Towards an integrated control of peach powdery mildew (*Podosphaera pannosa*) through the application of molecular tools in epidemiological and genetic resistance studies

Neus Marimon de María

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Doctoral thesis

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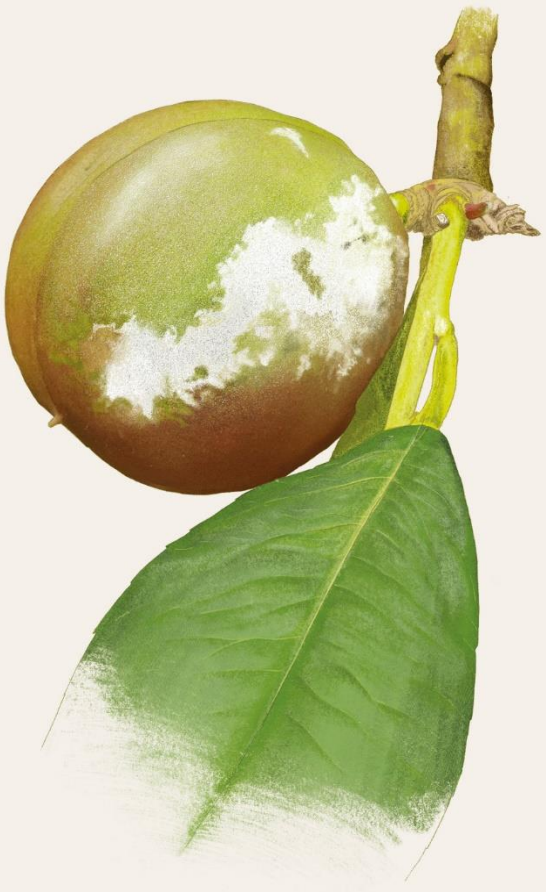

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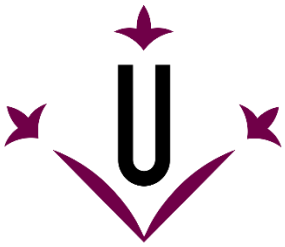

Universitat de Lleida

TESI DOCTORAL

Towards an integrated control of peach powdery mildew (*Podosphaera pannosa*) through the application of molecular tools in epidemiological and genetic resistance studies

Neus Marimon de María

Memòria presentada per optar al grau de Doctora per la Universitat de Lleida

Programa de **Doctorat en Ciència i Tecnologia Agrària i Alimentària**

Directors

Dr. Jordi Luque i Font

Dr. Iban Eduardo Muñoz

Tutor

Dr. Joan Segarra Bofarull

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List of abbreviations

ADD – Accumulated degree-days

AFLP – Amplified fragment length polymorphism

BAM – Compressed SAM (Sequence Alignment Map)

bp – Base pair

cDNA – Complementary DNA

CRAG – Center for Research in Agricultural Genomics

CS – Conidial suspension

CTAB – Cetrimonium bromide

DNA – Deoxyribonucleic acid

dNTPs – Deoxy-Nucleoside triphospate

g – Gram

G – Linkage group

GC – Guanine-cytosine

ha – Hectare

HPLC – High performance liquid chromatography

Indels – Insertion/deletion polymorphism

IL – Introgression line

ITS – Internal transcribed spacer

IRTA – Institut de Recerca i Tecnologia Agroalimentàries

kb – Kilobase pairs

kg – Kilogram

L – Liter

LOB – Limit of blank

LOD – Limit of detection

LOQ – Limit of quantification

µ – Micro- (10^{-6})

M – Mega / million (10^{6})/ Molar [concentration]

MAI – Marker-assisted introgression

MAS – Marker-assisted selection

Mbp – Mega base pairs

NIL – Near isogenic line

°C – Celsius degrees

PCR – Polymerase chain reaction

Pp – Peach chromosome

PPM – Peach powdery mildew

qPCR – Real time quantitative polymerase chain reaction

QTL – Quantitative trait locus

QTLs – Quantitative trait loci

rDNA – Ribosomal DNA

RFLP – Restriction fragment polymorphism

RNA – Ribonucleic acid

SAM – Sequence alignment map

SNP – Single nucleotide polymorphism

SSR – Single sequence repeats

T×E – ‘Texas’ × ‘Earlygold’ population

T1E – (‘Texas’ × ‘Earlygold’) × ‘Earlygold’ population

U – Enzyme International Unit

Abstract

Peach powdery mildew (PPM), caused by the ascomycete fungus *Podosphaera pannosa*, is one of the major diseases of peach, which may cause significant decreases in yield and fruit quality. Powdery mildew is currently managed through calendar-based fungicide applications. Adverse effects resulting from pesticide applications on human health and the environment have raised a great social awareness, which has driven the development of new integrated strategies more respectful to both environment and human health status. These disease management strategies are strongly depending on a deep knowledge of key aspects of the disease, which includes the pathogen and the host, and their relationship with the environment.

In this thesis, the influence of some environmental variables on the disease progress of PPM have been studied. Furthermore, a strategy was designed to delay the onset of fungicide applications (**Chapter 3**). Specifically, the delayed initiation of fungicide programs at 220 accumulated degree-days after flowering reduced the number of fungicide applications by 33% while keeping effectiveness in the disease control. Several traits related to the latency of the pathogen primary inoculum and the aerobiology of the pathogen propagules during the infectious period were also studied (**Chapter 4**). Thus, by using molecular techniques, it was confirmed that the primary inoculum is mainly present as overwintering mycelium on the surface of the affected twigs. The detection and quantification of airborne *P. pannosa* propagules during the growing season confirmed that those propagules can be detected from April to July.

The development of resistant cultivars is considered an alternative to disease control that is only based on fungicide treatments. In this thesis, the resistance gene *Vr3*, inherited from almond, was characterized (**Chapter 5**). We were able to locate it in a very specific region of the genome spanning 27 candidate genes. Through the expression analysis of candidate genes and an analysis of polymorphisms from parental resequences, it was concluded that the *RGA2* resistance gene could be the best *Vr3* candidate gene, assuming that a future functional validation is still required. Finally, in order to obtain resistant varieties to *P. pannosa*, the *Vr3* resistance is currently being introgressed into high-quality peach varieties (**Chapter 6**), by crossing individuals with one or two almond introgressions which included the *Vr3* gene.

Resum

La malaltia de l'oïdi o cendrosa del presseguer, causada pel fong ascomicet *Podosphaera pannosa*, pot arribar a causar pèrdues greus en la producció i en la qualitat del fruit. Actualment, el control de l'oïdi es basa en aplicacions periòdiques de fungicides. Els efectes derivats de l'ús d'aquests productes en la salut humana i en el medi ambient han provocat una gran conscienciació de la societat, tot afavorint el desenvolupament de noves estratègies basades en el maneig integrat de plagues i malalties. Aquestes estratègies han de comptar necessàriament amb un coneixement profund dels agents implicats en la malaltia; és a dir, del patogen, de l'hoste i de les condicions ambientals.

En aquesta tesi doctoral s'han estudiat algunes variables ambientals que descriuen el progrés de la malaltia de l'oïdi i s'ha dissenyat una estratègia per a retardar l'inici de l'aplicació dels tractaments fitosanitaris (**Capítol 3**). En concret, l'inici retardat del programa de protecció, a partir dels 220 graus-dia acumulats després de la floració, ha permès reduir el nombre de tractaments fitosanitaris en un 33%, sense perdre eficàcia en el control de la malaltia. També s'han estudiat aspectes relacionats amb l'inòcul primari de *P. pannosa* i l'aerobiologia del fong durant l'època infectiva (**Capítol 4).** Mitjançant tècniques moleculars, s'ha confirmat que l'inòcul primari es troba principalment en forma de miceli hivernant en la superfície dels branquillons afectats. També s'ha pogut quantificar els propàguls de *P. pannosa* presents en l'aire durant el període infectiu, d'abril a juliol.

Una alternativa al control de l'oïdi que requereix més temps en la seva aplicació, però que evitaria el tractament amb fungicides, és el desenvolupament de cultivars resistents de presseguer. En aquesta tesi s'ha caracteritzat el gen de resistència *Vr3*, procedent de l'ametller (**Capítol 5**). Ha estat possible localitzar-lo en una regió molt específica del genoma, que inclou 27 gens candidats a conferir la resistència. Mitjançant una anàlisi d'expressió dels gens candidats i l'anàlisi dels polimorfismes de les reseqüències dels parentals, s'ha pogut determinar que el gen de resistència *RGA2* podria ser el gen candidat *Vr3*, a falta d'una futura validació funcional. Finalment, i per tal d'obtenir noves varietats resistents a *P. pannosa*, s'ha introgressat la resistència *Vr3* en cultivars comercials de presseguer (**Capítol 6**), mitjançant els creuaments d'individus amb una o dues introgressions d'ametller que incloïen el gen *Vr3*.

Resumen

El oídio del melocotonero, enfermedad debida al hongo ascomiceto *Podosphaera pannosa*, puede llegar a causar pérdidas graves en la producción y en la calidad del fruto. Actualmente, el control del oídio se basa en aplicaciones periódicas de fungicidas. Los efectos derivados del uso de estos productos en la salud humana y en el medio ambiente han tenido como consecuencia una mayor concienciación social, lo que ha llevado al desarrollo de nuevas estrategias basadas en el control integrado de plagas y enfermedades. Este tipo de estrategias deben contar con un conocimiento profundo de los agentes implicados en la enfermedad; esto es, del patógeno, el huésped y el ambiente.

En esta tesis doctoral se han estudiado algunas variables ambientales que describen el progreso de la enfermedad del oídio y se ha diseñado una estrategia para retardar el inicio de las aplicaciones fitosanitarias (**Capítulo 3**). En concreto, el retardo en el inicio del programa de protección, a partir de los 220 grados-día acumulados después de la floración, ha permitido reducir hasta un 33% el número de tratamientos fitosanitarios sin perder la eficacia en el control de la enfermedad. También se ha estudiado el inóculo primario de *P. pannosa* y la aerobiología del mismo durante la época infectiva (**Capítulo 4**). Mediante el uso de técnicas moleculares se ha confirmado que el inóculo primario se encuentra principalmente en forma de micelio latente en la superficie de las ramillas afectadas. También se ha logrado cuantificar los propágulos de *P. pannosa* presentes en el aire durante el período infectivo, que se extiende principalmente de abril a julio.

Una técnica alternativa en el control de la enfermedad, que requiere más tiempo en su aplicación pero que puede evitar el uso de fungicidas, es el desarrollo de cultivares resistentes de melocotonero. En esta tesis se ha caracterizado el gen de resistencia *Vr3* procedente del almendro (**Capítulo 5**). Ha sido posible localizarlo en una región muy específica del genoma, que incluye 27 genes candidatos. Mediante un análisis de expresión de los genes candidatos y un análisis de los polimorfismos de las resecuencias de los parentales, se ha podido determinar que el gen de resistencia *RGA2* podría ser el gen candidato *Vr3*, aunque ello depende de una futura validación funcional. Finalmente, y con el fin de obtener nuevas variedades resistentes a *P. pannosa*, se ha introgresado la resistencia *Vr3* en cultivares comerciales de melocotonero (**Capítulo 6**), mediante el cruce de individuos con una o dos introgresiones de almendro que incluían dicho gen.

1. Introduction

1. Introduction

1.1. The peach

1.1.1. Botanical aspects

Peach, *Prunus persica* (L.) Batsch, is a tree species belonging to the *Prunus* L. genus, which includes other several important stone fruit crop species such as almond (*P. dulcis* (Mill.) D. A. Webb), apricot (*P. armeniaca* L.), cherry (*P. avium* L.), and plum (*P. domestica* (L.) (Potter, 2012). *Prunus* belongs to the *Rosaceae* family, which besides *Prunus* includes over 200 species of deciduous and evergreen trees, shrubs and herbs (Chin et al., 2014), some of them with agricultural interest such as apple (*Malus domestica* Borkh.), pear (*Pyrus communis* L.), rose (*Rosa* spp.), and strawberry (*Fragaria* ×*ananassa* Duch.). *Prunus* is classified within the subfamily *Amygdaloideae* (Juss.) Arn. (Potter et al., 2007) and included in tribe *Amygdaleae* Juss. Infrageneric classification of *Prunus*, as proposed by Rehder (1940), is still widely accepted. Thus, peach resides with almond within the *Amygdalus* subgenus.

Peach is a vigorous deciduous tree species with a root system developed within the first 50 to 60 cm depth, depending on the type of soil. Nevertheless, in commercial orchards is usually grafted using rootstocks from peach hybrids or other compatible *Prunus* species (Monet and Bassi, 2008). Peach trees can live up to 20-30 years, but commercial plantations are mostly limited up to half of this period due to a reduction in the productivity along time (Monet and Bassi, 2008). The development of new shoots and leaves usually occurs after bloom. Shoots are reddish-green when emerging and turn to grey-silver with time. Lanceolate leaves (5-12(15) x 2-4 cm) (Fig. 1.1) are flat or wavy in shape, with two deciduous stipules and sometimes showing slightly serrated margins (Blanca and Díaz de la Guardia, 1998). The leaves emerge from buds distributed in alternate nodes along the branches. Three buds per node can be usually found: a middle-placed vegetative bud surrounded by two flower buds. Sometimes, up to four or five buds can be found in a node (Bassi and Monet, 2008).

Figure 1.1. Drawing of half of a peach fruit and leaves from a young shoot. (Drawings by Quim Pallarès, 2020).

Peach fruit is a drupe (Fig. 1.1), from 40 to 90 mm in size (Blanca and Díaz de la Guardia, 1998), and a weight range from 80-110 g to over 680 g (Bassi and Monet, 2008). The endocarp, with a round or flat shape, is lignified and it contains one or two seeds. Seeds germination rate in fruit stone species is variable, depending on the genotype, seed size and environment conditions (Malcom et al., 2003).

Depending on the cultivar, a certain number of hours below 7 ºC (chilling) are required in winter for the flower buds to later achieve a normal blooming. After the chilling takes place, a certain accumulation of degree-days is needed to bloom (Okie, 1998). Normally, chill requirements are variable among cultivars and can range between 50 to 1500 chilling units, as described by Richardson et al. (1974) using the 'Utah model' (Bassi and Monet, 2008). The final selection of cultivars for a given area is usually highly dependent on the average chilling requirements.

1.1.2. Peach production

Peach is considered a typical crop of the temperate and subtropical regions, as it is adapted to various ecological conditions around the world. This characteristic allowed

peach to be adapted to different areas, those including humid, cool, subtropical, and even very dry climates (Faust and Timon, 1995). In 2018, the area grown for peach in the world was 1.7 M ha, which yielded a production of 24.4 M tonnes (Mt) (Table 1.1) (FAOSTAT, 2020). Asia accounts for 74% of the world production, while China stands for the world largest peach producer, with around 15.5 Mt in 2018 (FAOSTAT, 2020). Peach production in Europe is about 14% of the world total production. Main producer countries in Europe are located in the Mediterranean Basin, such as Italy (1.1 Mt), Greece (1.0 Mt) and Spain (0.9 Mt) (FAOSTAT, 2020). In 2017, Spain was the major exporter country of peach in the world, with a value nearly 1 billion US $ (FAOSTAT, 2020).

Peach cultivated area in Spain was around 50,000 ha in 2018 (FAOSTAT, 2020). This area has remained stable over the past decade but an increase of production around 30% has been recorded in the last twenty years (FAOSTAT, 2020). Occasional decreases in production have been recorded, e.g. 10% in 2018, probably due to inappropriate meteorological conditions such as persistent rainfall and frost (MAPA, 2019). In 2019, peach cultivated area was similar in relation to previous years; while it slightly decreased for nectarine; however, mean whole peach production increased around 11% (MAPA, 2019). In Spain, main peach cultivated areas are located in the Ebro river valley, being Aragon and Catalonia the two most important regions in terms of productivity, with

Table 1.1. Main world countries producing peach and nectarine in 2018 (FAOSTAT, 2020).

Country	Area harvested (ha)	Production (t)	Yield (hg/ha)
China	824,253	15,195,291	184,352
Italy	61,897	1,090,678	176,209
Greece	42,650	968,720	227,132
Spain	49,868	903,809	181,240
Turkey	46,361	789,457	170,285
USA	35,815	700,350	195,547
Iran	49,318	645,499	130,886
Chile	15,755	319,047	202,507
India	38,547	278,417	72,227
Egypt	20,341	246,742	121,303
Argentina	11,700	226,000	193,162
Brazil	17,605	219,598	124,736
Korea	18,184	205,742	113,141
Algeria	19,005	190,420	100,194
France	9,096	184,064	202,357

around 430 and 409 thousand of tonnes, respectively (MAPA, 2019). Specifically, peach represents 47% of cultivated fresh fruit tree species in Catalonia.

Several traits are used to characterize peach cultivars (Byrne et al., 2012): fruit size, shape (round or flat), epidermis surface (fuzzy for peach and glabrous for nectarine), flesh color (yellow or white, with different patterns of reddish flesh), stone adherence (freestone and clingstone), flesh texture (melting and non-melting), and flavor (acid and sub-acid). In Catalonia, different fruit morphologies of peach are cultivated, as follows: 40% round nectarines, 25% flat peaches, 24% of round peaches and 11% including less abundant clingstone peaches and flat nectarines (DARP, 2020). Mostly, peach orchards are located in Lleida province, accounting for nearly 93% of the Catalonian production (DARP, 2020).

The average annual consumption of peach in Spain is around 300 thousand tonnes during the last five years. Nevertheless, peach consumption in Spain is decreasing as also occurs in other European countries and the USA (Llácer et al., 2012). Peach annual consumption per person in Spain is 3.07 kg, which is lower than in other European countries, with a mean peach annual consumption of 6.98 kg per person (MAPA, 2019).

Peaches have a short shelf life, only 2-3 days at 25 to 32 ºC (Du et al., 2017). Nevertheless, shelf-life could be increased by previously placing fruits on cold-storage and through the application of pre-storage treatments. One of the major concerns about fruit storage is the preservation of fruit firmness; some nectarine cultivars like ‘Big Top’ could preserve fruit firmness up to 40 days through cold storage, hence obtaining consumer acceptance (Cano, 2012). Besides fruit preservation, other peach production handicap is the short availability of varieties along the season. As each cultivar is available during a short production season, the production of multiple cultivars is required to provide fruit from April to October in many areas and conditions (Iglesias and Casals, 2015). In addition, final consumer preferences, which are constantly evolving, demand peach market to be in constant adaptation (Iglesias and Casals, 2015).

1.1.3. Peach origin, distribution and domestication

Peach is a species native from China (Faust and Timon, 1995), where it has been cultivated for more than four millennia (Wang, 1985). Yu et al. (2018) described peach origin in the Southwest region of China, at the humid environment originated in the lowland glacial refuges at the Tibetan Plateau about 2.47 Mya. Fruit fossils from that age showed the same endocarp characteristics as modern peach cultivars, and it is suggested that fleshy mesocarp appeared much before domestication, probably due to frugivorous primates (Yu et al., 2018).

