the case in fruit size. The fruit size increased, decreased at a time and finally increased as the fruit develops. The pH dynamics exhibited non-sinusoidal waveform through the growth and development of the fruit. In all these physicochemical variations, *M. laxa* could not develop infection or shown any brown rot incidence in the fruit until the period of commercial maturity.

In conclusion, on the basis of this study it can be concluded that pH and titratable acidity have great impacts on the growth activity of *M. laxa* in a host-pathogen association both in solid PDA substrate and in peach fruit growth and development. And as intrinsic organic acids and genetic traits, pH and titratable acidity could constitute imperative antibrown rot determining elements to be given adequate attention in peach breeding program, as highlighted in this thesis.

The tolerance of commercial peach cultivars to brown rot by *Monilinia laxa* is modulated by its antioxidant content?

In the third paper, we have studied eight commercial peach cultivars with different fruit characteristics and tested their tolerance to brown rot caused by *Monilinia laxa* after artificial inoculation. As it was reported in our previous study, symptoms of infection in peach fruits were only developed after inoculation on commercial maturity (Obi et al., 2017, 2018). A similar approach and protocols are routinely used to phenotype tolerance to brown rot caused by *M. fructicola* in peach

germplasm (Bostock, 2018). These cultivars based on the pH can be classified as acid (pH<4) or non-acid fruits (pH>4) (Dirlewanger et al., 1999). However, the range of pH (3.7-4.3) found among cultivars has no effect on the *in-vivo Monilinia* growth. Obi et al., (2018) demonstrated a similar *in-vitro* mycelial growth at these pH ranges. Values found for pH, TA, FF1 and SSC1 were within the range reported in other studies in peach germplasm (Abidi et al., 2015; Font i Forcada et al., 2013; Saidani et al., 2017). The differences found among cultivars in firmness (FF1) are mainly related to factors, such as harvest date or fruit type. Firmness of ripe peaches tended to be higher for the late cultivars as we have found in 'Calanda Tardio' and 'Calante' (Iglesias and Echeverria, 2009; Montevecchi et al., 2012).

Similarly, the content for Ascorbic acid varied widely (from 4.09 in 'Big Top' to 14.91 mg/100 g FW in 'Andross') and fell within the ranges previously reported for ascorbic acid in peach pulp (Abidi et al., 2015; Font i Forcada et al., 2013, Saidani et al., 2017). Regarding NCG and CG acids content, 'Calanda Tardio' and 'Calante' showed the highest levels for both hydroxycinnamic acids. These cultivars also showed the highest relative antioxidant capacity (RAC), total polyphenols (TPP), total hydroxycinnamic acids (HA) and total flavanols (FA) contents, as reported by previous authors (Saidani et al., 2017) in peel and flesh tissue within a parallel study. The wide variation found among these cultivars has been attributed specially to seasonal influences. The cultivars late harvested contain higher contents on relative antioxidant capacity, flavonoid and TPP than cultivars harvested in earlier season (Saidani et al., 2017). The levels of antioxidants gradually increases as the harvest date progresses throughout the year. This is consistent with the high and positive correlation ($p \leq 0.01$) found between harvest date and relative antioxidant capacity (r = 0.836), total hydroxycinnamic acids (r = 0.742), total flavanols (r = 0.874) and total polyphenols (r = 0.761) (Saidani, 2016).

Based on the results found here, the degree of susceptibility to brown rot on these peach cultivars was not associated to the large differences in harvest dates. The cultivars were harvested from mid - June to early October but no correlation was found between harvest date and brown rot tolerance as was reported before in other peach germplasm (Obi et al., 2017). All pathological traits (brown rot incidence, colonization and lesion and colonization severities) were highly correlated among them (Obi et al., 2017). After 5 days of incubation no significant differences were found between the soluble solid contents (SSC) of control fruits (SSC2=14.0 ºBrix) with inoculated fruits (SSC3 =13.5 ºBrix). Therefore, there is no credible evidence that the activities of *M. laxa* depleted soluble solid contents in the peach as it was found previously in other progenies (Obi et al., unpublished results).