Peach was spread from Asia through the ancient trade routes, e.g. the Ancient Silk Road, between the East and the West. It would have reached Europe around the second or first century BC from Persia (current Islamic Republic of Iran) (Monet and Bassi, 2008). It seems that peach arrived in Europe before the Christian era through the Balkan route to France and Italy. Once in Spain, peach was brought to Central America in the first half of the XVIth century. It has been reported several additional arrivals to America from China that occurred in the mid-1850s (Monet and Bassi, 2008).

Genetic variability studies of many peach accessions from China and Europe, and closely related *Prunus* species have been performed using single nucleotid polymorphisms (SNP) arrays (Akagi et al., 2016; Micheletti et al., 2015). One of the conclusions of these studies (Micheletti et al., 2015) was the description of three main clusters: (i) Eastern peach accessions which represent the origin of peach in China and which include different landraces and the current wild peaches originated in a first general domestication (Akagi et al., 2016; Faust and Timon, 1995), (ii) Western accessions including traditional European pre-breeding accessions, coinciding with materials that were seed propagated from Central Asia towards Europe, that were mostly non-melting and highly homozygous peaches, and (iii) new Western accessions derived from a few founders used at early XXth century by U.S. breeding programs (Aranzana et al., 2003; Li et al., 2013; Micheletti et al., 2015; Scorza et al., 1985). These founders included a few accessions coming directly from China and other taken to America by European settlers. Aranzana et al. (2010) described that these founders were genetically related to most of current melting-flesh cultivars. Within this group, it has been described that peaches and nectarines breeding approaches have been performed independently (Akagi et al., 2016; Aranzana et al., 2010; Micheletti et al., 2015), so they are currently considered as a

subdivision within the Western populations. All these fruit cultivars, together with those for ornamental usage, were clearly derived from Asian landraces (Akagi et al., 2016). These landraces include a clade of eastern Asian cultivars, coinciding with the origin in China, and are related with some wild species, such as *P. davidiana* and *P. mira*.

Recently, studies on the genome sequencing of some peach accessions have been conducted to clarify the specific processes of peach domestication and improvement (Cao et al., 2019; Li et al., 2019; Yu et al., 2018). It was concluded that fruit size was probably the first trait selected in peach domestication (Li et al., 2019; Yu et al., 2018), which occurred more than 5,000 years ago in China (Cao et al., 2016). Other traits related to seed and fruit taste were selected in that period (Cao et al., 2019; Li et al., 2019), as well as sugar content and flesh softening (Akagi et al., 2016), and adaptation to low-chill regions (Li et al., 2019) and self-compatibility (Yu et al., 2018). These traits were strongly selected in domestication process and are related to specific regions of peach genome. For fruit size and maturity date, several quantitative trait loci (QTLs) were detected in chromosome 4 (Quilot-Turion et al., 2004). Evidence for selection was also observed in chromosome 6, which contains a genomic region controlling for self-compatibility (Akagi et al., 2016). These features represent an important first bottleneck for peach genome variability (Cao et al., 2016), as they resulted in a low overall variability in peach as compared to other *Prunus* species (Li et al., 2013; Scorza et al., 1985). A second genetic bottleneck occurred at the beginning of modern breeding activities conducted in USA and Europe (Micheletti et al., 2015), and was corroborated by Aranzana et al. (2010) by studying the genetic distances among peach populations from Europe and North America. Fruit-taste related QTLs were selected mainly throughout this improvement process, leading to a decline in genetic diversity related to fruit taste (Li et al., 2019).

1.1.4. Peach breeding

Plant breeding is driven by the improvement of desirable characteristics in plant products for both the market and industry, which in turn are based on customers' preferences. First breeding programs were started at the beginning of XXth century in USA using a few cultivars (Monet and Bassi, 2008). Traditional peach breeding programs have focused on tree- and fruit-related traits, e.g. tree architecture and productivity,

fruiting season length, adaptation to chill requirements, fruit size, skin color, fruit shelf-life, and resistance to biotic and abiotic stresses (Bielenberg et al., 2009; Byrne, 2005; Byrne et al., 2012; Eduardo et al., 2014; Yu et al., 2018). Nowadays, fruit quality is fundamental for the acceptance of consumers, and thus the whole chemical and physical fruit characteristics are considered in breeding programs (Cantín et al., 2010).

Breeding for new peach cultivars promotes a progressive substitution of varieties and the establishment of a continuous provisioning of new peach cultivars into the market, including different fruit morphologies and flesh and skin colors. In the last two decades, several new cultivars with 'Big Top'-like fruit traits has been released (Iglesias and Casals, 2015). These traits include sweet-flavored and firm flesh fruits. In addition, numerous peach breeding programs around the world are conducted to ensure the availability of cultivars during the whole peach season and its adaptation to constantly-evolving consumer and market needs. In 2013 there were described over 70 peach breeding programs in the world (Reig, 2013). Most of them were established in U.S.A, from which 52% of new peach cultivars were released; 30% of new cultivars were released from Europe (mostly from Italy and France, and in a less proportion from Spain), and the remaining new cultivars coming from South Africa, Australia, China, Japan, Mexico and Brazil (Byrne, 2002; Reig, 2013). More than 1,000 new cultivars have been released from 1970 to date, and from the beginning of XXIth century, an average of 100 new cultivars are being released every year (Badenes et al., 2006).

In Spain, as described by Iglesias and Chacón (2018), the most significative innovation in the last decade has been that the origin of new cultivars is increasingly made by Spanish breeding programs, most of them initiated from the last two decades. More than 30% of new cultivars introduced in the market in the last five years come from more than ten spanish breeding programs. Regarding these breeding programs, mostly are private (Provedo, Fruitaria-ALM, PBS Producción Vegetal, Proseplan, Planasa, Selection Plants Sevilla (SPS)), and other are public (CITA, IVIA) or with public and private participation (IMIDA-NOVAMED, IRTA-ASF-FruitFutur). New cultivars obtained from Spanish breeding programs currently already represent 36% of the cultivars evaluated at IRTA, being a larger proportion that ones from US (33%) or France (20%) (Iglesias, 2016).

As peach cultivars are tolerant to inbreeding (Aranzana et al., 2012), peach breeding programs are based on sequential cycles of crosses among peach cultivars with desired traits and the further identification and selection of the best phenotypes from the variants obtained. Interspecific crosses have been attempted mostly for the development of new rootstock cultivars, or when the desired trait is absent or poorly expressed in peach species (Scorza and Okie, 1990). Interspecific breeding confers an opportunity to introduce new genetic variability into peach genome (Byrne, 1990; Yu et al., 2018), thus improving peach agronomical traits of interest. However, the genes controlling for desired traits may be closely linked with those of undesirable traits and may require several generations to remove them. Further, peach tree size and juvenility period (from two up to four years) increase the time required to phenotype some traits, and therefore are considered limiting factors in peach classical breeding. The identification of traits that are depending on variable environmental conditions, including disease and pest occurrence that can be phenotyped only when favorable conditions are met, is also an important constrain in *Rosaceae* breeding (Ru et al., 2015). In order to overcome all those constrains, strategies based on the use of molecular markers, as detailed below, could be followed to save economical and time costs in peach breeding activities (Dirlewanger et al., 2004; Eduardo et al., 2015).

1.1.5. Marker-assisted breeding

Marker-assisted breeding (MAB) involves all the strategies and methods available that use different types of markers for a breeding purpose. Markers known as classical or morphological markers were the first used in traditional breeding approaches and include some traits as flower color or seed shape (Collard et al., 2005). These morphological markers, rarely segregated in natural populations and studying them was complex from their limited availability (Arús, 2017).

A substantial improvement was achieved in 1965, with the application of isozymes as markers for plant breeding. Isozymes are dominant markers that allowed the comparison between different populations and species, but its number was very limited. Later appeared the first markers based on the DNA variation. The first ones were the restriction fragment length polymorphisms (RFLPs) developed at the beginning of 1980s.

RFLP markers were codominant markers with time-consuming protocols (Arús, 2017; Staub et al., 1996), but that were able to identify many variants along the genome, were transferable among species, and allowed the construction of the firsts saturated genomic maps (Dirlewanger et al., 2004). In 1990 other markers as random amplified polymorphic DNAs (RAPDs) were developed based on polymerase chain reaction (PCR). The main inconvenient for RAPDs were their dominance and the low levels of reproducibility. In the mid-1990s, amplified fragment length polymorphisms (AFLPs) were developed, combining enzyme restriction used for RFLPs and PCR. Detection is performed through the combination of two restriction enzymes and then the fragment derived is amplified through PCR. AFLPs have a high degree of reproducibility and availability (Jones et al., 1997), but the main handicap resided in their dominance, being impossible to differentiate among homozygous and heterozygous individuals.

Currently the main type of molecular markers being used in peach breeding applications are based on DNA polymorphisms and include Microsatellites or simple sequence repeats (SSRs), which are highly conserved tandem repetitions of motifs from 1 to 6 nucleotides and present in all the organisms (Zane et al., 2002), and SNPs which detect single nucleotide substitutions (Arús, 2017). The use of molecular markers, as compared to morphological and biochemical markers, is considered more convenient considering that i) DNA polymorphisms exist all over the genome, ii) the genotyping results are reproducible and not influenced by the environment or intergenic interactions (non-epistatic) (Collard et al., 2005; Tanksley et al., 1989), iii) can be codominant, thus allowing to discriminate between heterozygous and homozygous genotypes, and iv) are currently affordable and the analysis time required is progressively decreasing.

Molecular markers have been used to develop several saturated genetic maps for *Prunus*, and more than 30 maps have been developed in different populations, including 'T × E' map, from almond 'Texas' and peach 'Earlygold' (Arús et al., 2012). Considering that genomes from different *Prunus* species are highly conserved, and that SSR (and also RFLP) were codominant and transferable among species, it was possible to integrate the data from different linkage maps in a reference linkage map (Dirlewanger et al., 2004). The *Prunus* reference map included the position of 28 major genes affecting different agronomic traits (Dirlewanger et al., 2004).

The release of the reference peach genome in 2010 (Verde et al., 2013) was another important step to decipher the genetic basis of the peach agronomic traits. The genome of the peach cultivar 'Lovell' was the first free available genome within the *Rosaceae* family (Aranzana et al., 2019). In 2017, the version 2.0 of *P. persica* reference genome was assembled (Verde et al., 2017), using high-throughput DNA sequencing and high-density linkage maps and (Aranzana et al., 2019). This version is considered a high-quality reference genome for *Prunus* (Aranzana et al., 2019). Genome sequence provide a template to which one or a set of resequences could be aligned, allowing the detection of a high number of markers, usually SNPs (Arús, 2017). In 2012, Verde et al. (2012) developed a 9K SNP array from 56 peach accessions, which cover the entire genome. More recently, millions of SNPs were obtained through genome-wide sequencing of 480 peach accessions. These data enabled to perform analysis of different agronomic traits to decipher its origin and changes through domestication process (Li et al., 2019). This large set of markers facilitated the saturation of specific map regions, thus facilitating the implementation of fine mapping and marker-assisted selection approaches (Arús, 2017; Verde et al., 2012).

Marker-assisted selection (MAS) is the process based on the early selection of the individuals using molecular markers. The main advantages of MAS are the reduction of the phenotypic costs, the exclusion of genome and environment interactions in the evaluation, an increased selection precision and a reduction of the time required in a traditional evaluation process. Improvement in terms of time-consuming is especially important for traits that appear late on plant's life cycle, such as fruit traits in tree species (Byrne et al., 2012). Since it is difficult to obtain a marker specifically located in the genetic variant of the trait of interest, a feasible alternative is to develop markers keeping the lowest possible distance with the target region corresponding to the trait of interest. The use of molecular markers genetically linked to desirable traits enables to perform an early selection of the seedlings, much before the phenotyping could be performed by visual evaluation. This strategy has been incorporated as a routine very recently in some peach breeding programs (Eduardo et al., 2015).

Most of reviewed peach traits are quantitatively inherited and have been described as QTLs (Eduardo et al., 2011). Conversely to other *Prunus* species, where most of QTL studies were performed on intraspecific crosses, for peach were available also studies

performed with interspecific crosses (Salazar et al., 2014). Some examples in intraspecific populations from different peach accessions include fruit quality, agronomical and fruit quality traits (Abbott et al., 1998; Eduardo et al., 2011; Zeballos et al., 2015), rootstock characters (Abbott et al., 1998) and biotic resistances (Abbott et al., 1998; Dirlewanger et al., 1996). In addition, traits related with tree architecture, blooming and ripening time were described from interspecific populations with *Prunus ferganensis* (Kost. and Riab.) Kov. and Kost (Quarta et al., 2000), and total sugar content in fruit from interspecific populations from *Prunus davidiana* (Carr.) Franch (Quilot-Turion et al., 2004), among others. Arús et al. (2012) reviewed many quantitative traits for peach affecting morphological or agronomic characters that were on the *Prunus* reference map.

To date, more than 50 monogenic genes linked to interesting agronomical traits in different *Prunus* species have been mapped (Serra, 2017). However, only a few monogenic traits are currently used in peach breeding programs (Arús et al., 2012), which are related mainly to fruit traits: skin glabrourness (*G/g*) (Bliss et al., 2002; Dirlewanger et al. 1998, 1999;), fruit flesh color (yellow/white, *Y/y*) (Bliss et al., 2002), fruit shape (flat/round, *Sh/sh*) (Fan et al., 2010), and slow ripening (Meneses et al., 2016), stone adhesion (freestone/clingstone, *F/f*) (Gu et al., 2016; Yamamoto et al., 2001) and fruit acidity (Eduardo et al. 2014). Other peach major genes have also been described for flower traits like male sterility (*Ps*), leaf traits like evergrowing (annual/perennial) (*Evg*) (Wang et al., 2002), and plant structure traits as plant height (normal/dwarf) (*Dw*) (Yamamoto et al., 2001; Cantín et al., 2018). Markers associated to a major gene are relatively easier to obtain as only exist one or few causal alleles in one locus. Conversely, markers associated with QTLs are more challenging as more than one locus are involved. Thus, QTL-associated markers are difficult to identify and thus to integrate them into selection programs (Byrne et al., 2012).

MAS had a limited impact on peach breeding, and one of the possible causes for that was the low availability of efficient markers linked to characters of interest (Lambert et al., 2016). Nevertheless, recently high throughput screening markers as SNPs have reduced the economic cost of MAS application (Verde et al., 2012). Moreover, to bridge the gap among genomic and practical breeding approaches, some international projects have been implemented in the last decade on fruit tree breeding, as European FruitBreedomics (http://www.fruitbreedomics.com/) focused on apple and peach

cultivars, RosBREED (https://www.rosbreed.org/breeding) in USA for *Rosaceae* cultivars, or Freeclimb in the Mediterranean Basin cultivars as peach, almond, citrus, olive and grape. Therefore, in the last decades breeding programs are making a distinct effort to increase breeding efficiency facing agricultural challenges in different fruit tree species, including peach.

Other breeding strategies involving the use of molecular markers includes marker-assisted introgression (MAI), which is based on the introgression of alleles of interest from an exotic line into a genetic recurrent species, keeping its genome background as unaltered as possible. The aim of MAI is to increase variability in the recurrent species genome. An example of a MAI is peach is presented in Chapter 6 of this thesis. This method was firstly proposed by Tanksley et al. (1981) and established a feasible alternative to introgress genes of interest in a short period of time, compared with traditional approaches. Later, this strategy led to the development of near-isogenic lines (NILs), which constitute a valuable resource for dissection the genetic base of quantitative traits or QTLs. This strategy was adapted to fruit species by Serra et al. (2016) using almond as the exotic species and peach as the recurrent. The authors developed a strategy consisting in the obtention of individuals with a unique almond chromosome fragment in the peach background in only two generations after obtaining the interspecific hybrid. MAI strategy was initiated in 2006 obtaining a backcross 1 (BC1) progeny from 'Texas' almond and 'Earlygold' peach. Then, a reduced number of individuals containing few introgressions from almond were selected and phenotyped for many agronomic traits. In that phase, some QTLs and major genes could be mapped (Donoso et al., 2016). Those selected lines were further backcrossed or selfed to obtain lines with a single introgression from the almond donor into the peach genetic background. These lines are called near isogenic lines (NILs). In the Chapter 6 of this thesis some introgression lines were used to introgress the PPM resistance gene *Vr3* into elite parentals of the IRTA-ASF-FruitFutur peach breeding program.

1.1.6. Peach breeding for biotic resistance

The development of new resistant cultivars to pests and diseases through plant breeding could be a challenging option to respond to the consumers' concerns on

environmental and human health issues, as applications of chemical products could be reduced using resistant cultivars (Byrne, 2002; Pascal et al., 2010). Moreover, the potential increase in incidence of pests and diseases in peach crops due to climate change could be overcome using controlled crosses to introgress resistance (Llácer et al., 2012). In relation to biotic resistances in peach cultivar, many quantitative but only a few monogenic characters have been previously described (Byrne et al., 2012) (Table 1.2). Often these resistances are found in wild species or exotic germplasm with low fruit quality, therefore MAI is a very appropriate strategy to get rid of all the undesired traits in a fast an efficient way. Peach is affected by various pest and diseases that can differ across geographical locations. Some of the main pests and diseases include fungal diseases, such as brown rot (*Monilinia* spp.), powdery mildew *(Podosphaera pannosa* (Wallr.) de Bary), leaf curl (*Taphrina deformans* (Berk.) Tul.), and fungal gummosis (*Botryosphaeria dothidea* ((Moug.:Fr.) Ces. et De Not.); bacteria causing bacterial spot (*Xanthomonas campestris* pv. *pruni* (Smith) Vauterin, Hoste, Kersters and Swings); virus such as sharka (Plum Pox Virus, PPV); insects such as aphids (*Myzus persicae* Sulz.); and root-knot nematodes (*Meloidogyne* spp.). Previously described QTLs and resistance genes for these pests and diseases of peach are summarized in Table 1.2.

Table 1.2. Disease and pest resistance sources for peach cultivars.

Disease	Pathogen	Source of resistance	Type of inherintance	Gene name	LG	Reference
Brown rot	*Monilinia* spp.	Contender	quantitative	QTLs	2, 3, 4	Pacheco et al. (2014)
Brown rot	*Monilinia* spp.	Almond × peach (clone F8,1-42)	quantitative	QTLs	1, 4	Martínez-García et al. (2013)
Brown rot	*Monilinia* spp.	Texas (*P. dulcis*)	quantitative	QTLs	4	Baró-Montel et al. (2019)
Powdery mildew	*Podosphaera pannosa*	*P. ferganensis*	two loci	*Vr* and *Sr*	Not mapped	Dabov (1983)
Powdery mildew	*Podosphaera pannosa*	*P. davidiana* (clone P1908)	quantitative	QTLs	1, 2, 4, 6, 8	Foulongne et al. (2003)
Powdery mildew	*Podosphaera pannosa*	Pamirskij 5 (clone S 6146)	single dominant gene	*Vr2*	8	Pascal et al. (2017)
Powdery mildew	*Podosphaera pannosa*	Texas (*P. dulcis*)	single dominant gene	*Vr3*	2	Donoso et al. (2016)
Fungal gummosis	*Botryosphaeria dothidea*	Tardy Nonpareil *(P. dulcis)*	single dominant gene	*Botd8*	6 or 8	Mancero-Castillo et al. (2018)
Bacterial canker	*Pseudomonas syringae*	*P. armeniaca*	-	-	5, 6	Omrani et al. (2019)
Xanthomonas	*Xanthomonas arboricola* pv. *pruni*	Clayton (*P. persica*)	quantitative	QTLs	1, 4, 5, 6	Yang et al. (2013)
Sharka	Plum pox virus (PPV)	*P. davidiana* (clone P1908)	quantitative	QTLs	-	Pascal et al. (1998)
Sharka	Plum pox virus (PPV)	*P. davidiana* (clone P1908)	quantitative	QTLs	1, 2, 4, 5, 6, 7	Rubio et al. (2010)
Sharka	Plum pox virus (PPV)	Garrigues (*P. dulcis*)	-	-	-	Dehkordi et al. (2018)
Sharka	Plum pox virus (PPV)	Del Cid (*P. dulcis*)	-	-	-	Cirilli et al. (2016)
Green peach aphid	*Myzus persicae*	Rubira®	single dominant gene	*Rm2*	1	Lambert and Pascal (2011)
Green peach aphid	*Myzus persicae*	Weeping Flower Peach	single dominant gene	*Rm1*	1	Pascal et al. (2017)
Green peach aphid	*Myzus persicae*	*P. davidiana* (clone P1908)	quantitative	QTLs	1, 2, 3, 4, 5, 6, 8	Sauge et al. (2012)
Root-knot nematode	*Meloidogyne* spp.	Myrabalan (*P. cerasifera*)	single dominant gene	*Ma*	7	Claverie et al. (2011); Duval et al. (2019)
Root-knot nematode	*Meloidogyne* spp.	Almond × peach (clone GN22)	single dominant gene	R_{MiaNem}	2	Claverie et al. (2004)
Root-knot nematode	*Meloidogyne* spp.	Almond × peach (Nemared)	single dominant gene	*RMia*	2	Jáuregui (1998); Duval et al. (2019)
Root-knot nematode	*Meloidogyne* spp.	Alnem1 (*P. dulcis*)	single dominant gene	*RMja*	7	Van Ghelder et al. (2010); Duval et al. (2019)
Root-knot nematode	*Meloidogyne sp*	*P. kansuensis*	major gene	*PkMi*	2	Cao et al. (2014)
Root-knot nematode	*Meloidogyne sp*	*P. kansuensis*	single gene	-	Not mapped	Maquilan et al. (2018)

Several sources of complete resistance have been identified for peach powdery mildew, and some of them have already been mapped (Donoso et al., 2016; Pascal et al., 2017). The main resistance genes major genes for PPM resistance are *Vr3*, that has been identified in the linkage group G2 of the almond cultivar 'Texas' (Donoso et al., 2016), and *Vr1* and *Vr2* located in G8 of the peach rootstock 'Pamirskij 5' and 'Malo Konare', respectively (Lambert, 2018; Pascal et al., 2017). Other sources for peach powdery mildew tolerance have been described previously in some peach cross-compatible *Prunus* species, as in *Prunus davidiana* (Carrière) Franch., and *Prunus ferganensis* (Kostina and Rjabov) Y.Y. Yao. Regarding *P. davidiana*, several quantitative trait loci (QTLs) conferring resistance to PPM have been described, including two major QTLs in linkage groups G6 and G8 (Dirlewanger et al., 1996, Foulongne et al., 2003). Furthermore, two QTLs were detected in *P. ferganensis* as a source of PPM resistance in G7 and G8, although this could not be confirmed (Verde et al., 2002).

1.2.The peach powdery mildew: *Podosphaera pannosa*

1.2.1. Introduction to powdery mildews

Powdery mildew was firstly described by Theophrastus around 300 BC (Horst, 1983) and is one of the most widespread plant diseases caused by fungi. Powdery mildew fungi affect approximately 10,000 plant species of more than 1,600 genera, including all kind of plants but gymnosperms (Agrios, 2005). Powdery mildew fungi belong to Phylum Ascomycota, O. Erysiphales. The taxonomy of powdery mildew fungi has been extensively revised within the last two decades (Braun and Takamatsu, 2000; Saenz and Taylor, 1999; Takamatsu, 2013), mainly based on DNA sequence data. Previously, identification was based largely on teleomorph (sexual stage) morphology (Fig. 1.2). The morphological traits of chasmothecia, that were traditionally used to distinguish genera, are now used to distinguish species, whereas DNA sequence data and characteristics of the anamorph (asexual stage) are currently used to delineate genera (Glawe, 2008). Powdery mildew genera are currently grouped into five tribes, namely *Blumerieae*, *Cystotheceae*, *Erysipheae*, *Golovinomyceteae*, and *Phyllactinieae* (Glawe, 2008). Main genera of powdery mildews in terms of economic importance to agriculture are *Blumeria*, *Erysiphe*, *Leveillula*, and *Podosphaera* (Agrios, 2005). Nowadays, about 900 known species are included in 16 genera of powdery mildews (Braun and Cook, 2012; Takamatsu, 2013).

Powdery mildews are obligate plant pathogens (biotrophs) that cannot be isolated and maintained on synthetic culture media, as they must obtain nutrients exclusively from the living host plant tissues (Agrios, 2005). It has been proposed that biotrophism might appeared only once in powdery mildew ancestry and it has been retained ever since (Takamatsu, 2013). Most of powdery mildew genera are ectotrophic, i.e. they develop mycelia on the surface of plant organs and only the haustorium, a specially designed

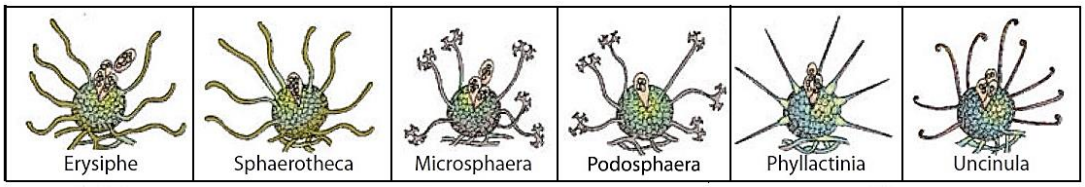

Figure 1.2. Traditional arrangement of powdery mildews genera according to the morphology of chasmothecia (Agrios, 2005).

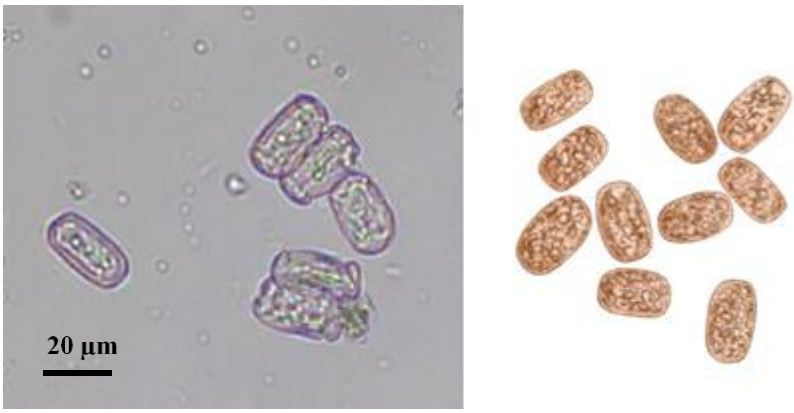

Figure 1.3. Conidia of *Podosphaera pannosa* showing fibrosine bodies (refractive bodies) from a peach infected leaf (left, microscope; right, drawing). (Drawing by Quim Pallarès, 2020.)

organ, gets inside the host epidermis cells of infected plants to obtain nutrients. Powdery mildew fungi commonly infect leaves, shoots, buds, flowers, and immature fruits of susceptible hosts. Mycelium on those plant parts is shown as white to greyish powdery spots on their surface, where conidiophores and chasmothecia fruiting bodies are developed (Figs. 1.2 and 1.3). Propagules of powdery mildews can germinate and successfully develop an infection at low relative humidity conditions (Yarwood, 1957).

Takamatsu (2004, 2013) suggested that powdery mildew fungi and their host have coevolved (Fig. 1.4). Evidence based on molecular analysis suggest that powdery mildews firstly infected tree species, and later they adapted onto herbs (Takamatsu et al., 2000). Moreover, this transition is believed to have occurred also in *Rosaceae* hosts (Leus et al., 2006). From ancestral powdery mildews, the evolution of different phenotypic characters based on molecular phylogenetic analyses indicates that some characters have evolved in specific directions. Main ancestral characteristics were ectoparasitism, large-sized chasmothecia with many asci and ascospores, dichotomic branched apices emerging from chasmothecia, and conidia produced in chains and containing fibrosin bodies (Fig. 1.4, according to Takamatsu, 2013).

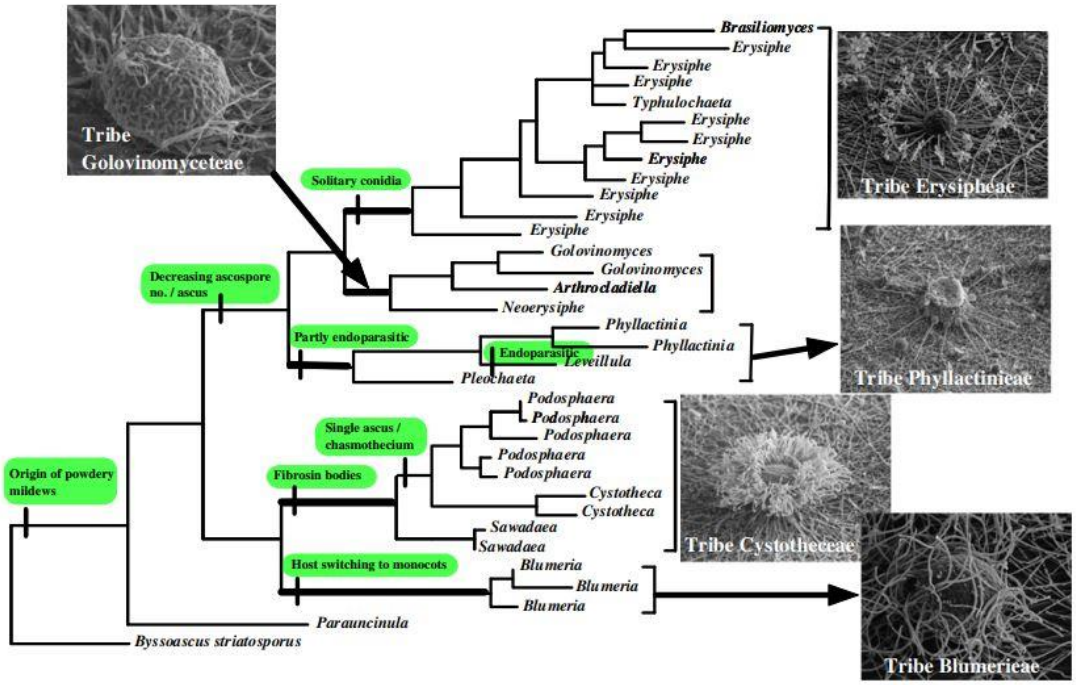

Figure 1.4. Phenotypic evolution of the powdery mildews (from Takamatsu, 2013).

1.2.2. Taxonomy and description

Podosphaera pannosa is one of the main powdery mildew species affecting *Rosaceae* hosts included in the genera *Prunus* and *Rosa* (Agrios 2005; Takamatsu et al., 2000). *Podosphaera pannosa* belongs to tribe *Cystotheceae* (Braun, 1987), subtribe *Cystothecinae* (Braun and Cook, 2012). This pathogen was synonymyzed for many decades under the name *Sphaerotheca pannosa* (Wallr.) Lév. until molecular studies based on the rDNA ITS region (Braun and Takamastu, 2000; Saenz and Taylor, 1999) suggested to merge both genera, and *Podosphaera* Kunze predated over *Sphaerotheca* Lév. as it was earlier validly published. Old taxonomic criterion to differentiate *Sphaerotheca* from *Podosphaera* was based on the branching patterns of chasmothecia appendices (Fig. 1.2), as they show unbranched or dichotomously branched hyphal appendices, respectively. However, this morphological character was not further considered as a valid taxonomic feature after DNA-based phylogenetic studies (Braun and Takamastu, 2000; Saenz and Taylor, 1999; Takamatsu, 2013).

Species belonging to the genus *Podosphaera* show flexuous but strong hyphae-forming mycelium, and haustoria occur in the epidermal cells of the host (Braun and Takamatsu, 2000). Conidiophores are generally simple and produced from external

mycelium, at right angles to the host surface and branching occasionally (Braun and Takamatsu, 2000). Conidia are produced in basipetal chains, and they contain fibrosine bodies (Yarwood, 1957), which are defined as reserve bodies (Fig. 1.3). Boesewinkel (1980) described morphological traits distinctive for *P. pannosa* as follows: mycelium normally dense and well-developed, formed by hyphal cells measuring (12-)25 × 40(-120) µm. The conidiogenous cell is at the same time the foot cell. Conidiophores wider than the mycelium from which they emerge, measuring measure 70-80 µm, with foot-cells relatively long (45-75 µm), more or less straight, but slightly swollen at the base (up to 12.5 µm wide at the base but with parts measuring from 7.5 to 11 µm). Conidia developed from conidiophores formed in chains, with immature (base) and mature ones (top) at the same time and chain, measuring 25-30 × 13.5-17.5 µm, from which emerge simple and straight germ tubes.

Podosphaera pannosa has been reported from over 40 peach-growing countries in the world (Amano, 1986; Farr and Rossman, 2020). The pathogen infects fruit, leaves, buds, shoots, and twigs (Grove, 1995; Ogawa and English 1991) of all known peach fruit morphology cultivars, including nectarine and flat fruit species. Some common infection symptoms on fruit (Fig. 1.5), and leaves (Fig. 1.6) are shown.

Figure 1.5. Symptoms of powdery mildew on a nectarine fruit, as shown by a grey-whitish spot on fruit surface. (Drawing by Quim Pallarès, 2020.)

Figure 1.6. Healthy peach leaves (left) and leaves with severe infection of *Podosphaera pannosa* (right). (Drawings by Quim Pallarès, 2020.)

The main economic impact due to *P. pannosa* infection relates to fruit infection, which results in unacceptable fruit for the industry. As fruit deformation and premature fruit fall may occur when infection is severe (Dirlewanger et al., 1996), a yield reduction can be usually recorded (Jarvis et al., 2002; Weinhold, 1961). In the Cape province of South Africa, severe outbreak from peach powdery mildew was reported by Burchill (1978), as for Japanese plums, apricots, nectarines, and peaches, where fruit infection reached almost 50% fruit (Grove, 1995). Furthermore, as this pathogen infects leaves and shoots, a reduction of photosynthetic capacity has also been reported (Agrios, 2005). Seriously infected leaves may shrivel and deform (Fig. 1.6), and early defoliation may occur (Dirlewanger et al., 1996).

1.2.3. Life cycle

The life cycle of *P. pannosa* in *Rosa*, as described by Agrios (2005), can be well fitted into peach life cycle (Fig. 1.7). The pathogen overwinters as dormant mycelium in buds (Ogawa and English, 1991; Weinhold, 1961; Yarwood, 1957) or as chasmothecia

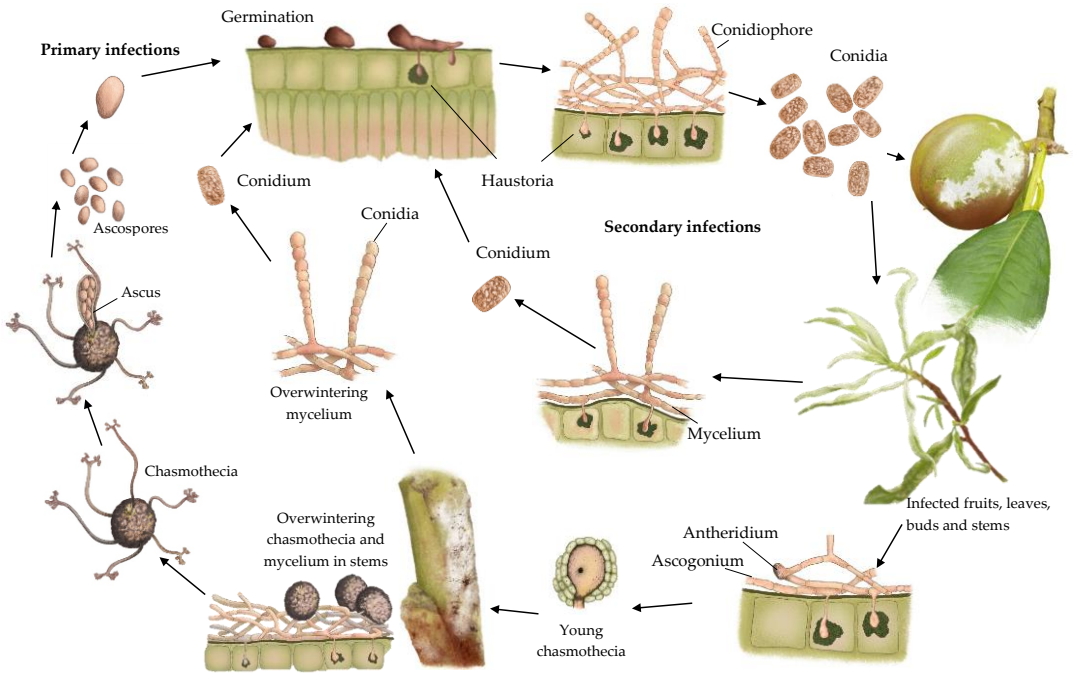

Figure 1.7. Life cycle of *Podosphaera pannosa* (modified from Agrios, 2005). (Drawings by Quim Pallarès, 2020.)

developed in the epiphytic mycelium of infected twigs and leaves (Butt, 1978). In spring, chasmothecia discharge ascospores (i.e., the primary inoculum) that, in favorable humidity and temperature conditions but mainly depending on water-related variables, can infect susceptible fruit and young leaves. Primary infections from overwintering mycelium in buds have also been reported (Weinhold, 1961). The developing germ tube of an ascospore grows into the epidermal cells of the host forming a haustorium inside the host cell. Alongside with the haustorium development, the germ tube also grows on the surface of the host, producing a mycelial network that forms more haustoria into other epidermal cells. Besides the pathogen expansion over the host surface, the aerial mycelium produces conidia at the end of erected, single hyphal conidiophores, which are then released into the air to initiate secondary infections (Grove, 1995). Thus, the life cycle of peach powdery mildew shows a clear polycyclic development, which is characterized by an exponential growth and explosive epidemics (Agrios, 2005). The production of conidia ends when temperature decreases in autumn, and new chasmothecia begin to develop as resistance structures for the winter survival of the pathogen.