In this work we have showed that only AsA content presented an inverse correlation with lesion severity (r = -0.555, $p \leq 0.01$). On the contrary, none of the disease parameter correlated with any other bioactive compounds, neither the RAC nor NCGA, CGA or TPP. In agreement with these findings, no relation was detected between BR resistance to *M. fructicola* and concentration of phenolic compounds in Californian peach germplasm (Gradziel and Wang, 1993). Apparently, phenolic compounds were not specifically involved in the cultivar tolerance to BR caused by *M. fructicola* or *M. laxa*. Nevertheless, we could suggest that a combination of different antioxidant compounds may confer partial immunity as was found in 'Andross' (this study, Gradziel and Wang, 1993). This cultivar showed the highest level of ascorbic acid, high CG acid and moderate levels of RAC that may contribute to its tolerance. 'Andross' also presented moderate levels of flavonoids and TPC in peel and pulp tissues (Saidani et al., 2017). On the contrary, the levels of ascorbic, NCG and CG acids found in 'Calante', and the highest contents in RAC, NCGA and CGA and TPP found in 'Calanda Tardio' cannot explain its susceptibility to brown rot.

As it was mentioned above, the role of plant phenolic acids in fungal inhibition has been widely discussed by several authors (Oliveira-Lino et al., 2016 and references therein). CG and caffeic acids at levels similar to or in excess of those in the exocarp of immature resistant fruits did not affect *M.*

fructicola growth (Bostock et al., 1999), however, these acids down regulate cutinase production in *M. fructicola* cultures (Wang et al., 2002) and markedly inhibited the production of the cell wall polygalacturonase and cutinase (Lee and Bostock, 2007). In the same direction, Villarino et al., (2011) found that NCGA and CGA contents in immature fruits were negatively correlated to BRI and *in vitro* demonstrated that high CGA concentration modified fungal melanin production that might interfere to *M. laxa* penetration. These studies suggested that phenolic acids may suppress the cellular activities in the fungal pathogens that may be crucial for its growth and colonization on a host. However, in this study the correlation analysis revealed that NCGA and CGA contents or total polyphenols in fruits had not effect on fungal lesion after artificial inoculation. In other words, contents of NCG and CG acids at harvest probably are independent and do not indicate cultivar tolerance.

On the contrary, the ascorbic acid that is considered one of the most important antioxidants in plant tissues was negatively correlated with fungal growth after artificial inoculation. Our results are in agreement with Wang et al., (2002) who demonstrated that other antioxidants such as glutathione and lipoic acid significantly attenuate cutinase production in *M. fructicola* and discuss that the effect of phenolics is due to a general antioxidant effect rather than a specific chemical interaction. Lee and Bostock, (2007) also mentioned that phenolics in plant tissue may influence the antioxidant level in the pathogen and, as a consequence, the expression of genes associated with infection. The ascorbic acid, which concentration has been considered as an important nutritional quality indicator for peach, can be considered relevant in plant breeding for its antioxidant role conferring *in vivo* brown rot tolerance. These findings will open new research to test the effect of ascorbic either *in vivo* on brown rot tolerance or *in vitro* on microbial growth.

In conclusion the result of evaluation of tolerance in the eight commercial cultivars to brown rot disease demonstrates variability in the genetic susceptibility *to Monilinia laxa*, with 'Andross' and 'Baby Gold 9' the more tolerant but not significantly different from 'Tebana' and 'Miraflores'. 'Crown Princess', 'Big Top' 'Calante' and 'Calanda Tardío' were the less tolerant peach cultivars. The content of ascorbic acid can only partially explain the tolerance-susceptibility observed, indicating that other factors are probably involved in the response. Identification of these factors will be fundamental for breeding programs focused on improving resistance.

In addition, neither neochlorogenic nor chlorogenic or TPP contents at harvest can explain the differences in tolerance to brown rot found in these peach cultivars. We suggest that together with the ascorbic acid, other physico-chemical factors may confer the tolerance to 'Andross', 'Tebana', 'Baby Gold 9' and 'Miraflores'. To our knowledge this is the first study that correlates the ascorbic acid contents in mature peach fruit with *Monilinia laxa* brown rot tolerance. From a practical point of view application of ascorbate based formulae may be promising. Furthermore, we may speculate that the complex interaction *Monilinia-Prunus* establish the redox environment that modulate or restrict the fungal infection. Nonetheless, further studies need to be done in order to know the effect of AsA as a curative or preventive treatment to control *M. laxa* infections and/or to disentangle the role of each specific antioxidant compound in the genetic brown rot tolerance in peach.