1.2.4. Epidemiology

The infection of peach powdery mildew depends largely on the growth stage of the plant host, as it mainly occurs on immature fruits and young leaves (Toca et al., 2017). Thus, it is said that no infection occurs after pit hardening (Ogawa and English, 1991). Regarding the environmental factors affecting disease development, temperature has been described to be one of the main factors affecting the disease progress of powdery mildews (Yarwood, 1957). Relative humidity appears to be also correlated with disease incidence, being temperature and water-related parameters closely related (Linde and Shishkoff, 2003). These two variables are components of water pressure deficit, a parameter which usually explains most host-parasite systems (Anderson, 1936). Nevertheless, ascospore germination and infection in the case of powdery mildews are described to be inhibited in the presence of free water (Agrios, 2005; Jarvis et al., 2002). Thus, powdery mildew incidence and severity are limited by extreme wet weather, resulting in less prevalent disease as the rainfall increases. In contrast, powdery mildews are common in dry climate areas of the world (Agrios, 2005). However, most infections occur after a wetness period followed by a dry one (Jarvis et al., 2002).

Little information is available regarding the specific temperature and relative humidity requirements for the development of *P. pannosa* (Grove, 1995; Toma et al., 1998). Optimal temperature was described to be in the range from 20 ºC to 23 ºC (Grove, 1995; Longrée, 1939; Xu, 1999), or even higher, until 27 ºC (Weinhold, 1961). Optimal relative humidity for conidia germination and infection was established from 90 to 99% (Horst and Cloyd, 2007; Longrée, 1939), although other reports indicate lower levels of optimal relative humidity. Thus, Grove (1991) indicated a maximum relative humidity of 90% for an optimal ascospore release and germination, or a minimum of 70-75% for conidia germination (Grove, 1995).

1.2.5. Control

A disease could be explained as the outcome of the interaction of three components, thus: i) the susceptibility of plant host, ii) the virulence and quantity of a virulent pathogen, and iii) favorable environment conditions (Fig. 1.8), which is usually represented and referred as the "disease triangle" (Agrios, 2005). Each component contributes to a proportional potential to cause disease. If any of those three components

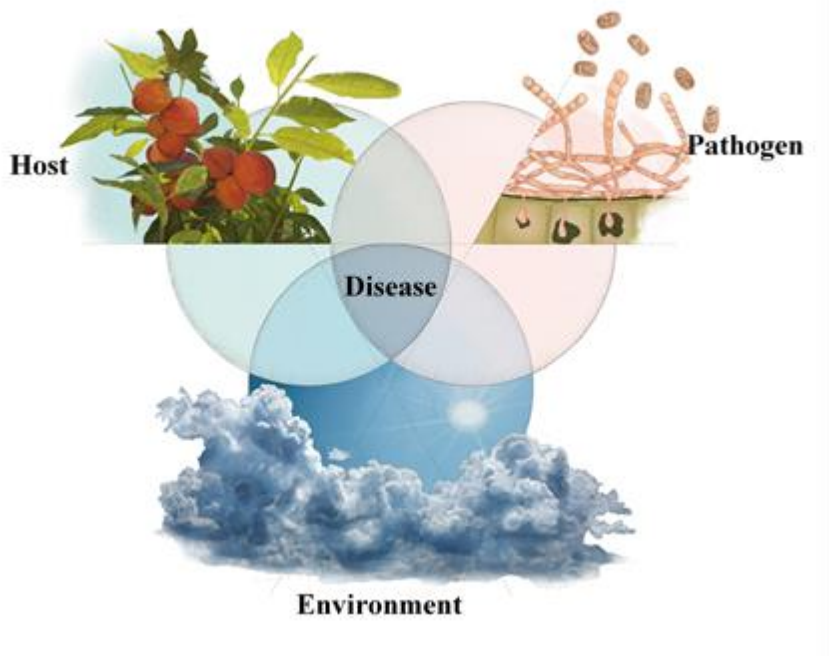

Figure 1.8. Disease triangle adapted from Agrios (2005). (Drawings by Quim Pallarès, 2020.)

is zero, then the disease is absent. Controlling a disease can be achieved in an effective way by using strategies to mitigate or eliminate the influence by any of the three components or any combination of them. Thus, three types of strategies could be applied based on i) the control of the pathogen itself, through chemical, biological, and cultural methods; ii) breeding of resistant cultivars, and iii) determining the influential environmental conditions which favor disease to further implement prevention strategies able to reduce the infection risk.

The control of peach powdery mildew is usually achieved through the application of foliar fungicides on a calendar basis. Fungicide programs start in spring at petal fall or the beginning of fruit set (Grove, 1995; Ogawa and English, 1991) until summer. In some Mediterranean countries, including Spain, a common fungicide application program may consist of four to seven applications each season (Reuveni, 2001). Fungicide applications are conducted every 7 or 14 days (Xu, 1999), depending on environmental conditions, which are mainly related to potential rain episodes that may reduce fungicide effectiveness. Commonly used fungicides in Spain are sterol biosynthesis inhibitors (SBI), quinone outside inhibitors (QoI), protein synthesis inhibitors, and several inorganic multi-site activity products including sulfur derivatives (MAPA, 2020).

Fungicide programs provide a high effective control of peach powdery mildew (Ogawa and English, 1991). As this strategy has been proven effective for decades, no other alternative control methods for peach powdery mildew have been reported. However, currently fungicide applications are performed whether the pathogen is present or not in peach crops, and under each farmer's criteria. This lack of previous information about the disease pressure in the orchard could indicate that more pesticide treatments than strictly necessary are applied. Current global framework about sustainable use of pesticides aims to reduce the amount of pesticide applications, or at least to promote more rational pesticide applications. Therefore, new management strategies based on disease prevention, anticipating the initiation of the infection, would be convenient. Moreover, a comprehensive information about the presence of the pathogen at different disease stages could promote an effective control of peach powdery mildew.

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2. Objectives

2. Objectives

The main objective of this thesis was to develop new alternative tools for the integrated management of the peach powdery mildew disease, caused by the fungus *Podosphaera pannosa*. Two complementary approaches were considered in this global strategy: *i*) to expand the current knowledge on the biology of the pathogen and the disease progress, to further optimize fungicide programs; and *ii*) to characterize the genetic basis of disease resistance gene *Vr3* coming from almond, to further introgress this character into peach commercial cultivars. In both cases, different DNA molecular-based techniques were used.

To achieve the main goal of this thesis two objectives were proposed:

1. To study key aspects on the biology and control of *Podosphaera pannosa* including:

    1.1. The development of a decision support system to optimize the initiation of fungicide programs.

    1.2. The development of a qPCR-based protocol for the detection and quantification of the pathogen at different stages of its life cycle in biological samples.

2. To characterize the peach powdery mildew resistance gene *Vr3* coming from almond including:

    2.1. The fine mapping of resistance gene *Vr3* and the analysis of candidate genes.

    2.2. The introgression of peach powdery mildew resistance gen *Vr3* into peach elite cultivars using marker-assisted selection.

3. A decision support system based on degree-days to initiate fungicide spray programs for peach powdery mildew in Catalonia, Spain

3. A decision support system based on degree-days to initiate fungicide spray programs for peach powdery mildew in Catalonia, Spain

3.1. Abstract

The incidence of peach powdery mildew (PPM) on fruit was monitored in commercial peach orchards to: i) describe the disease progress in relation to several environmental parameters, and ii) establish an operating threshold to initiate a fungicide spray program based on accumulated degree-day (ADD) data. A beta-regression model for disease incidence showed a substantial contribution of the random effects orchard and year, whereas relevant fixed effects corresponded to ADD, wetness duration, and ADD considering vapor pressure deficit and rain. When beta-regression models were fitted for each orchard and year considering only ADD, disease onset was observed at 242 ± 13 ADD and symptoms did not develop further after 484 ± 42 ADD. An operating threshold to initiate fungicide applications was established at 220 ADD, coinciding with a PPM incidence in fruit around 0.05. A validation was further conducted by comparing PPM incidence in: i) a standard, calendar-based program, ii) a program with applications initiated at 220 ADD, and iii) a non-treated control. A statistically relevant reduction in disease incidence in fruit was obtained with both fungicide programs, from 0.244 recorded in the control to 0.073 with the 220-ADD alert program, and 0.049 with the standard program. The 220-ADD alert program resulted in 33% reduction in fungicide applications.

3.2. Introduction

The fungus *Podosphaera pannosa* (Wallr.) de Bary is one of the causal agents of the powdery mildew which occurs on peach, nectarines and flat fruit (Farr and Rossman 2019). Other powdery mildew species can be found on this fruit tree species, such as *P. clandestina, P. leucotricha,* and *P. tridactyla* (Farr and Rossman, 2019), but *P. pannosa* is widely recognized as the main causal agent of the peach powdery mildew (PPM). The species *P. pannosa* is a cosmopolitan biotrophic pathogen that has been reported from over 40 peach-growing countries in the world (Amano, 1986; Farr and Rossman, 2019).

It is also known to affect other Rosaceae species, mainly included in the genera *Prunus* and Rosa (Farr and Rossman, 2019). On peach, the fungus infects fruit, leaves, buds, shoots and twigs (Grove, 1995; Ogawa and English, 1991), showing a distinguishable white-greyish mycelium developing on the surface of the affected parts. The pathogen overwinters as dormant mycelium in latent buds (Ogawa and English, 1991; Weinhold, 1961; Yarwood, 1957), and in chasmothecia produced in the epiphytic mycelium of infected twigs and leaves (Butt, 1978). Primary infections on the tree green parts occur in spring, when primary inoculum (ascospores) is available and favorable conditions are met. Infections from latent mycelium that overwintered in buds have also been reported (Weinhold, 1961). Conidia released from these primary colonies disperse in air and initiate secondary infections throughout the season (Grove, 1995; Jarvis et al., 2002). Infection of fruit, if severe, makes the fruit commercially unacceptable (Weinhold, 1961), thus causing important economic losses.

Data on potential yield reduction by PPM have been previously reported in some countries. In California, Ogawa and Charles (1956) reported that the amount of marketable peaches from fungicide-sprayed trees was about 20% greater than those from unsprayed trees. Grove (1995) reported that crop losses resulting from fruit infections may reach 50% on Japanese plums, apricots, nectarines and peaches. Unfortunately, no data on potential production losses are available in Spain, where this study has been carried out. Spain ranks as the second country in the world, after China, in terms of cultivated area (86,000 ha) and annual fruit production of peaches (1,5 M tons in 2016), followed by Italy, USA and Greece (FAO, 2019; MAPA, 2019). These figures account for about 6% of the total world crop area and 7% of world production. In Spain PPM is endemic but quantitative data on potential production losses are not available.

The control of PPM is usually achieved through the applications of fungicides (Grove, 1995; Hollomon and Wheeler, 2002; Ogawa and English, 1991). Most used fungicides are sterol biosynthesis inhibitors (SBI), quinone outside inhibitors (QoI), protein synthesis inhibitors, and various inorganic multi-site activity products including sulfur derivatives. Foliar fungicides, starting at petal fall or the beginning of fruit set, are sprayed routinely to protect peach fruit from infection (Grove, 1995; Reuveni, 2001), as fruit are susceptible from the early stages of fruit growth to the beginning of pit hardening (Ogawa and English, 1991). In Spain, four to seven fungicide applications in a season are

generally needed, which is comparable to other Mediterranean countries where peaches are grown (Reuveni, 2001). In California, it has been reported that three applications are enough to control the disease (Ogawa and Charles, 1956; Ogawa and English, 1991). However, fungicide applications are made on a calendar basis (Ogawa and English, 1991) since, to our knowledge, no epidemiological models to predict the risk infection of PPM are currently available.

Disease prediction is required to apply plant protection products in rational, sustainable integrated strategies, which are intended to keep control effectiveness against plant diseases while reducing the application costs and the potential risks to the environment and public health (Jørgensen et al., 2017). Thus, optimizing timing of fungicide application is fully desirable for economic and environmental reasons. Several epidemiological models have been developed for powdery mildews in different crops, including apple, barley, grape, rose, rubber, sugar beet and tomato, as reviewed by Jarvis et al. (2002), cherry (Grove et al., 2000), cucurbits (Sapak et al., 2017), mango (Nasir et al., 2014), and wheat (Cao et al., 2015). In general terms, models focus on the prediction of 1) the critical date for a single fungicide application, 2) the date to initiate the fungicide program, or 3) the timing of fungicide applications in intensive spray programs, as reviewed by Butt (1978).

Empirical (i.e. correlative) and mechanistic (i.e. process-based) modeling approaches have been used to develop decision support systems (DSSs) for plant disease management. Empirical models are correlative in nature, so their predictive ability is limited by the scope of the data (Madden and Ellis, 1988). Mechanistic models are developed from controlled experiments to quantify the effects of environmental factors on the different components of the disease cycle (De Wolf and Isard, 2007). Mechanistic models are generally considered more robust for extrapolation, but epidemics are sometimes more complex than a simple combination of their monocyclic components.

We aimed at acquiring new knowledge on the disease onset and progress of PPM under the crop conditions in Catalonia, Northeast Spain, and to develop and validate a DSS adapted to this area. In a field survey conducted in 2015, *P. pannosa* was the only powdery mildew species detected on peach in the study area. The specific objectives of this study were therefore: i) to describe the disease onset and progression of PPM caused by *P. pannosa* on peach and nectarine fruit in terms of incidence along the season, ii) to

develop a simple epidemiological model to estimate the disease incidence in relation to temperature; and iii) to evaluate the performance of this empirical model as a DSS to initiate the fungicide spray program for PPM management.

3.3.Materials and methods

3.3.1. Experimental sites

The incidence of powdery mildew on peach and nectarine fruit was monitored yearly along the growth season in the period 2013-2015 in eight commercial orchards (1 to 8) located in Lleida, Catalonia, Spain and aged 4 to 8 years at the beginning of the experiment (Table 3.1). Most orchards were nectarine crops whereas only one was cultivated for peach, and an additional one for platerine. The commercial validation of the DSS, as described by Magarey and Sutton (2007), for the onset of fungicide applications was conducted in 2017 in six orchards, namely 2, 8 and four additional ones, 9 to 12 (Table 3.1). All orchards (1 to 12) were located within a radius of approximately 10 km. All varieties in the orchards were grafted onto 'GF-677' rootstock except for

Table 3.1. Characteristics of the commercial orchards used in this study and years corresponding to symptom monitoring, model fitting (train dataset), model evaluation (test dataset) and commercial validation.

Orchard no.	UTM Coordinates (WGS 84, 31 T)		Crop	Cultivar	Symptom monitoring (year)	Train dataset (year)	Test dataset (year)	Commercial validation (year)
	X	*Y*						
1	287680	4602661	Nectarine	'Red Jim'	2013-15	2013-15	-	-
2	297674	4602928	Nectarine	'Red Jim'	2013-15	2014	2013	2017
3	289237	4613448	Peach	'Albesa Red'	2013-14	2013	-	-
4	288554	4613923	Platerine	'ASF 07.78'	2015	-	-	-
5	283489	4619988	Nectarine	'Venus'	2013	-	2013	-
6	302991	4627916	Nectarine	'Nectareine'	2014-15	2014	2015	-
7	287918	4597751	Nectarine	'Venus'	2013-14	2013-14	-	-
8	287141	4609517	Nectarine	'Autumn free'	2013-15	2013-15	-	2017
9	287972	4603490	Nectarine	'Tarderina'	-	-	-	2017
10	286696	4605773	Nectarine	'Independence'	-	-	-	2017
11	289380	4612041	Nectarine	'Extreme Red'	-	-	-	2017
12	282806	4614805	Nectarine	'Nectatinto'	-	-	-	2017

orchard 10, that was grafted onto 'Garnem'. Trees in the orchards were arranged around 4-5 x 2-3 m, trained in 4-scaffolds open vase and drip-irrigated, which is locally common in the area. The climate in the area is BSk (Tropical and Subtropical Steppe Climate), according to Köppen-Geiger's climate classification system (Kottek et al., 2006).

3.3.2. Dynamics of powdery mildew symptoms on fruit

For each growing season and experimental plot, symptoms of PPM were recorded on fruit starting from the 50% blossom biofix (BBCH scale 65, see Meier, 2001) occurring in mid-March, until no further disease progression was noticed for up to 2-3 weeks (BBCH scale 77 to 79), which occurred in mid-June to early July depending on the year. Observations of PPM symptoms were carried out on a weekly basis but twice a week in some sites and seasons, especially when incidence progressed rapidly. The observations were conducted on five contiguous trees, which were not treated with fungicides during the growing season, thus allowing for a natural progress of disease. Monitored trees were surrounded by 1-2 rows of non-treated trees to avoid spray drift, as confirmed in earlier observations. In each tree, 3-4 scaffolds were selected and the central third of each branch was marked to set homogeneous sampling conditions within trees and among experimental sites. All the fruit in the selected branch sections were recorded as either symptomatic or not and those showing symptoms were individually labelled. At the end of the monitoring period, all fruit in each monitored branch sections were counted, and disease incidence was calculated as the proportion of symptomatic fruit (0 to 1) for each monitoring period, branch, tree and experimental site combination. Any diseased fallen fruit during the monitoring period was considered as a diseased fruit to avoid underestimates of disease incidence (i.e., decrease) with time.

3.3.3. Environmental data

A wireless cellular data-logger (model Em50G, from Decagon Services, Pullman, WA, USA) was located in each experimental site, less than 50 m from the marked trees. The data-logger was used to measure the air temperature, relative humidity, rainfall and

wetness duration at 1-hour intervals during the whole experimental period. Environmental variables were summarized for each period between two consecutive symptom evaluations as follows: mean values of temperature and relative humidity, and accumulated values of rainfall and leaf wetness duration, the latter either expressed as total number of minutes or time proportion within the whole interval. In addition, degree-days (DD) were calculated according to Zalom et al. (1983), by using the single-sine method and setting 10 °C and 35 °C as the lower and higher thresholds, respectively. Thresholds were determined from the values reported for *P. fuliginea* (Jarvis et al., 2002). Accumulated degree-days (ADD) for each monitoring date were calculated starting from the 50% blooming biofix date. Finally, combined environmental variables were included in the analyses (Table 3.2).

Table 3.2. Name and description of the environmental variables used for model fitting.