Breeding strategies for identifying superior peach genotypes tolerant to brown rot

In the **fourth paper**, we have screened a total of 68 individuals from the population of 'Babygold 9' × 'Crown Princess'. From the results, we have found that genotypes with smaller diameter of the fungus injury correspond to the smaller diameter of colonization. In addition, these genotypes are also associated with a lower incidence, that is, a lower percentage of damaged fruits (susceptibility).

The annual disparity found in the genotypes response to brown rot after inoculation may be due to different levels of cuticular cracking or fractures as other authors have reported for stone fruits (Gradziel et al., 2003 b; Kappel and Sholberg, 2008). Cuticular cracks are considered as preferential entry spots for fungi pathogens of the *Monilinia* species (Gibert et al., 2007) and incidence of fruit infection increased with increasing surface area of fruit cuticular cracks (Borve et al.,2000; Gibert et al., 2009).

In this experiment fruits were not wounded before they were inoculated; therefore, the brown rot pathogen would require naturally occurring wounds or micro-cracks in the cuticle to gain entry into the fruit (Oliveira-Lino et al., 2016). Secondly since we used a uniform quantity of artificial inoculum density throughout our experiment the yearly variation would be attributed to the natural differences in surface cuticular cracks. Gradziel et al., (2003 b) reported that cuticular and epicuticular waxes of peaches were influenced by the environment during the growing season. Ágreda, (2016) has also reported yearly variation in the *in-vitro* infection of brown rot in another peach population evaluated under the same conditions.

In the scrutiny of the brown rot tolerance along three years, the seventeen genotypes showed a wide range of variability in most of the pathogenic parameters studied. The lowest BRI (73.3 %) and colonization (51.7%) occurred in genotype BC67, while the lowest LD (41.98 mm), LS (31.75 mm), CE (39.05 mm) and CS (21.75 mm) were observed in genotype BC58. The highest values for brown rot incidence (BRI, 100%) occurred in four different genotypes (BCs:11, 24, 61 and 66), for LD (52.34 mm), LS (50.27 mm) and CS (42.51 mm) highest values were recorded in BC11, while for colonization (91.7%) and CE (49.47 mm) were observed in BC61 and BC60 genotypes, respectively. Consequently, the level of susceptibility to brown rot depends largely on peach genotype (Gradziel, 1994).

The positive and significant correlation between pairs of pathological traits in our study is rather a typical trend. This undoubtedly inclines to indicate that the level of infection significantly influenced the lesion diameter and colonization extent including severities in the diseased situation (Michailides et al., 2000). Thus lesion and colonization are usually two associated brown rot parameters evaluating brown rot tolerance in peach. Susceptibility information from the two traits is important in the evaluation for tolerance considering their genetic and pathogenic points of view, respectively (Xu et al., 2008; Burnett et al., 2010).

Considering the physicochemical variables, the observations in harvest date (HD) of our work are in agreement with the report of Giménez, (2013) within the entire population which was harvested during 2009, 2010 and 2011 between 163 to 248 JDs. And considering the fact that we obtained infection in all the sixty eight genotypes spread between late June and middle of August it tends to suggest, therefore, that peach susceptibility to brown rot of *M. laxa* occur even in early or late harvest season (Obi et al., 2017). Though when fruits are harvested late they are sweeter, larger, and have higher total phenolic, flavonoids and total sugar concentration (Font i Forcada et al., 2013), both very early-maturing, as well as very late-maturing peach genotypes are of significant interest for the peach industry, particularly, in the Mediterranean area (Cantín et al., 2010). The fruit weight (FW) range of143 g and 241 g obtained in the 17 genotypes is similar to the range reported, in the same population, by Giménez, (2013).

Though mean FF at harvest (32.47 N) was lower than mean FF at storage (33.06 N) in the 17 genotypes, there was no significant differences, indicating that the incubation conditions did not affect fruit firmness (data not showed). On the contrary, there was a significant decrease ($P \leq 0.05$) at storage in FF no-inoculated (33.06 N) vs FF inoculated (30.49 N), indicating that fruit firmness in inoculated fruit is due to the activity of *M. laxa* and that may have affected the surrounding tissues. Our findings may explain the observation of Yaghmour et al., (2011) that found more severity as the state of FF decreases.