Variable	**Description**
Tm	Mean temperature (°C)
Rain	Rainfall (mm)
RH	Relative humidity (%)
VPD	Vapor Pressure Deficit, as described by Martínez-Minaya et al. (2019). Used to calculate ADDvpd
WetnessD	Leaf wetness duration (minutes)
WetnessP	Leaf wetness duration expressed as percentage of time
ADD	ADD calculated by the simple sinus method (Zalom et al., 1983)
ADD2	ADD calculated as described by Martínez-Minaya et al. (2019)
ADDrh70-90	ADD of days with 70 < RH < 90%
ADDno_rain	ADD of days with Rain < 2 mm
ADDno_wet	ADD of days with 70 < RH < 90% and Rain < 2 mm, based on Toma et al. (1998)
ADDno_wet2	ADD of days with WetnessP < 70%, based on Grove (1995)
ADDvpd	ADD2 of days with VPD < 4 (Martínez-Minaya et al., 2019)
ADDwet	ADD2 of days with VPD < 4 and Rain > 2 mm (modified from Martínez-Minaya et al., 2019)
ADDwet2	ADD of days with VPD < 4 and Rain > 2 mm

3.3.4. Disease progress modeling

Beta regression is commonly used for variables that assume values in the unit interval (0,1). This method overcomes the drawbacks of the traditional data transformations, so it allows a direct interpretation of model parameters in terms of the original data. The analysis is not sensitive to the sample size and posterior distributions are expected to concentrate well within the bounded range of proportions (Ferrari and Cribari-Neto, 2004; Martínez-Minaya et al., 2019).

As in generalized linear models, the mean (μ_i) is linked to the linear predictor using the logit link function:

$$logit(\mu_i) = \beta_0 + \sum_{j=1}^{N_\beta} \beta_j x_{ji} + \sum_{k=1}^{N_v} v_{ki} \quad i = 1, \dots, n$$

where β_0 is the intercept of the model, β_j are the parameters corresponding to the fixed effects of the model, and v_{ki} represent *k* unstructured error terms (random effects).

3.3.5. Commercial validation of the DSS to initiate fungicide applications

From the field observations, early primary PPM symptoms were observed at approximately 240 ADD in average (actually, 241.2 ± 13.1 ADD). Moreover, an average incidence of 0.05 was estimated at 239.1 ± 18.1 ADD with the beta regression model described here. Thus, an operating alert threshold to initiate fungicide applications was chosen at 220 ADD. This value was chosen considering logistic constraints at the farm level to provide growers with a reasonable period to initiate fungicide sprays. Roughly, this 20 ADD difference was equivalent to approximately 2 days, as DD values observed in this period were about 10 DD a day.

According to Magarey and Sutton (2007), commercial evaluation considers if the model can predict the appropriate deployment of disease management measures. Commercial validation is usually performed by comparing disease incidence and/or severity of a model-driven fungicide spray schedule with that of a routine calendar

program. Six orchards, namely 2, and 8 to 12 (Tables 3.1 and 3.3), were used in this study. In each orchard, three fungicide programs were evaluated: i) the standard, calendar-based, fungicide program, which was applied under farmers' criteria and coinciding with the European Directive on Sustainable Use of Pesticides (2009/128/EC). This program was applied in all orchards after petal fall, well before the 220-ADD alert; ii) the fungicide program starting at the 220-ADD alert, which was further continued on a calendar basis, and with same applications and dates as the standard; and iii) the control, non-treated group of trees. Each experimental unit consisted of five contiguous trees which were surrounded by 1-2 rows of untreated trees to avoid spray drift. The selection of fungicides to be used in each application time, as well as the application calendars, were left to each farmer's criteria, but were the same in the calendar-based and after the 220-ADD alert spray program conducted in each orchard. Fungicides used in the orchards during the commercial validation were included in the chemical groups of triazoles, dithiocarbamates, benzamides, strobilurins, pyrimidines, quinolines and inorganic fungicides.

Table 3.3. Most relevant dates and accumulated degree days (ADD) values recorded during the commercial validation of the 220-ADD alert spray program for the control of peach powdery mildew in 2017 in six nectarine orchards.

Orchard no.	50% bloom date	Petal fall	220-ADD alert Pre-evaluation			Application at 220-ADD alert		Final evaluation	
			Date	*ADD*	*Incidence*	*Date*	*ADD*	*Date*	*ADD*
2	8 Mar	15 Mar	21 Apr	214.9	0.000	22 Apr	219.4	9 Jun	654.2
8	7 Mar	13 Mar	18 Apr	207.9	0.001	21 Apr	222.7	9 Jun	636.3
9	7 Mar	15 Mar	19 Apr	228.6	0.000	20 Apr	232.8	8 Jun	675.2
10	7 Mar	29 Mar	21 Apr	213.5	0.006	22 Apr	219.4	12 Jun	648.4
11	9 Mar	21 Mar	21 Apr	222.7	0.009	20 Apr	216.9	12 Jun	758.9
12	8 Mar	30 Mar	24 Apr	208.1	0.000	27 Apr	217.8	8 Jun	572.8

The ADD values were calculated daily as described above for all experimental orchards starting at 50% blooming date, the latter being in the range 7 to 9 March 2017. When the 220-ADD alert was approaching (i.e., around 200 ADD; from 18 to 24 April 2017), PPM incidence was evaluated in all combinations of fungicide programs and orchards. At the end of the experimental period, when no further disease progression was observed (values from 570 ADD to 760 ADD; from 8 to 12 June 2017), disease incidence was again assessed in all experimental sites and trees.

3.3.6. Statistical analyses

The beta regression to model PPM disease dynamics was fitted following a Bayesian hierarchical approach with the INLA methodology (Rue et al., 2009). This methodology uses Laplace approximations (Tierney and Kadane, 1986) to get the posterior distributions in Latent Gaussian models (LGMs) (Rue et al., 2009). Vague Gaussian distributions were used here for the parameters involved in the fixed effects $\sim N$. Precision of the beta distribution (ϕ) was reparametrized as $\phi = exp\ (\alpha)$ to ensure that ϕ was a positive parameter. We assumed pc-priors on the log-precision for both parameters. The computational implementation R-INLA (Rue et al., 2009) for R (R Core Team, 2018) was used to perform approximate Bayesian inference. In order to conduct the analysis in our data, values of the response variable were transformed to be included in the interval (0,1) dividing by the maximum PPM incidence recorded in each orchard and year combination. As a common practice in beta regression, 0s and 1s were settled to 0.01 and 0.99, respectively.

A joint analysis including all orchards and years was conducted. The dataset including all orchards and years (n = 14) was split into a train dataset (n = 11) and a test dataset (n = 3) (Table 3.1). Pearson correlations among covariates were calculated, and those greater than 0.7 were not further considered to minimize potential multicollinearity issues (Dormann et al., 2013). Thus, variables for final analyses were restricted to seven, namely ADD, ADDvpd, ADDwet, Rain, RH, Tm, and WetnessP (Table 3.2). Two additional random independent effects, year and orchard, were included. All possible models (n = 512) were fitted to the train dataset and the best models were selected based on the Watanabe Akaike Information Criterion (WAIC) (Watanabe, 2010), which is the sum of two components, one quantifying for the model fit and the other one evaluating model complexity. Models with the lowest WAIC values were selected. The importance of the covariates in the models was checked based on the value of their coefficients. Median values of the posterior predictive distribution were linearly regressed against the observed values and R2 of models were computed. The mean absolute error (MAE), mean square error (MSE) and root mean square error (RMSE) were also calculated. The best model was then evaluated using the test dataset. Linear regression of predicted vs.

observed values including R2, MAE, MSE and RMSE values were also calculated. Finally, data from each separate orchard-year combination were analyzed similarly but including only ADD as a covariate.

In the commercial validation experiment, disease incidence data at the end of the experimental period were analyzed with a logistic regression and binomial distribution. Fungicide programs (i.e., calendar-based, 220-ADD alert and non-treated control) were considered as a fixed factor and orchards as a random blocking factor. The non-treated control was used as the reference level and the odds ratios for the calendar-based and 220-ADD alert spray programs were calculated including their corresponding 95% credibility intervals. R-INLA for R was used to perform approximate Bayesian inference with the prior distributions provided by default.

3.4.Results

3.4.1. Dynamics of powdery mildew symptoms on fruit

Only datasets with final PPM incidence on fruit equal or higher than 0.05 in the orchards were used in this study, i.e., a total of 14 datasets resulting from the combination of the experimental orchards and monitored years (Fig. 3.1). Final incidence values ranged among orchards and years between 0.05 and 0.96. Four orchard-year combinations were in the range 0.05-0.20 final PPM incidence, eight in the range 0.20-0.60, and two over 0.80 (Fig. 3.1). Moreover, first symptoms were noticed at variable dates and their equivalent ADD values among orchards and years. Field observations revealed that first PPM occurrences on fruit were noticed on average at 240 ADD after the 50% blooming biofix (mean ± std. err.: 242.0 ± 13.1 ADD; median: 241; range: 144 to 311). At this stage, first infection signs were noticed at 0.045 incidence on average (range: 0.010 to 0.115). On a calendar basis, most of these primary infection symptoms were noticed between the last week of April and the two first weeks of May (range: Apr 18 to May 14). PPM incidence increased in the experimental orchards roughly until June, and last new symptoms were mostly detected at 460-480 ADD (median: 460 ADD; mean 484 ± 42.2; range 283 to 833). Last new symptoms on fruit were early detected in May (first to third

week) in some orchard-year combinations, whereas in other cases they were detected as late as in July (first week).

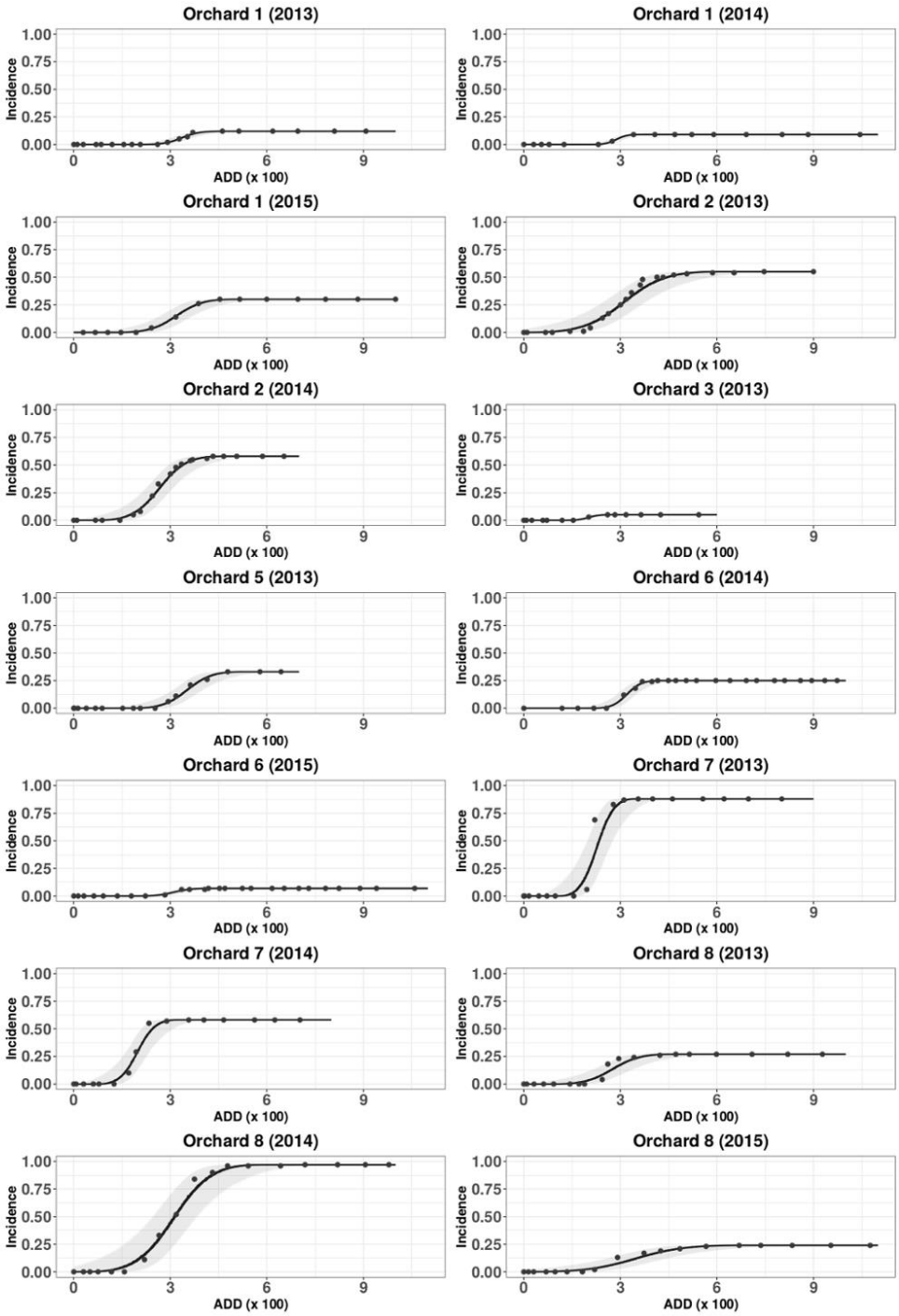

Figure 3.1. Dynamics of peach powdery mildew incidence in fruit (solid dots) and accumulated degree-days in the orchards evaluated from 2013 to 2015. Median posterior distribution (solid line) and 95% credibility interval (shaded area) obtained with the beta regression models.

The best models for PPM incidence fitted to the train dataset are shown in Table 3.4. Models not including the random effects year (v) and orchard (w) were ranked very low based on their WAIC values. Four out of the five best models included the fixed effects ADD, ADDvpd, ADDwet and WetnessP. The finally selected model, with the lowest WAIC value, included those fixed effects and the random effects year and orchard. Linear regression of the median posterior predictive distribution against observed values accounted for more than 84% of the total variance (R2 = 0.842) (Figure 3.2). The MAE for this model was 0.090, the MSE was 0.014 and the RMSE was 0.119. In the selected model, ADD, ADDvpd, ADDwet and WetnessP were relevant. The parameter for the fixed effect ADD had a mean posterior distribution of 0.668 with a 95% credible interval [0.442, 0.902] (Table 3.5). The parameter for the fixed effect ADDvpd had a mean posterior distribution of -2.294 with a 95% credible interval [-3.187, -1.459]. The parameter for the fixed effect ADDwet had a mean posterior distribution of 4.881 with a 95% credible interval [3.035, 6.824]. The parameter for the fixed effect WetnessP had a mean posterior distribution of -1.891 with a 95% credible interval [-3.063, -0.711]. None of the credible intervals overlapped with zero.

Table 3.4. Beta regression models for peach powdery mildew incidence based on environmental variables and their associated WAIC1 values. 1 Watanabe-Akaike information criterion (Watanabe 2010). 2 Random effects year (v) and orchard (w).

Model	**WAIC**
With random effects[2]	
Intercept + ADD + ADDvpd + ADDwet + WetnessP + v + w	-131.34
Intercept + ADD + ADDvpd + ADDwet + WetnessP + Tm + v + w	-129.97
Intercept + ADD + ADDvpd + ADDwet + WetnessP + RH + v + w	-129.32
Intercept + ADD + ADDvpd + ADDwet + WetnessP + Rain + v + w	-129.19
Intercept + ADD + ADDvpd+ ADDwet + Tm + Rain + v + w	-128.67
Without random effects	
Intercept + ADD	-69.98
Intercept + ADD + Tm	-69.91
Intercept + ADD + ADDvpd + Tm	-69.18
Intercept + ADD + RH	-68.59
Intercept + ADD + ADDvpd	-68.42

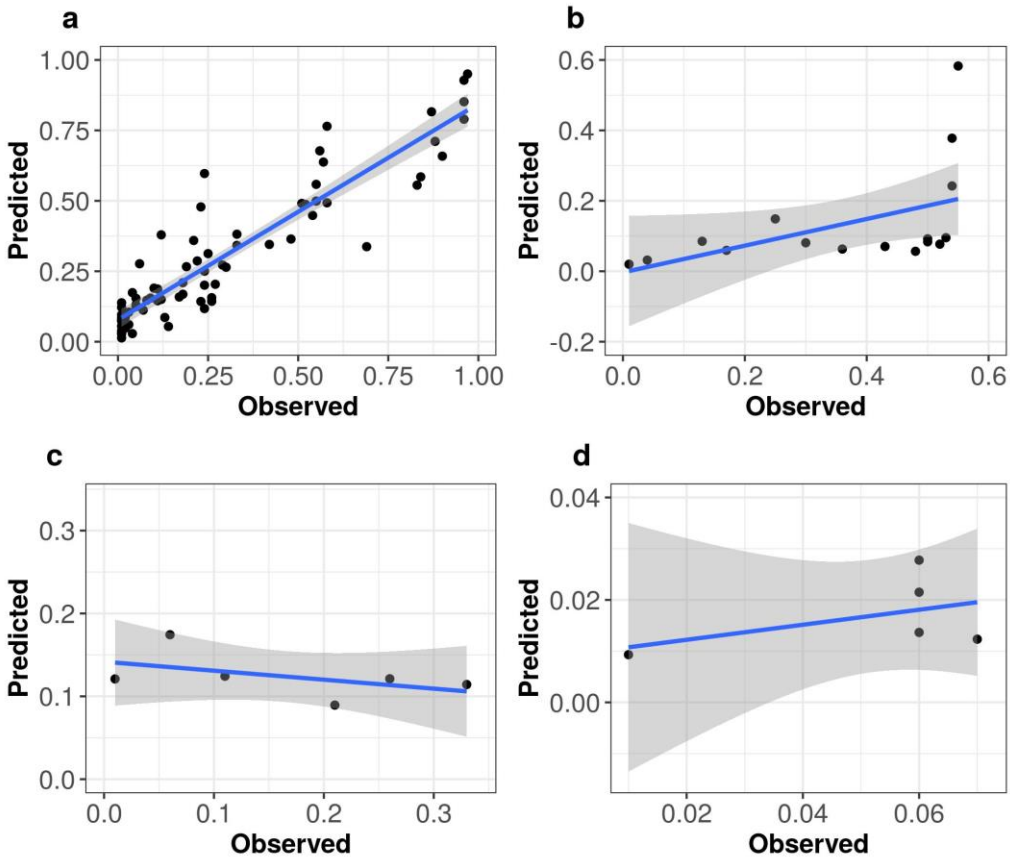

Figure 3.2. Linear regression between observed values and the median of the posterior predictive distribution for the model of the peach powdery mildew incidence. Model fitted to the train dataset (a). Model applied to the test dataset: orchard 2 in 2013 (b), orchard 5 in 2013 (c), and orchard 6 in 2015 (d). Blue line is the regression line, shaded area is the 95% credibility interval.

Posterior distribution of the hyperparameters are displayed in Table 3.5, showing that random effects are explaining some of the variability of the response variable, and it is important to consider them in the model. The fixed effects ADD and ADDwet had positive effects on the expected incidence of PPM whereas ADDvpd and WetnessP had negative effects. When the selected model was applied to the test dataset, MAE ranged from 0.035 to 0.235, MSE from 0.002 to 0.082, RMSE from 0.040 to 0.286 among datasets. When the median of the posterior predictive distribution was linearly regressed against the observed data, values of R2 ranged from 0.215 in orchard 6 in 2015 to 0.236 in orchard 2 in 2013. In general, residuals showed a poor graphical fit (Figure 3.2).