Our analysis revealed a broad range of FF of 17.51 to 47.51 N within the genotypes with LS below 40 mm, indicating that brown rot is not dependent on fruit firmness.

Our study also revealed that there is loss in SSC as fruit is stored, inoculated or no inoculated, from harvest. In the seventeen peach genotypes mean SSC at harvest was 9.3 ^{o}Brix which ranged from 7.7 in BC 58 to 11.1 ^{o}Brix in BC 48. Within the stored peaches, mean SSC in fruits no inoculated was 8.7 ^{o}Brix, while in inoculated was 8.3 ^{o}Brix. After storage significant differences were found in SSC among the 17 selected genotypes. The fact that we have found significant differences in SSC at harvest (9.3 ^{o}Brix) vs SSC after storage (8.5 ^{o}Brix) ($P \leq 0.001$) indicates that the decrease in SSC might be due to the effect of storage including the pathogenic activities of the fungus on the inoculated host. We can speculate with our results that in inoculated fruit that SSC influences pathogenic activities of *M. laxa* in peach inferring that as the pathogen preys on the host, the interaction leads to the depletion of SSC as sugars are used for its mycelia biosynthesis, growth and development. There were significant differences ($P \leq 0.001$) in SSC mean of the seventeen genotypes in 2014 (8.2 ^{o}Brix) comparing to 2013 (8.9 ^{o}Brix) and 2015 (8.8 ^{o}Brix), but any difference between 2013 and 2015. This indicates that SSC were significantly lower in fruits with less pathogenic damage in the population.

Concisely, our findings in the effect of storage and inoculation on fruit properties are in agreement with the reports of Biggs and Miller, (2004) that showed positive correlations between *Botryosphaeria dothidea* pathogen activity and sugar content. However, under storage, SSC in inoculated peach presented an inverse significant correlation with CE, LD and LS ($r = - 0.273$, $P \leq 0.01$ and $r = - 0.236$, $P \leq 0.01$; $r = - 0.178$, $P \leq 0.05$, respectively), which is in concordance with Gradziel, (1994) that associated less susceptibility to brown rot with high SSC content as the peach fruit ripens.

In relation to disease parameters with FF within the seventeen genotypes, BC58 at harvest recorded one of the lowest FF (23.79 N) was associated with the lowest disease parameters in LD (41.98 mm), LS (31.75 mm), CE (39.05 mm) and CS (21.75 mm), while genotype BC11 recorded the highest FF at harvest (51.16 N) and the highest disease parameters in LS (50.27 mm) and CS (42.51 mm). However, genotype BC44 which demonstrated FF of 20.35 N) did not correspond to tolerant or susceptible genotype (LS = 43.64 mm and CS = 34.96 mm).

Hence state of FF, especially at harvest, does not seem to influence brown rot development significantly. In the same manner the genotype BC58 which registered the least SSC at harvest (7.7 ^{o}Brix) was associated with the lowest disease parameters as well as the other genotype BC67 (6.9 ^{o}Brix), however, the genotype BC48 with the highest SSC (11.1 ^{o}Brix) reflected low damage too. Other genotypes with intermediate SSC content at harvest as BC 44 (9.8 ^{o}Brix) showed the highest brown rot severities.

In furtherance the positive and significant correlation of HD with FW, FF, SSC and pH in our test is compatible with the report by other authors (Cantín et al., 2010; Giménez, 2013; Font et al., 2014; Ágreda, 2016). The correlation found in FF and SSC at harvest with same parameters after storage ($r = 0.418$, $P \leq 0.01$) is similar to the report of Giménez (2013) ($r = 0.226$, $P \leq 0.01$) studying one hundred progenies of the same population. The positive association between FF and SSC in the tolerant genotypes is important since the selection of genotypes with high SSC will aim first at higher firmness and second lower susceptibility to pathogen predisposing mechanical damage during handling and package (Crisosto et al., 2001).

The variation of pH from 3.62 to 3.89, in our six tolerant genotypes, indicates values of normal acidity fruits since pH lower than 4.0 at maturity are considered as acidic (Abidi et al., 2015). The inverse and significant correlations found between pH and TA ($r = - 0.327$, $P \leq 0.01$) and TA vs RI ($r = - 0.665$, $P \leq 0.01$), are similar to the report by other authors (Giménez, 2013; Abidi et al., 2015). We have observed in previous experiments that the pH of the fruit increased with progress in fruit maturity while the

titarable acidity (TA) decreased (Obi et al., 2017). These parameters can be important since it has been reported that acidity preserve fruits from pathologic damage (Hajilou and Fakhimrezaei 2011; Cropotova et al., 2013; Tarabih and El-Metwally, 2014).

Regarding the bioactive compounds, ascorbic acid (AsA) content in flesh ranged from 2.55 to 9.19 mg of AsA/100 g of FW as reported by Giménez, (2013) in the same population. However, the total phenolic content (27.49 to 61.71 mg of GAE/100 g of FW) among the selected 17 genotypes was over the range found by Giménez, [(2013) (11.22 to 37.42 mg of GAE/100 g of FW)] in the same progeny studied during three years (2009-2011). The differences found here can be due to the screening of genotypes with LS lower than 40 mm. Concerning flavonoids contents varied from 9.57 to 35.45 mg of CE/100 g of FW, being also higher than those obtained in previous studies in different peach progenies by Giménez, 2013, (1.6 to 13.7 mg of CE/100 g of FW); Abidi et al., 2015, (2.3 to 18.0 mg of CE/100 g of FW) and Ágreda, (2015) (3.79 to 27.63 mg of CE/100 g of FW). Regarding anthocyanins, particularly in fruit flesh, the variation was from 0.09 to 0.40 mg of C3GE/100 g of. These values were below those reported by other authors [(0.7 to 12 mg of C3GE/100 g of FW) in a broad germplasm collection (Font i Forcada et al., 2013); (0.23 - 11.83 mg of C3GE/100 g of FW), in the same progeny (Giménez, 2013)]. The differences can be due to the flesh-colorless of these seventeen 'B9' × 'CP' genotypes and/or for the different method used for the quantification.

Furthermore, in peel a wide range of antioxidant contents was found in the seventeen studied genotypes. In general vitamin C, total phenolic and flavonoid contents were higher in peel than contents found in the flesh in agreement with previous reports (Ágreda, 2016; Saidani et al., 2017). We have found around 65% of vitamin C, 75% of total phenolics, 81% of flavonoid and 96% of anthocyanin contents are concentrated in peel in our progeny. Contents for total phenolics in BC67, ascorbic and anthocyanins in BC1 and BC67 contained significantly higher values comparing with the other genotypes. However, flavonoid contents were not significantly different in the tolerant compared to non-tolerant genotypes. The ascorbic acid (AsA) content in peel of the 17 genotypes ranged from 5.89 to 16.29 mg of AsA/100 g of FW, as other investigators have recently reported (Ágreda, 2016; Saidani et al., 2017).

The content of anthocyanins from 0.62 to 12.94 mg of C3GE/100 g of FW in peel tissue of the 17 selected genotypes reveals that most of the tolerant genotypes had 27-81 times higher contents of anthocyanins in peel compared to the flesh. These values are in agreement with previous reports (Prior et al., 1998; Gil et al., 2002; Saidani et al., 2017) inferring and supporting that anthocyanins are concentrated more in fruit peel than in the flesh.

Unequal distribution of vitamin C, total phenolic in flesh (≈ 25-30 %) and peel (≈ 65-70%) of peach has also been documented (Ágreda, 2016; Saidani et al., 2017). Of great significance, therefore, is that high content of these bioactive compounds in peel help the fruit to resist the forces of abiotic stress (Cantín et al., 2009), whose activities often predispose peach fruits to biotic inversion. And even directly fruit peel has been extensively indicated as an important broad range resistance source against opportunistic pathogens such as *Monilinia* spp. (Pacheco et al., 2014).