The beta regression models for each orchard-year combination which included only ADD as explanatory variable were able to accommodate dynamics of PPM incidence

at different degree, despite the large differences observed in disease progress and final incidences (Fig. 3.1).

Table 3.5. Parameters of the best beta regression model for peach powdery mildew incidence including the fixed effects accumulated degree-days (ADD), ADD considering vapor pressure deficit (ADDvpd), ADD considering vapor pressure deficit and rain (ADDwet), percentage of wetness duration (WetnessP) and the random effects year and orchard. Mean, standard deviation (sd), quantiles (Q) and mode for the parameters and hyperparameters (φ, τ, ρ).

Parameters and hyperparameters[1]	**Mean**	**sd**	**$Q_{0.025}$**	**$Q_{0.5}$**	**$Q_{0.975}$**	**Mode**
Intercept	-2.927	0.959	-4.841	-2.928	-1.013	-2.931
ADD	0.668	0.117	0.442	0.667	0.902	0.664
ADDvpd	-2.294	0.439	-3.187	-2.284	-1.459	-2.265
ADDwet	4.881	0.964	3.035	4.865	6.824	4.835
WetnessP	-1.891	0.599	-3.063	-1.892	-0.711	-1.896
ϕ	8.999	1.763	5.969	8.856	12.867	8.591
τ	1.091	0.922	0.156	0.841	3.518	0.429
ρ	2.008	1.361	0.449	1.676	5.532	1.113

The mean of the posterior distribution for the intercept (β0) ranged from -12.2 in orchard 3 to -4.9 in orchard 2 in 2013, from -16.8 in orchard 1 to -5.2 in orchard 7 in 2014, and from -11.7 in orchard 8 to -4.6 in orchard 6 in 2015 (Table 3.6). The mean of the posterior distribution for the parameter of ADD (β1) ranged from 1.6 in orchard 2 to 6.1 in orchard 3 in 2013, from 1.7 in orchard 7 to 5.9 in orchard 1 in 2014, and from 1.3 in orchard 6 to 3.8 in orchard 8 in 2015 (Table 3.6). Based on the beta regression models, between 107.2 ADD (orchard 2, 2013) and 278.1 ADD (orchard 1, 2013) were needed to reach PPM incidences of 0.01 in the 2013-15 monitoring period (Table 3.7). In addition, between 161.6 ADD (orchard 7, 2014) and 389.9 ADD (orchard 1, 2013) were needed to reach 0.10 PPM incidence in the same period. Highest annual mean values for ADD estimations at 0.01 to 0.10 incidence were obtained in 2015, whereas lowest estimates were obtained in 2014. On average, 187.1 to 264.0 ADD were needed to reach PPM incidences between 0.01 and 0.1, respectively, among orchards and years (Table 3.7). An average of 239.1 ADD for 0.05 PPM incidence was determined for all orchard and year

combinations, which was comparable with the first PPM occurrences visually noticed in the orchards.

Table 3.6. Accumulated degree-days calculated by the beta regression model for the studied orchards and years combinations when the incidence of peach powdery mildew in fruit was 0.01, 0.02, 0.05 and 0.1. n.a.: not applicable.

Year	Orchard	Disease incidence			
		0.01	**0.02**	**0.05**	**0.1**
2013	1	278.1	296.3	327.9	389.9
	2	107.2	138.0	181.0	230.0
	3	180.6	195.9	n.a.	n.a.
	5	246.1	264.1	293.4	327.5
	7	141.0	149.2	164.3	180.4
	8	166.4	187.0	221.6	261.6
Mean 2013		186.6	205.1	237.6	277.9
2014	1	255.7	267.6	291.2	n.a.
	2	131.2	146.7	177.6	208.4
	7	112.7	123.3	141.6	161.6
	6	260.0	271.2	291.6	315.0
	8	114.3	131.0	163.2	200.4
Mean 2014		174.8	188.0	213.0	221.4
2015	1	205.8	225.4	260.8	296.6
	6	270.4	290.8	336.0	n.a.
	8	150.4	188.4	257.7	333.0
Mean 2015		208.9	234.9	284.8	314.8
Total means		187.1	205.4	239.1	264.0

Table 3.7. Posterior distributions for the parameters (β0, β1) of the beta regression model on the peach powdery mildew disease progression modelling for different orchards and years, including mean, 95% credibility interval and standard deviation. 1Accumulated degree days.

Year	Orchard	β_0 (Intercept)				β_1 (ADD)[1]			
		Mean	**0.025 quant**	**0.975 quant**	**Std. deviation**	**Mean**	**0.025 quant**	**0.975 quant**	**Std. deviation**
2013	1	-12.0	-16.9	-7.7	2.3	3.6	2.3	5.0	0.7
	2	-4.9	-6.2	-3.6	0.7	1.6	1.2	2.0	0.2
	3	-12.2	-18.0	-7.6	2.7	6.1	3.7	9.0	1.3
	5	-9.2	-12.5	-6.3	1.6	2.6	1.8	3.6	0.5
	7	-8.3	-11.5	-5.4	1.5	3.6	2.4	5.1	0.7
	8	-6.4	-9.3	-3.9	1.4	2.3	1.4	3.4	0.5
2014	1	-16.8	-24.2	-10.7	3.5	5.9	3.7	8.5	1.2
	2	-6.4	-8.0	-4.8	0.8	2.4	1.8	3.0	0.3
	6	-7.1	-10.0	-4.5	1.4	3.6	2.3	5.1	0.7
	7	-5.2	-7.0	-3.6	0.9	1.7	1.2	2.2	0.3
	8	-13.7	-19.2	-9.0	2.6	4.3	2.9	5.9	0.8
2015	1	-7.7	-10.7	-5.2	1.4	2.4	1.7	3.3	0.4
	6	-4.6	-6.2	-3.1	0.8	1.3	0.9	1.8	0.2
	8	-11.7	-17.2	-7.2	2.6	3.8	2.3	5.5	0.8

3.4.2. Commercial validation of the DSS to initiate fungicide applications

Two of the six orchards evaluated in 2017, namely orchards 9 and 12, were excluded from the commercial validation as PPM symptoms recorded at the end of the experimental period were <1% and we thought that data from those orchards might not be adequate for the statistical analyses. Thus, only data from four orchards (2, 8, 10 and 11) were used in the analyses (Fig. 3.3). Disease incidence values recorded in the non-treated control ranged from 0.157 (orchard 8) to 0.411 (orchard 2). Mean PPM incidence recorded in the non-treated control was 0.244 ± 0.114 (std. dev.) (Fig. 3.4), with a total sample size of 5894 fruit. Mean PPM incidence recorded in the calendar-based spray program was 0.049 ± 0.032, with a total sample size of 5465 fruit. Mean PPM incidence recorded in the 220-ADD alert spray program was 0.073 ± 0.044, with a total sample size of 5883 fruit.

The odds ratio was 0.199 (credibility interval: 0.175-0.225) for the calendar-based spray program and 0.116 (0.099-0.135) for the 220-ADD alert spray program. The 95% credibility interval of the odds ratio was lower than 1, so both spray programs reduced

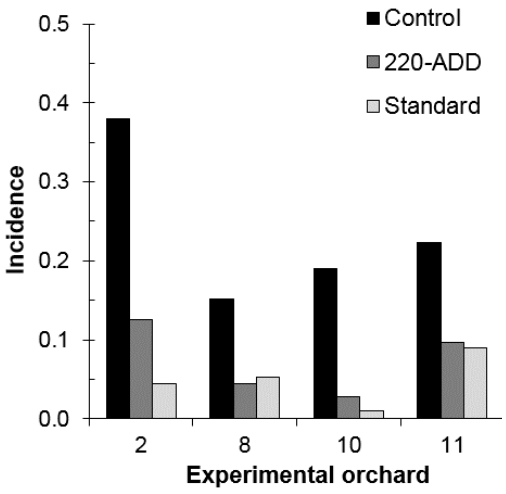

Figure 3.3. PPM incidence in four commercial orchards where three different calendar strategies for fungicide application were tested.

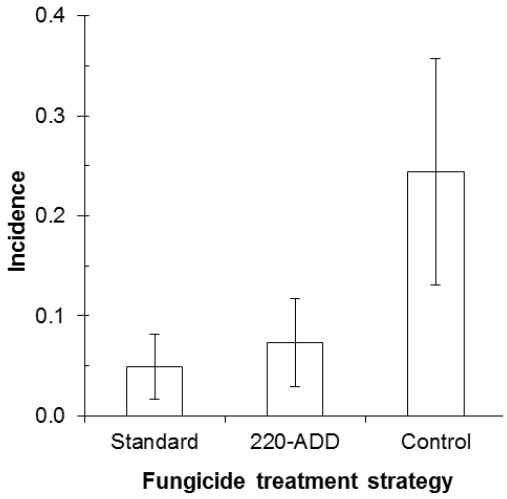

Figure 3.4. Peach powdery mildew incidence obtained with a calendar-based fungicide program, fungicide applications initiated after 220 accumulated degree days (ADD), and a non-treated control evaluated in 2017 in a commercial validation. Error bars stand for standard deviation of the mean.

PPM incidence compared with the reference level (non-treated control). The odds of PPM incidence in the calendar-based spray program were 8.63 times less than in the non-treated control, whereas the odds corresponding to the 220-ADD alert spray program were 5.02 times less than in the control. The 95% credibility intervals of the odds ratio for the calendar-based and the 220-ADD alert spray programs did not overlap, being lower for the calendar-based treatment. Therefore, higher reduction of PPM incidence compared with the non-treated control was obtained with the calendar-based spray program than with the 220-ADD alert spray program.

Regarding the total number of fungicide applications in the calendar-based program, it ranged from 4 (orchard 2 and 10) to 7 (orchard 8). Meanwhile, the number of fungicide applications in the 220-ADD alert spray program ranged from 2 (orchard 10) to 5 (orchard 8). This represents, in percentage, and compared with the calendar-based program, a reduction in the numbers of fungicide applications from 25% (orchard 2) to 50% (orchard 10) (mean: 33.3%) (Table 3.8).

Table 3.8. Number of fungicide applications before and after the 220-ADD threshold was reached in four experimental orchards evaluated for the model validation. The percentage of application reduction is indicated for each orchard.

Orchard no.	**Applications**		**Application reduction (%)**
	Before 220-ADD	***After 220-ADD***	
2	1	3	25.0
8	2	5	28.6
10	2	2	50.0
11	2	4	33.3
Total	7	14	33.3

3.5.Discussion

The incidence of PPM in fruit was assessed in different commercial peach and nectarine orchards located in Catalonia, Northeast Spain, along several years. The beta-regression model selected for describing PPM epidemics included two random effects, namely orchard and evaluation year, which were highly relevant in the model, therefore indicating that unmeasured sources of variability were actually driving PPM disease

progress after symptom appearance. This was further supported by the poor performance of the model when evaluated with the test dataset. These random sources of variability are likely to be associated with different factors, including cultivar susceptibility, different inoculum levels and infection dynamics in the orchards among years. These variables were not measured in our study and further experiments would be needed to decipher the random effects and hence optimize the model, e.g. by including additional varieties and orchards under different environmental conditions, and the specific use of spore samplers and trap plants to monitor inoculum and infection dynamics.

Regarding the fixed effects of the beta-regression model developed here, durable wetness and ADD recorded during low VPD conditions (i.e. humid days) had a negative effect on the disease incidence progression. A negative effect of water on the disease progress has been reported for powdery mildews (Jarvis et al., 2002; Yarwood, 1957), which is specifically related to the inhibition of conidia germination in free water (Perera and Wheeler 1975; Sivapalan 1993; Yarwood, 1957), and the washing off of airborne spores during rain episodes (Blanco et al., 2004). Sutton and Jones (1979) reported that amounts of airborne ascospores of *P. leucotricha* are increased at the beginning of rain episodes but decreased rapidly with continuous rain. Similarly, Grove et al. (2000) reported that rain favors ascospore release of *P. clandestina*. However, conflicting reports on the effects of rain on powdery mildews are notably. Thus, Yarwood (1957) described favorable effect of rain episodes on the incidence progression due to a possible removal of protective applications of fungicides. Other authors pointed out that rainfall induces growth of new susceptible plant tissues (Grove, 1995; Ogawa and English 1991). Glawe (2008) and Grove and Boal (1991a,b) argued that dispersion of powdery mildew ascospores may occur after rain or during wetness periods initiated by rain. In our study, when considering ADD under >2 mm rain episodes, a significant positive effect in PPM incidence was obtained. Thus, wetness could be affecting differentially both primary and secondary infections within the pathogen cycle, i.e. by favoring ascospore release but inhibiting conidia germination and washing airborne propagules off from affected plat tissues and environment. In our study, monitoring of PPM incidence and its relationship with ADDwet was performed for the whole infection cycle, so it was not possible for us to evaluate the influence of this variable in each particular stage of PPM epidemics. When analyzing each orchard-year combination separately, ADD was able to successfully describe PPM progression. Air temperature has been previously reported to be one of the

main factors affecting the disease progress in powdery mildews (Trecate et al., 2019; Xu and Butt 1998; Yarwood, 1957).

Previous works on modeling *P. pannosa* progression on fruit are scarce in literature. Optimal temperature and relative humidity parameters for different phases of the disease cycle have been reported (Grove, 1995; Toma et al., 1998). However, Pieters et al. (1993) concluded that neither the temperature nor the relative humidity influenced the differentiation between the two epidemic phases (primary and secondary infections) that were described for *P. pannosa* progression on rose in greenhouse conditions. In contrast, we have shown that combined water and temperature parameters are needed to better explain PPM progression under field conditions.

An epidemiological model for the cherry powdery mildew has been developed (Grove, 1991, 1998; Grove and Boal, 1991a; Grove et al., 2000). These authors studied the effects of several environmental factors on the development of *P. clandestina* on cherry, such as the release and germination of ascospores depending on temperature and wetness duration (Grove, 1991), the germination of conidia on leaves and fruit depending on the temperature and VPD (Grove and Boal, 1991a), and the availability of the secondary inoculum based on temperature, relative humidity and wind speed (Grove, 1998). As in the case of cherry powdery mildew, we think that more precise PPM epidemic drivers based on water and temperature can be obtained from future research.

When disease progress was analyzed separately in each orchard-year combination, a robust estimate for the onset of disease was obtained by including only ADD as covariate. We were further able to establish a fungicide program based on a degree-day monitoring with an operating threshold of 220-ADD to initiate fungicide applications, providing growers a reasonable period to mobilize application logistics before the onset of the risk period for PPM. Similarly, Carisse et al. (2009) developed and validated a degree-day model to initiate a fungicide spray program for the management of grapevine powdery mildew. They concluded that fungicide sprays could be initiated when 1% to 5% of the total seasonal airborne inoculum was reached, which was depending on the grape variety about 500-600 ADD after vines reached the 2–3 leaf phenological stage. According to this degree-day model, fungicide applications were initiated 30 to 40 days later (just at the 3–4 leaf phenological stage) than those in the standard program. This resulted in a 40-5%. Reduction in fungicide applications.

For the defined 220-ADD operating threshold, the beta regression model estimated a PPM incidence between 0.02 and 0.05 (with ADD ranging between 205.3 and 239.1 ADD). Thus, the 220-ADD alert spray program is based on synchronizing the initiation of fungicide applications with the detection of the first PPM symptoms. The 220-ADD alert spray program resulted in an increase of 2.4% final PPM incidence as compared to the calendar-based program. Although statistically significant because of the relatively large sample size, the size effect of this difference was not relevant in our opinion and, thus, we consider the 220-ADD alert spray program as effective as the current calendar-based spray program. Fungicide sprays in the 220-ADD alert spray program were initiated 24 to 39 days later than in the calendar-based spray program, resulting in an overall reduction of 33% in the number of fungicide applications. Estimated local cost per each fungicide application (including fungicide, machinery and personnel costs) in the commercial orchards of our study ranged from 70 to 90 $ per ha and application (Marimon, *unpublished*). Thus, the 220-ADD alert spray program could be a useful tool to optimize PPM control by reducing both production and environmental costs. Further validations would be needed to transfer the 220-ADD alert spray program for PPM management to other cultivars and growing areas with different environmental conditions, including different inoculum potential levels.

We aimed at describing the PPM progress by using a simple model with few variables. We focused on air temperature as this variable is widely available and can be easily recorded at orchard level. Also, DSSs based on this environmental variable are more accessible and easier to implement by growers (Jarvis et al., 2002). Despite of the potential advantages foreseen by the implementation of the 220-ADD alert spray program, we assume that epidemiological models including only one or few components of the disease cycle may limit, to some extent, model transferability and robustness. Therefore, further work is needed to develop PPM models including additional environmental predictors for the primary and secondary infections on peach fruit. In this sense, the 220-ADD operating threshold described here may be considered as the first component of a future, more complete, DSS for powdery mildew control on peach.

Diversification of fungicides and use of resistant cultivars are the main management strategies used for powdery mildew management worldwide (Cao et al., 2015; Wolfe, 1984). Epidemiological models and derived DSSs are also important in

integrated disease management. Combining the use of tolerant cultivars with effective DSSs would certainly reduce the amount of fungicides applied while maintaining optimal disease control levels.

3.6.References

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4. A qPCR-based method for the detection and quantification of the peach powdery mildew (*Podosphaera pannosa*) in epidemiological studies

4. A qPCR-based method for the detection and quantification of the peach powdery mildew (*Podosphaera pannosa*) in epidemiological studies

4.1. Abstract

A molecular qPCR-based method was developed to detect and quantify *Podosphaera pannosa*, the main causal agent of peach powdery mildew. A primer pair was designed to target part of the ITS region of the fungal ribosomal DNA, which proved to be highly specific and sensitive. A minimum of 2.81 pg μL^{-1} of *P. pannosa* DNA and 6 conidia mL^{-1} in artificially-prepared conidia suspensions were found to be the limit of detection. Moreover, a quantification of conidia placed on plastic tapes commonly used in volumetric air samplers was performed. Regression equations on conidia quantification obtained either from aqueous conidia suspensions or conidia placed on plastic tapes were similar. The protocol was further validated in field conditions by estimating the number of *P. pannosa* conidia obtained with an air sampler, by both microscopical and molecular quantification. Both techniques detected simultaneously the peaks of conidia production during a 4-month sampling period, and a significant correlation ($r = 0.772$) was observed between both quantification methods. Additionally, the molecular method was applied to detect the latent fungal inoculum in different plant parts of peach trees. The pathogen was detected mainly on the bark of affected twigs, and to a lesser extent, in foliar buds. The method developed here can be applied in the study of *P. pannosa* epidemiology and can help in improving the management of this pathogen through its early detection and quantification.