Interestingly, pathologic variables, brown rot incidence and colonization (%BRI, %C) and lesion and colonization severities (LS, CS) correlated inversely with peel anthocyanin contents ($r = -0.551$, $r = -0.552$, $r = -0.481$, $r = -0.491$, $P \leq 0.05$ respectively. However, only %BRI negatively correlated with anthocyanin content from fruit flesh ($r = -0.219$, $P \leq 0.05$). Anthocyanin is the most common pigment in nature (Khoddami et al., 2013), a phytochemical that gives plants their colour and protects tissues from oxidative abiotic stress, which invariably extends the life span of the plant organ. Hence it is found more concentrated in the skin portion of fruit, particularly near maturity (Prior et al., 1998) for protective

barrier against potential phytopathogenic invaders and that, in our genotypes, could be an advantage to tolerance.

Nevertheless, it was only TPC from flesh that correlated inversely and significantly in this progeny with LD, LS and CE (r = - 0.282, r = - 0.279, and r = - 0.225, all at $P \leq 0.05$, respectively) as found by Ágreda (2016). Other authors also have reported significant inverse correlation between phenolic acids and BRI in peach and nectarine cultivars (Villarino et al., 2011). Apparently high contents of antioxidants tend to influence brown rot negatively by reducing pathogenic activities; however, in this work the genotypes with LS < 40 mm were not those with the highest TPC content and vice-versa. In particular, major phenolic acids, such as chlorogenic and neochlorogenic acids (Villarino et al., 2011) of high and potent antioxidant properties (Dai and Mumper, 2010; Khoddami et al., 2013), may protect the plant and plant materials against fungi and other phytopathogenic organisms (Prasad et al., 2014; Spadoni et al., 2014). However, at harvest fruit phenolic contents has decreased and their effectiveness to control brown rot infection can vary with peach cultivar (Obi et al., 2017; Cindi et al., 2016).

Pearson's correlation coefficients among bioactive compounds were between 0.790 and 0.506. TPC flesh correlated positively and significantly with flesh and peel flavonoids (r = 0.790, r = 0.718, respectively) all at $P \leq 0.01$. Moreover, TPC and flavonoid contents in peel were also highly correlated (r = 0.722, $P \leq 0.01$). The results found in this progeny were in agreement with studies carried out previously in this o other progenies or peach germplasm (Giménez, 2013; Font et al., 2014; Abidi et al., 2015). Furthermore, Ágreda, (2016), Saidani et al., (2017) found flesh ascorbic positively and significantly correlated with peel anthocyanin (r = 0.797, $P \leq 0.01$), and Obi et al., (2017) found flesh ascorbic negatively and significantly correlated with disease incidence. This sort of high association found in this study within the biochemical compound implies that they are important antioxidant phytochemicals acting in coordination inducing tolerance to brown rot in peach but further studies need to be done in order to determine this.

Infection or incidence, sporulation and dissemination make up the three major components of a fungal pathogen life cycle in a disease situation (Agrios, 2005). From the genetic point of view, lesion severity is a good parameter to consider in selection for breeding because though there is damage from the fungi, dispersion of pathogen is limited due to lack of sporulation in the incidence. But from the pathogen point of view colonization severity is a better option to be considered because there is the possibility of sporulation due to colonization which leads to spore dispersion within the environment for further damage.

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Conclusions

Optimizing protocols of the methodology to screen for tolerance in peach brown rot susceptibility in peach population

1. An efficient ex-situ procedure through artificial skin inoculation to assess the susceptibility to brown rot (*Monilinia laxa*) of commercially ripe peach fruits was established by modifying the existing protocols, shortening the inoculum production and also ensuring the accurate supply of inoculum. We used 625 spores of 25µL load / fruit, between 3-6 days of incubation, at temperature and relative humidity range of 20-26 ^{o}C and 40-60%, respectively.

2. This methodology was tested on four commercial cultivars ('Calante', 'Catherina', 'BigTop' and 'Venus') and six genotypes (descendants of three families) of peach and found possible to discriminate between highly and less susceptible peach germplasm at the EEAD, and subsequently applied in our breeding program.

The influence of acidity (pH and TA) on the subsistence and development of *M. laxa*, in both artificial media (*in-vitro*) and peach (*in-vivo*)

3. The study has shown that there is significant variation in the growth and sporulation capacities of *M. laxa* at different pH levels. The extent of mycelial growth or colonization in different pH levels does not actually correspond to the amount of sporulation capacity.

4. Nevertheless, pH and titratable acidity (TA) proved to possess great influence on the growth activity of *M. laxa* in a host-pathogen association both in solid PDA substrate and in peach fruit growth and development. Thus, pH 5.30 supported the highest sporulation while pH 6.40 encouraged the highest colonization extent or mycelia growth. And as organic acids and genetic traits, pH and TA constitute imperative antibrown rot determining elements to be given adequate attention in peach breeding program.

The tolerance of commercial peach cultivars to brown rot by *Monilinia laxa* is modulated by its antioxidant content?

5. The result of evaluation of tolerance in the eight commercial cultivars to brown rot disease demonstrates variability in the genetic susceptibility to *Monilinia laxa*, with 'Andross' and 'Baby Gold 9' the more tolerant but not significantly different from 'Tebana' and 'Miraflores'. 'Crown Princess', 'Big Top' 'Calante' and 'Calanda Tardío' were the less tolerant peach cultivars.

6. The content of ascorbic acid can only partially explain the tolerance-susceptibility observed, indicating that other factors are probably involved in the response.

7. To our knowledge this is the first study that correlates the ascorbic acid contents in mature peach fruit with *Monilinia laxa* brown rot tolerance. From a practical point of view application of ascorbate based formulae may be promising.

Breeding strategies for identifying superior peach genotypes tolerant to brown rot

8. Based on our three years screening protocol, we have identified and selected six genotypes (BC1, BC48, BC58, BC63, BC67, and BC68) of low brown rot susceptibility and high fruit quality within the population of 'Babygold 9' × 'Crown Princess' progeny in the germplasm of the Experimental Station of Aula Dei-CSIC.

9. Though we have found genotypes that possess bioactive compounds as total phenolic, ascorbic acids, flavonoids and anthocyanin compounds associated to potential brown rot tolerance, not all with lesion severity less than 40 mm contained the highest bioactive contents. BC1 and BC67 had significantly higher contents for ascorbic, total phenolics and anthocyanins. However, flavonoid contents were not significantly different in the tolerant compared to non-tolerant genotypes.

10. The progeny BC1 and BC67 had significantly higher contents for ascorbic, total phenolics and anthocyanins. However, flavonoid contents were not significantly different in the tolerant compared to non-tolerant genotypes.

11. The inverse correlations observed between anthocyanin and brown rot severity highlight their potential effect on tolerance to *M. laxa*. The selection of genotypes with bioactive contents in peach breeding in combination with brown rot tolerance may avoid dramatic consequences in commodities safe alternative to control measures, via inherence health-enhancing properties and good postharvest performance.

12. The identification and characterization of peach genotypes of the 'Baby gold' x 'Crown Princess' tolerant to *M. laxa*, makes available parental for new improvement and breeding programs, and initial material to carry out agronomic trials in different localities for their transfer to the industrial sector. Interestingly, this is the first work to study tolerance / susceptibility to *M. laxa* in this population.

Optimización de los protocolos de la metodología para detectar la tolerancia en la susceptibilidad a la podredumbre parda del melocotón en la población de melocotonero

1. Un procedimiento *ex-situ* eficiente a través de la inoculación artificial de la piel para evaluar la susceptibilidad a la podredumbre parda (*Monilinia laxa*) de melocotones madurados se estableció modificando los protocolos existentes, acortando la producción del inóculo y asegurando también el suministro preciso del inóculo. Se utilizó 625 esporas de 25 µL de carga / fruto, entre 3-6 días de incubación, a una temperatura y rango de humedad relativa de 20-26 ^{o}C y 40-60%, respectivamente.

2. Esta metodología fue probada en cuatro cultivares comerciales ('Calante', 'Catherina', 'BigTop' y 'Venus') y seis genotipos (descendientes de tres familias) de melocotonero y se encontró la posibilidad de discriminar entre germoplasma de melocotón altamente y menos susceptible en el EEAD, y posteriormente se aplicó en nuestro programa de mejora genética.

La influencia de la acidez (pH y TA) en la subsistencia y el desarrollo de *M. laxa*, tanto en medios artificiales *(in-vitro*) como en melocotoneros *(in-vivo*).