4.2. Introduction

The ascomycete *Podosphaera pannosa* (Wallr.) de Bary is one of the causal agents of powdery mildew, which occurs mainly on the *Prunus* and *Rosa* genera of Rosaceae (Farr and Rossman, 2019; Takamatsu et al., 2010). Other powdery mildew species are rarely found on peach, such as nectarines and flat fruits albeit rarely, such as *P. clandestina*, *P. leucotricha*, and *P. tridactyla* (Farr and Rossman, 2019). However, *P. pannosa* is widely recognized as the main causal agent of the peach powdery mildew

(PPM). *Podosphaera* species infect green parts of the tree, e.g. fruits, leaves, buds, and twigs (Grove, 1995; Ogawa and English, 1991), where a distinguishable white-greyish mycelium develops on the surface of the affected part. Severe infections of *P. pannosa* on fruits makes them unacceptable to industry, thus causing significant economic losses. This species has been reported from over 40 peach-growing countries in the world (Amano, 1986; Farr and Rossman, 2019). The fungus overwinters in peach as dormant mycelium in latent buds (Ogawa and English, 1991; Toma et al., 1998; Weinhold, 1961; Yarwood, 1957), and the ascocarps (chasmothecia) are usually found in the mycelium infecting twigs and leaves (Butt, 1978). Primary PPM infections occur in spring, when primary inoculum is available under favourable weather conditions are met. However, precise experimental data on the environmental conditions needed for primary PPM infections are scarce (Toma et al., 1998; Weinhold, 1961). Air-dispersed conidia released from primary-established colonies are responsible for secondary infections that extend over the vegetative growing season of peach tree (Grove 1995; Jarvis et al. 2002). In general, PPM spreads rapidly in seasons when a relatively cold and humid spring is followed by a dry summer (Toma et al., 1998). Previous studies reported the optimal temperature and relative humidity (RH) for pathogen development to be at approximately 21 °C and 70-95% RH, respectively (Grove, 1995; Toma et al., 1998). Regarding the infection of *P. pannosa* on *Rosa*, Longrée (1939) described similar optimal conditions for *P. pannosa* infection (21 °C and between 75-99% RH). The control of PPM can be achieved efficiently through periodical applications of foliar fungicides (Grove, 1995; Hollomon and Wheeler, 2002; Ogawa and English, 1991), which usually starts at petal fall or the beginning of fruit set and continues periodically (Grove, 1995; Reuveni, 2001). These fungicide applications are done on a calendar basis (Ogawa and English, 1991) since epidemiological models on PPM infection risk are scarce. Recently, a decision support system to initiate fungicide applications programs has been proposed (Marimon et al., 2020).

Rapid and reliable detection and quantification of *P. pannosa* in biological samples might contribute to a better understanding of its life cycle and therefore to improve its management. The detection of airborne inoculum of powdery mildews has been made traditionally through air-sampling devices combined with microscopical observations (Cao et al., 2015; Grove, 1991). However, this method is time-consuming and non-specific for the identification and quantification of airborne plant pathogens

(Dung et al., 2018; Falacy et al., 2007). Otherwise, coupling spore traps with DNA-based analytical techniques is faster, more specific and sensitive, and a reliable alternative to the conventional detection of airborne plant pathogens through microscopical observations (Kunjeti et al., 2016), including powdery mildews (Falacy et al., 2007; Thiessen et al., 2016).

The main objective of the current study was to develop a real-time qPCR assay for the detection and quantification of *P. pannosa* in biological samples, including the design of a species-specific primer pair. In addition, two further practical applications were conducted in peach orchards to detect and quantify (*i*) the airborne inoculum of *P. pannosa* in spore traps, and (*ii*) the primary inoculum of *P. pannosa* in host plant material. The protocol reported here could be used in future applied studies, e.g. those including the need for a rapid and accurate detection and quantification of *P. pannosa*.

4.3.Materials and methods

4.3.1. Experimental orchards

Three experimental peach and nectarine orchards owned by IRTA and located in Catalonia, Spain, were used in this study (Alcarràs, 41°36’33’’N, 0°26’45’’E; Cabrils, 41°31’7”N, 2°22’34”E; and Mollerussa, 41°37’8’’N, 0°52’2’’E). The orchard located in Alcarràs was an ‘Autumn free’ nectarine orchard, whereas orchards in Cabrils and Mollerussa were planted with ‘Early Gold’ peach and ‘Texas’ almond interspecific progenies that are known to be susceptible to PPM (Donoso et al., 2016). These orchards were managed using cultural practices, such as pruning, soil management and nutrient supply, according to the guidelines of Spanish Integrated Production Management practices (MAPA, 2002). No fungicide treatments were applied during the experimental period (spring to summer) to allow natural infections of *P. pannosa*, which were known to occur in the orchards.

4.3.2. Plant material

Specificity and sensitivity tests. In order to obtain conidia suspensions of *P. pannosa*, symptomatic peach fruits and leaves were collected in summer 2017 in the Mollerussa orchard. Samples were stored in a portable cooler and taken to the laboratory for further processing. All field samples were processed in the laboratory within 48 h after collection. For the specificity experiment, fresh leaves of apple and plum trees infected with powdery mildew (one sample each) were obtained and treated similarly as the peach samples to get conidia suspensions. Additional herbarium material used in this experiment, consisting of six powdery mildew species phylogenetically close to *P. pannosa* and occurring on various hosts, was kindly provided by Dr Josep Girbal (Universitat Autònoma de Barcelona, Bellaterra, Spain) as follows: three samples of *Podosphaera aphanis*, collected on *Alchemilla alpina*, *Alchemilla vulgaris*, and *Potentilla reptans*, respectively; one sample of *P. clandestina* from *Crataegus monogyna*; one sample of *P. fusca* from *Cucurbita pepo*, and two samples from *Cucumis sativus*; six samples of *P. leucotricha* from *Malus domestica*; two samples of *P. macularis* from *Humulus lupulus*, and five of *P. tridactyla* from *Prunus cerasifera*.

Latent mycelium detection. Five trees per each experimental orchard located in Alcarràs and Mollerussa, and three trees from the orchard located in Cabrils were used. At the end of summer 2016, eight sight-heighted branches (1.3 to 1.9 m above ground level) preferably showing PPM symptoms were selected and marked in each tree. The apical part of each branch (about 40 cm) was covered with a plastic mesh to retain leaves from falling, and the mesh was tied to prevent its accidental opening. In February 2017, all selected branches were collected and kept at 4 ºC until further processing.

4.3.3. Fungal material

Powdery mildew conidia were collected from the symptomatic plant parts by repeatedly washing away the plant infected surface with 1.5 mL of sterile 5% Chelex-100 (Bio-Rad, Hercules, CA, USA) aqueous suspension. Each sample volume was collected separately in 1.9 mL Eppendorf tubes and conidia concentration was measured using a Neubauer haemocytometer. Samples were stored at 4°C for further DNA extraction.

4.3.4. DNA extraction

Conidia suspensions. DNA was extracted from conidia suspensions using the short protocol of the E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA), with modifications described by Zúñiga et al. (2018) as follows: 0.15 g of 500-750 μm glass beads (Acros Organics, Geel, Belgium) were added to 700 μL of the extraction buffer in each sample, and the samples were vortexed for 15 min at 50 Hz. DNA quality and concentration were checked and measured with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA samples were stored at -20 °C until further use.

Spore trap samples. DNA was extracted from the air-exposed plastic tapes used in the spore-trapping device (see below) by following the short protocol of the E.Z.N.A. Plant DNA Kit (Omega Bio-tek). Extraction, and DNA quantity and quality checking were conducted as described above and DNA was stored at -20 °C until further use.

Plant tissues. Before DNA extraction, all fresh peach samples (i.e. leaf and flower buds, leaves, and bark from twigs) were oven-dried at 35 °C until constant weight. Herbarium samples were processed for DNA extraction with no previous oven-drying. Fungal DNA was extracted from those plant tissues using the E.Z.N.A. Plant DNA Kit (Omega Bio-tek), following the dried plant samples protocol and the sample homogenization step with glass beads. DNA checking was also conducted as earlier described and DNA was stored at -20 °C until further use.

4.3.5. Primer design

Primers were designed to target the Internal Transcribed Spacer (ITS) in the ribosomal DNA region. Two representative ITS sequences of *P. pannosa* samples, namely ‘Ppan53’ and ‘Ppan92’, were used in this study (Table 4.1). These sequences were selected from a previous screening analysis involving 31 *P. pannosa* samples obtained from *P. persica* and *Rosa* (Luque, *unpublished*). Sequences were included in a matrix together with 29 additional sequences retrieved from GenBank (Table 4.1), as follows: 4 from *P. pannosa*; 10 from phylogenetically closer species such as *P. aphanis* (n = 3), *P. clandestina* (n = 4), and *P. spiraeae* (n = 3); and 15 sequences from other

Table 4.1. GenBank accession numbers of sequences used to design a specific primer pair for the detection and quantification of *Podosphaera pannosa*.

Fungal taxa	Sample	Host	Country	GenBank ITS[a]
Podosphaera aphanis	S_Italy3	*Fragaria* sp.	Italy	GU942447
Podosphaera aphanis	R_Eng_Kent2	*Rubus* sp.	UK	GU942461
Podosphaera aphanis	R_Sco1b	*Rubus* sp.	UK	GU942462
Podosphaera clandestina	MUMH 1868	*Crataegus* sp.	Argentina	AB525932
Podosphaera clandestina	30111	*Phlox drummondii*	Italy	HQ844621
Podosphaera clandestina	P-G	*Prunus avium*	Belgium	DQ139434
Podosphaera clandestina	BC-1	*Prunus serotina*	Mexico	KJ158161
Podosphaera fusca	Unknown	*Cucurbita pepo*	USA	AF011321
Podosphaera fusca	SqPl-1	*Eupatorium fortunei*	China	JX546297
Podosphaera fusca	MAY1	*Euryops pectinatus*	Spain	EU424056
Podosphaera fusca	UC1512289	*Taraxacum officinale*	USA	AF011320
Podosphaera fusca	PF001	*Trichosanthes kirilowii*	South Korea	HQ683746
Podosphaera leucotricha	MUMH 468	*Malus domestica*	Japan	AB027231
Podosphaera leucotricha	N4-08	*Prunus persica*	Serbia	HM579839
Podosphaera pannosa	Ppan53	*Prunus persica*	Spain	*MN796128*
Podosphaera pannosa	R-A	*Rosa* sp.	Belgium	DQ139410
Podosphaera pannosa	R-D	*Rosa* sp.	Belgium	DQ139430
Podosphaera pannosa	Ppan92	*Rosa* sp.	Spain	*MN796129*
Podosphaera pannosa	UCB	*Rosa* sp.	USA	AF011322
Podosphaera pannosa	UC1512288	*Rosa* sp.	USA	AF011323
Podosphaera spiraeae	TPU-1825	*Spiraea cantoniensis*	Japan	AB026143
Podosphaera spiraeae	HMQAU 13013	*Spiraea japonica*	China	KF500426
Podosphaera spiraeae	TPU-1877	*Spiraea thunbergii*	Japan	AB026153
Podosphaera tridactyla	MUMH 247	*Photinia beauverdiana*	Japan	AB026147
Podosphaera tridactyla	VPRI 19864	*Prunus armeniaca*	Australia	AY833657
Podosphaera tridactyla	UC1512290	*Prunus armeniaca*	USA	AF011318
Podosphaera tridactyla	VPRI 19238	*Prunus cerasifera*	Australia	AY833656
Podosphaera tridactyla	VPRI 22157	*Prunus laurocerasus*	Switzerland	AY833654
Podosphaera tridactyla	P-S	*Prunus lusitanica*	Belgium	DQ139435
Podosphaera tridactyla	VPRI 22158	*Prunus lusitanica*	Switzerland	AY833655
Podosphaera tridactyla	KUS-F26292	*Prunus salicina*	South Korea	JQ517296

[a]**:** Accession numbers obtained in this study are shown in italics.

Podosphaera species, namely *P. fusca* (n = 5), *P. tridactyla* (n = 8) and *P. leucotricha* (n = 2). The identical sequences were grouped by Sequencher software 5.0 (Gene Codes Corp., Ann Arbor, Michigan), using the Assemble algorithm with the 100% Minimum Match parameter. Sequences were aligned using ClustalW (Thompson et al., 1994) with default settings and posterior manual adjustments were made when necessary. Regions

with polymorphisms and suitable for specific primer design were identified, and later analysed with the PrimerQuest tool (IDT, https://eu.idtdna.com/PrimerQuest/Home/Index) using the default parameters. The primer pair PpanITS1-F/PpanITS1-R was obtained.

4.3.6. qPCR conditions

Optimal qPCR conditions were set up as follows: for a final volume of 20 µL each reaction, products and concentrations were: 10 µL SYBR Premix Ex Taq™ TliRNase H Plus (Takara), 0.4 µL of each specific forward and reverse primers (at 10 µM), 5 µL of template DNA, and HPLC-grade deionized water to reach the final volume. qPCR was carried out on a Rotor-Gene Q 5plex thermal cycler (Qiagen, Hilden, Germany) with the following temperature and timing profile: an initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. After the final amplification cycle, the temperature was held at 72 ºC for 90s. The melting curve analysis was performed raising the temperature from 72 ºC to 95 °C, increasing 1 °C every 5 s with continuous measurement of fluorescence at 510 nm wavelength. All reactions were run in triplicate and using genomic DNA extracted from *P. pannosa* conidia suspensions as positive controls, and negative controls with no DNA template.

4.3.7. Analytical specificity and sensitivity tests

The primer pair specificity was checked *in silico* and *in vitro*. *In silico*, specificity for the primer pair PpanITS1-F/PpanITS1-R was evaluated with the Primer-BLAST tool (htpps://www.ncbi.nlm.nih.gov/tools/primer-blast/). *In vitro*, specificity was tested by analysing qPCR amplifications of 28 DNA samples obtained from six *Podosphaera* species other than *P. pannosa* occurring on several Rosaceae and non-Rosaceae species, which included 20 samples from the earlier described herbarium material, and fresh samples of *P. leucotricha* (n = 5, from apple), and *P. tridactyla* (n = 3, from plum), both collected at IRTA Cabrils facilities. Identity of the fungi that were different from *P. pannosa* was confirmed by sequencing their rDNA ITS region using the forward primer ITS1F (Gardes and Bruns, 1993) and the reverse primer ITS4 (White et al., 1990) using

the methods described by Luque et al., 2005. All qPCR reactions involved in the specificity test were carried out in triplicate and included negative and positive (Ppan53) controls of *P. pannosa*.

The primer pair sensitivity was evaluated according to the protocols described by Armbruster and Pry (2008). Two independent DNA samples (DNA 1 and DNA 2) and three independent conidia suspensions (CS 1, CS 2 and CS 3) were prepared and used in the experiments. The DNA samples were obtained from conidia suspensions and later serially-diluted, whereas the CS samples were serially-diluted before DNA extraction. In both cases, DNA was extracted from the resulting conidia suspensions using the method described above. The measured DNA concentrations for DNA 1 and DNA 2 samples were (mean ± std. error) 25.4 ± 3.8 ng μL^{-1} and 33.9 ± 4.6 ng DNA μL^{-1}, respectively. Ten-fold dilutions series down to 10^{-5} were prepared and subsequently used in the qPCR assays. For each CS sample, amounts of conidia were determined from four measurements with five pseudoreplicates using a haemocytometer. Initial conidia concentrations for CS1 to CS3 samples were 5.87 ± 0.212 × 10^5 conidia mL^{-1}, 3.13 ± 0.136 × 10^5 conidia mL^{-1}, and 8.06 ± 0.274 × 10^5 conidia mL^{-1}, respectively. For each suspension, ten-fold dilution series down to 10^{-5} were prepared. The DNA from each dilution point was extracted as described earlier. All DNA samples were amplified with the primer pair designed in this study and using the qPCR conditions described above, and by additionally including 0.4 µL of ROX Reference Dye in each reaction. All qPCR reactions were performed using a StepOne™ Real-Time PCR System thermal cycler (Life Technologies, Carlsbad, CA, USA). Three technical replicates were run for each biological sample, and three replicates of deionized water template were included in each reaction plate as negative controls. After each qPCR, a melting curve was performed to verify the targeted amplification product. A homogeneous melting peak at 88°C indicated that the amplified targeted ITS1 region was specific for *P. pannosa*. For each DNA and CS samples, a standard curve was calculated by plotting the quantification cycle values (C_q) against the logarithm of the DNA or conidia concentration at each dilution point. The amplification efficiency (AE), intercept, slope, and determination coefficient (r^2) were calculated for each standard curve obtained in this study. Then, the limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ) were calculated according to the EP17 guideline of the Clinical and Laboratory Standards institute (Armbruster and Pry, 2008).

4.3.8. Validation of the specific qPCR primer pair PpanITS1-F/PpanITS1-R

Case 1: Detection of P. pannosa *airborne inoculum in spore traps*

Starting from a conidia suspension (CS 4) containing $7.47 \pm 0.45 \times 10^4$ conidia mL^{-1}, two independent 10-fold dilution series were prepared until 10^{-5} of the initial concentration, with three replicates per dilution. For the first dilution series, DNA for each dilution and replicates was extracted as described earlier. Regarding the second dilution series, 500 µL from each dilution and replicate was placed on a Melinex (Tekra, New Berlin, WI, USA) polyester plastic strip (19 x 48 mm) previously treated with silicone solution (Lanzoni, Bologna, Italy) on one side. Plastic strips were dried overnight in a laminar airflow cabinet at room temperature. Finally, DNA was extracted and amplified according to the protocol described in this study. Three technical replicates were run per sample. Standard curves for each of two replicates were obtained and used in further quantification of *P. pannosa* conidia trapped on plastic tapes.

In a subsequent experiment, daily airborne conidia of *P. pannosa* were tracked in the peach orchard located in Mollerussa using a Hirst-type, 7-day recording volumetric spore sampler VPPS 2000 (Lanzoni) (Fig. 4.1). The spore sampler was placed from 6

Figure 4.1. Hirst-type, 7-day recording volumetric spore sampler VPPS 2000 (Lanzoni) (Drawing by Quim Pallarès, 2020).

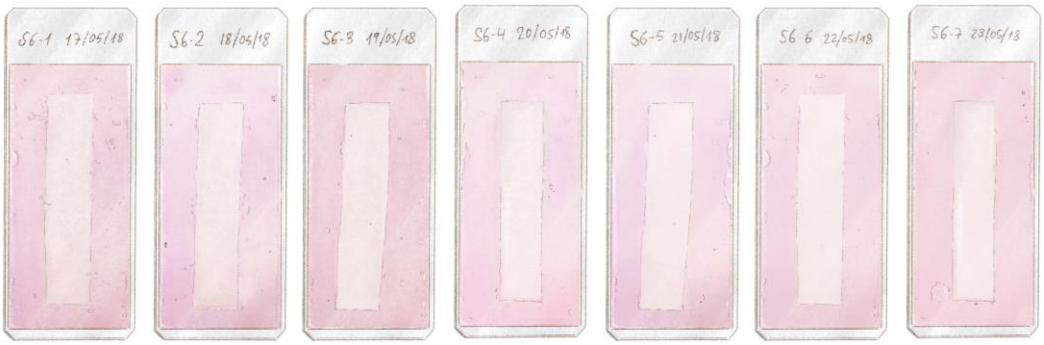

Figure 4.2. Daily fragments from aerobiological sampler used for microscopic observation (Drawing by Quim Pallarès, 2020).