3. El estudio ha demostrado que hay una variación significativa en las capacidades de crecimiento y esporulación de *M. laxa* a diferentes niveles de pH. El grado de crecimiento micelial o colonización en diferentes niveles de pH en realidad no se corresponde con la cantidad de capacidad de esporulación.

4. Sin embargo, el pH y la acidez titulable (AT) demostraron tener una gran influencia en la actividad de crecimiento de *M. laxa* en una asociación huésped-patógeno tanto en sustrato de PDA sólido como en el crecimiento y desarrollo de melocotón. Por lo tanto, el pH 5,30 soportó la mayor esporulación mientras que el pH 6,40 estimuló la mayor extensión de colonización o crecimiento de micelios. Y como ácidos orgánicos y rasgos genéticos, el pH y la TA constituyen elementos determinantes de la podredumbre antibrown imperativa para recibir la atención adecuada en el programa de mejora de melocotoneros.

La tolerancia de los cultivares comerciales de melocotonero a la podredumbre parda por *Monilinia laxa* está modulada por su contenido de antioxidantes?

5. El resultado de la evaluación de la tolerancia en los ocho cultivares comerciales a la enfermedad de podredumbre parda demuestra la variabilidad en la susceptibilidad genética a *Monilinia laxa*, con 'Andross' y 'Babygold 9' el más tolerante pero no significativamente diferente de 'Tebana' y ' Miraflores'. 'Crown Princess', 'Big Top' 'Calante' y 'Calanda Tardío' fueron los cultivares de melocotoneros menos tolerantes.

6. El contenido de ácido ascórbico solo puede explicar parcialmente la tolerancia-susceptibilidad observada, lo que indica que probablemente intervienen otros factores en la respuesta.

7. Hasta donde sabemos, este es el primer estudio que correlaciona el contenido de ácido ascórbico en melocotones maduros con tolerancia a la podredumbre parda de *Monilinia laxa*. Desde un punto de vista práctico, la aplicación de fórmulas basadas en ascorbato puede ser prometedora.

Estrategias de mejora genética para identificar genotipos superiores de melocotonero tolerantes a la podredumbre parda

8. Con nuestro protocolo de evaluación de tres años, hemos identificado y seleccionado seis genotipos (BC1, BC48, BC58, BC63, BC67 y BC68) de baja susceptibilidad a la podredumbre parda y alta calidad del fruto dentro de la población de 'Babygold 9' × 'Crown Princess' en el germoplasma de la estación experimental de Aula Dei-CSIC.

9. Aunque hemos encontrado genotipos que poseen compuestos bioactivos como fenólicos totales, ácidos ascórbicos, flavonoides y compuestos de antocianinas asociados a la posible tolerancia a la podredumbre parda, no todos con una severidad de lesión inferior a 40 mm contenían los contenidos bioactivos más altos. BC1 y BC67 tenían contenidos significativamente más altos para ascórbicos, compuestos fenólicos totales y antocianinas. Sin embargo, los contenidos de flavonoides no fueron significativamente diferentes en los genotipos tolerantes en comparación con los no tolerantes.

10. La progenie BC1 y BC67 tenían contenidos significativamente más altos para ascórbicos, compuestos fenólicos totales y antocianinas. Sin embargo, los contenidos de flavonoides no fueron significativamente diferentes en los genotipos tolerantes en comparación con los no tolerantes.

11. Las correlaciones inversas observadas entre la antocianina y la severidad de la podredumbre parda muestran su efecto potencial sobre la tolerancia a *M. laxa*. La selección de genotipos con contenido bioactivo en la mejora genéticas de melocotoneros en combinación con la tolerancia a la podredumbre parda puede evitar consecuencias dramáticas en productos alternativos seguros a las medidas de control, a través de propiedades saludables que mejoran la salud y un buen rendimiento poscosecha a fin.

12. La identificación y caracterización de los genotipos de melocotonero del 'Babygold' × 'Crown Princess' tolerantes a *M. laxa,* se pone a disposición de los padres nuevos programas de mejoramiento y mejoramiento, y material inicial para llevar a cabo ensayos agronómicos en diferentes localidades para su transferencia a la sector industrial. Curiosamente, este es el primer trabajo para estudiar la tolerancia / susceptibilidad a *M. laxa* en esta población.