April to 10 July 2018 in the vicinity of trees that had shown PPM infections in previous years. Sampler orifice was located 0.5 m above ground level and the volumetric ratio adjusted at 10 L air min^{-1}.

Plastic tapes treated with the silicon solution were replaced weekly and taken to the laboratory for subsequent processing. Exposed tapes were cut into seven 48-mm pieces, each one corresponding to 1-day period. Each daily fragment was further cut longitudinally into two equal-sized segments: one half-part was used for microscopic observation whereas the other half was used for the qPCR analysis. For microscopic observation, samples were processed as proposed by the Spanish Aerobiological Network (REA) (Galán et al., 2007): each daily fragment was stained with acid lactofuchsin and mounted on a glass slide (Fig. 4.2). Microscope samples were examined using a microscope (model Eclipse E400, Nikon Corporation, Toquio, Japan) at 250× and only conidia that were morphologically compatible with *P. pannosa* were considered, i.e. conidia containing fibrosin refractive bodies (Braun et al., 2002), and measuring 12-15 × 20-27 µm (Horst and Cloyd, 2007). Final number of conidia per day was estimated from the examined surface (about 45% of the total strip surface) and expressed as conidia m^{-3}. For qPCR quantification, daily samples were cut into six equally-sized pieces and put into a 1.5 mL Eppendorf tube. DNA was extracted and amplified according to the protocols described in this study. Additionally, a positive control from CS 4 (dilution 10^{-2}) was included in the qPCR plate. The quantification of conidia for each daily sample was calculated using the standard curve obtained from the CS 4 suspension placed on a plastic tape. Samples matching at least one of the following criteria were excluded from further

conidia quantification: i) Only one technical replicate with acceptable values of C_q (C_q < 35 cycles) and melting temperature (T_m = 88 °C), ii) replicates with a mean T_m highly different from 88 °C, and iii) replicates with acceptable C_q and Tm values but showing a standard deviation (SD) higher than 0.5 between technical replicates. Cases i) and ii) resulted in a negative quantification (zero) whereas case iii) resulted in an undetermined value (missing). Quantification of trapped conidia using qPCR was expressed in conidia m^{-3} after proper conversion factors were applied on the values obtained from the standard curve analysis (expressed in conidia mL^{-1}). Conversion factors considered were: *i*) the volumetric ratio of sampler (10 L air min^{-1}), *ii*) the final volume of DNA extracted from daily samples (100 µL), and *iii*) the standard curve data obtained from the CS 4 conidial suspension placed on a plastic tape.

Case 2: Detection of the primary inoculum of P. pannosa *in host plant material*

Three biological replicates of different peach plant parts (leaves, leaf buds, floral buds and twig barks) were detached from each collected branch. Samples were carefully examined using a stereomicroscope (10×) to detect symptoms and signs compatible with *P. pannosa* infections. When those compatible structures were detected, an optical microscope was used to ascertain the presence of mycelium and chasmothecia, and a sample (about 12 mg) was taken for DNA extraction and further qPCR amplification. Sample weights according to sample origins were as follows: 11.97 ± 0.19 mg for leaves, 12.07 ± 0.23 mg for foliar buds, 12.43 ± 0.22 mg for floral buds, and 11.52 ± 0.16 mg for twig barks. Samples were separately put into 1.5 mL Eppendorf tubes and DNA extraction and qPCR quantification were done according to the methods described in this study. Three technical replicates per biological sample were run and two types of negative controls were used: DNA from *in vitro*, no symptomatic *P. persica* leaves and deionized water template. The quantification of DNA for each sample was calculated using the DNA 1 solution.

4.3.9. Statistical analyses

Output data corresponding to the fitted qPCR standard curves equations, including intercept, slope, r^2 and AE, were obtained from the software of the thermal cyclers used

in this study. Further statistical analyses were performed using the *stats* package included in R (R Core Team, 2019). The analysis of covariance was used to compare the regression equation slopes of the standard curves when appropriate. Lineal modelling including correlation and regression analyses was used to study the relationship between the amounts of trapped conidia in aerobiological samples estimated through either the microscopical or qPCR approaches. Statistical significance in all analyses was declared at $\alpha < 0.05$. Values of mean ± standard error of the mean are reported when appropriate.

4.4. Results

4.4.1. Primer design

The design of *P. pannosa* specific primers was performed through the alignment of the ITS region of 31 unique sequences of powdery mildew fungi (Table 4.1). Several nucleotide polymorphisms were detected among species at two polymorphic regions that allowed the design of forward and reverse primers at those sites. The forward and reverse primers were named PpanITS1-F and PpanITS1-R, respectively, and amplified a region of 155 bp at the ITS 1 region. The amplified product showed a melting temperature at 88 °C. Sequences for the PpanITS1-F and PpanITS1-R primers were 5'-CCACCCGTGTGAACTGAATT-3' and 5'-CCGTTGTTGAAAGTTTTACTTATTAAGTT-3', respectively.

4.4.2. Specificity and sensitivity of the primer pair PpanITS1-F/PpanITS1-R

Specificity tests were performed using the primer pair PpanITS1-F/PpanITS1-R for the amplification of several *Podosphaera* species. Only DNA from known *P. pannosa* positive controls (Ppan53 and Ppan92) were amplified with the specific primers, showing a single peak around 88°C in the melting curve analysis, whereas no amplification was observed for other non-*P. pannosa* samples. In order to discard false positives and to confirm *P. pannosa* identification, amplified products were checked in 2% agarose gels and further sequenced (*data not shown*).

P. pannosa was detected and quantified in two independent DNA samples (DNA 1 and DNA 2) obtained from *P. pannosa* conidia. A clear linear relationship was obtained between the C_q values and the logarithm of DNA concentrations (Fig. 4.3a). Parameters for the standard curves for DNA 1 and DNA 2 are described in Table 4.2. Both equations (Fig. 4.3a) had significant slopes ($P < 0.001$) of similar gradient ($P = 0.56$). Three independent conidia suspensions were also quantified using qPCR (Fig. 4.3b). The standard regression curve parameters for conidia suspensions CS 1, CS 2 and CS 3 are described in Table 4.2. Slopes for the equations of the three conidia suspensions did not show significant differences among them ($P = 0.72$). After these experiments, an arbitrary LOD was established at 2.81 ± 0.49 pg DNA μL^{-1} and 6 ± 2 conidia mL^{-1}. Estimated LOB

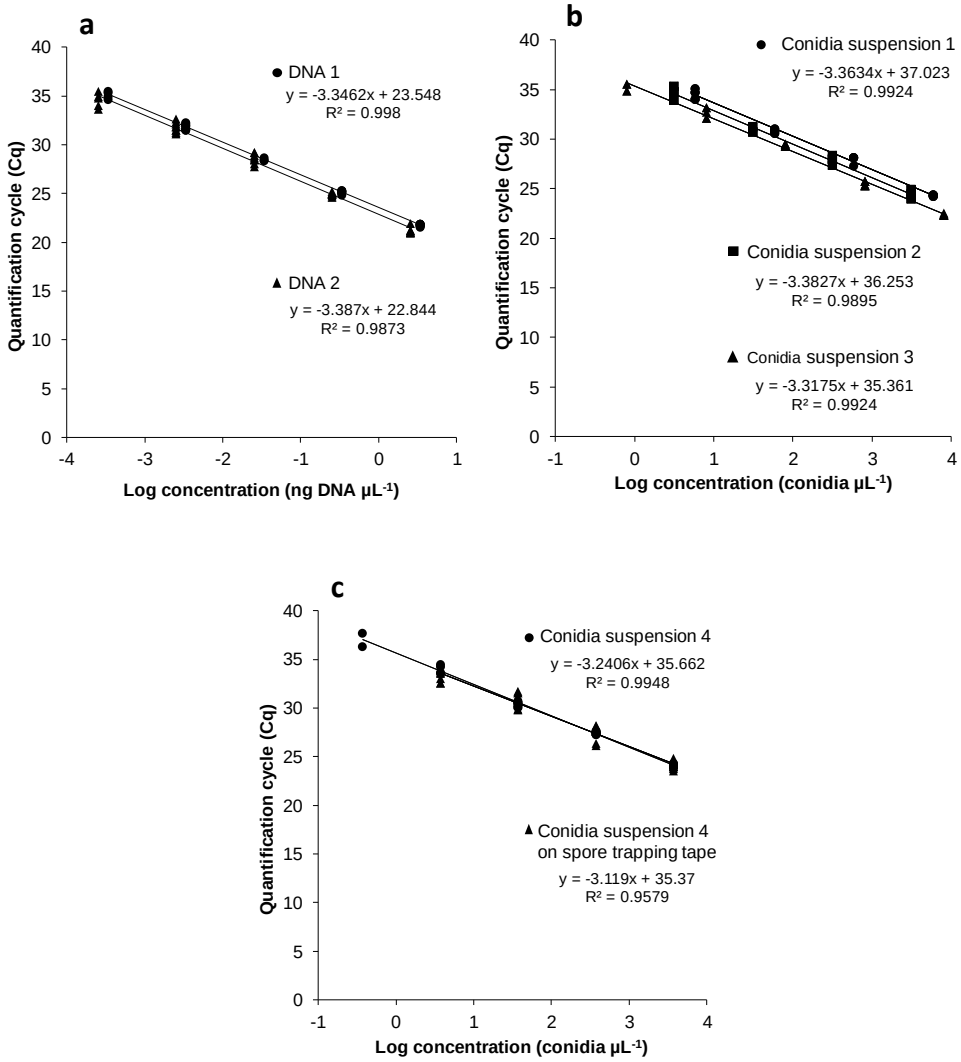

Figure 4.3. Standard regression curves obtained from qPCR assays involving 10-fold serial dilutions from a) DNA extracted from conidia suspensions, DNA 1 and DNA 2; b) conidia suspensions CS 1, CS 2 and CS 3; c) conidia suspension CS 4 either placed or not on a spore-trapping tape.

Table 4.2. Parameters for the standard curves obtained in this study (see text for details). LOD and LOQ parameters are expressed as pg DNA μL^{-1} for DNA 1 and DNA 2 samples, and as conidia mL^{-1} for conidia suspensions (CS). [a]: LOD, Limit of detection. [b]: Cq LOD, Quantification cycle at LOD. [c]: LOQ: Limit of quantification. [d]: Cq LOQ, Quantification cycle at LOQ.

Standard curve name	Intercept	Slope	r^2	Efficiency (%)	LOD[a]	C_q LOD[b]	LOQ[c]	C_q LOQ[d]
DNA 1	23.548	-3.346	0.998	98.99	2.31	31.78	6.86	30.79
DNA 2	22.844	-3.387	0.987	97.35	3.29	30.38	8.94	29.78
CS 1	37.023	-3.363	0.992	98.30	5.30	35.89	9.57	34.51
CS 2	36.253	-3.383	0.990	97.52	2.90	34.68	7.58	33.28
CS 3	35.361	-3.318	0.992	100.00	10.50	31.98	16.65	31.31
CS 4	35.683	-3.248	0.995	103.17	6.90	32.94	17.21	31.66
CS 4 tape	35.370	-3.119	0.958	109.22	7.20	32.69	40.57	30.35

values, as described by Armbruster and Pry (2008), are not reported for all the above qPCR assays since they were lower than LOD values in all cases. Mean C_q corresponding to LOB was established at 35 cycles for all the reactions performed in this study.

4.4.3. Validation of the specific primer pair PpanITS1-F/PpanITS1-R

Case 1: Detection of P. pannosa *airborne inoculum in spore traps*

Ten-fold dilution series from suspension CS 4, with or without placing on spore-trapping tapes, were successfully detected until 10^{-3} dilution. The standard regression curve parameters for both types of samples are described in Table 4.2. Slopes for both standard curves did not show significant differences ($P = 0.29$) (Fig. 4.3c). Regarding the detection of *P. pannosa* in periodical air samplings, 12 daily samples were discarded (10 samples with technical replicates showing SD > 0.5, and two samples with lesser than two acceptable technical replicate each), and 32 daily samples were negative, from a total of N = 96. The fungus was successfully detected and quantified in the spore-trapping tape samples collected from April to July 2018 (Fig. 4.4). Propagules of *P. pannosa* were firstly detected at the beginning of the third sampling week, corresponding to mid-April.

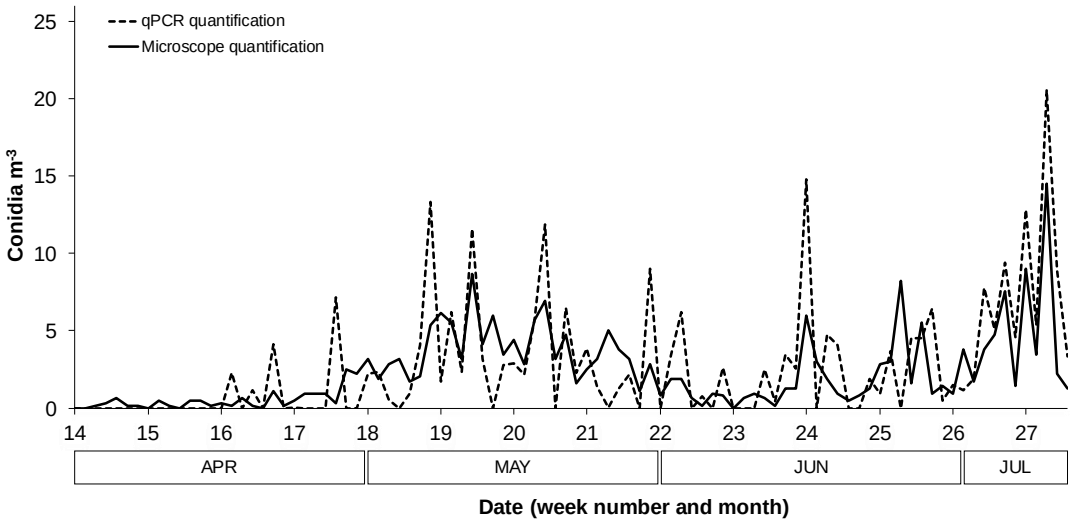

Figure 4.4. Daily values of airborne conidia trapped using a volumetric spore sampler (conidia m^{-3}), estimated either from microscopic examination (solid line) or qPCR quantification (dashed line). Time expressed as week number of the year (2018) and month.

Thereafter, abundance of airborne conidia was fluctuating throughout the season, with spontaneous peaks, and achieved the seasonal maximum (14.5 conidia m^{-3} from microscope observations and 21.0 conidia m^{-3} from qPCR analysis) by mid-July. Both estimation methods, either by microscope observation or qPCR analyses, followed a similar time pattern in conidia detection (Fig. 4.4.). Furthermore, a linear regression equation ($P < 0.001$, $r^2 = 0.5957$) was adjusted between the microscopic and qPCR variables (Fig. 4.5), with the following parameters: $y = 0.766 + 0.508x$, where y = conidia quantified through microscopical observation, and x = conidia quantified through qPCR. From the regression equation, lower levels of conidia were observed (about 50%) through microscope as compared to qPCR quantification. We hypothesize that those low recordings from visual identifications, as compared to molecular quantifications, could be explained by: *i*) large amounts of particles (dust, pollens, other fungal spores…) in the trapping tape which could have interfered with the microscopical identification of *P. pannosa* conidia in the samples, and *ii*) an eventual degradation of *P. pannosa* conidia, thus making difficult the morphological identification of the species.

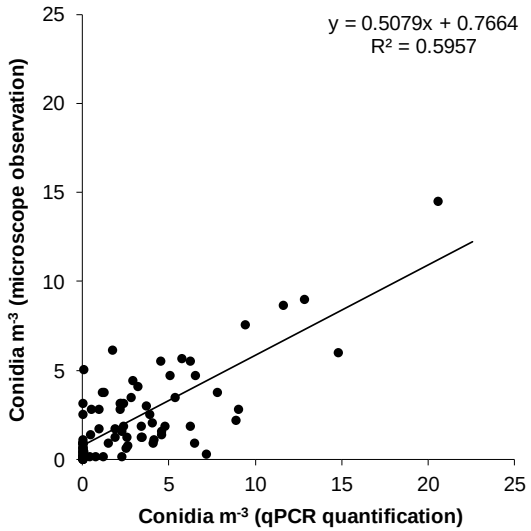

Figure 4.5. Correlation between the estimated amounts of conidia (conidia m^{-3}) obtained through qPCR quantification (x) and microscopy examination (y) of airborne conidia trapped in a peach orchard (Mollerussa, Catalonia, Spain) in the period April to July, 2018 (N = 96 days).

Case 2: Detection of the primary inoculum of P. pannosa *in host plant material*

The detection and quantification tests using leaves, twigs, and foliar and floral buds samples were conducted using the detection threshold C_q LOD = 30.79, as determined in the analytical sensitivity test. Trees in orchards located in Alcarràs and Cabrils did not show any visual symptom of PPM infection in 2017. Furthermore, none of samples collected in those orchards showed positive qPCR detections of *P. pannosa* (*data not shown*). Regarding the samples collected in Mollerussa, the pathogen was not detected from dried leaf and floral bud tissues (Table 4.3). In contrast, leaf buds showed to be infected with the pathogen on average in 42.5% cases (range: 25 to 75%), although this finding could not be confirmed through visual examination as no distinguishable fungal structures could be detected under the stereomicroscope. Mean DNA concentration of PPM in sampled leaf bud tissues ranged from 0.02 to 3.90 ng g^{-1} dried tissue. All twig samples from the orchard located in Mollerussa showed clear PPM symptoms on their surface. Examined samples showed one to seven visible lesions with symptoms, 0.6 to 216 mm in length, and with the presence of chasmothecia in 60% of samples (84 out of 140 total examined lesions). Mean DNA concentration of PPM in sampled twig tissues ranged from 37.74 to 96.27 ng g^{-1} dried tissue, about 50 times greater than in foliar bud tissues.

Table 4.3. Detection and quantification of *Podosphaera pannosa* in different plant tissues (N = 8 per tree) collected in a peach orchard located in Mollerussa, Spain.

Plant part	Tree	No. Positive detections[a]	C_q[b]	Fungal DNA biomass ($ng \cdot g^{-1}$ dry tissue)
Leaf bud	1	2	29.06 ± 0.13	0.02
	2	3	22.19 ± 0.50	2.11
	3	4	21.30 ± 2.80	3.90
	4	6	23.08 ± 3.24	1.15
	5	2	25.94 ± 2.39	0.16
Floral bud	1	0	> C_q LOD[c]	n.d.[d]
	2	0	> C_q LOD	n.d.
	3	0	> C_q LOD	n.d.
	4	0	> C_q LOD	n.d.
	5	0	> C_q LOD	n.d.
Twig	1	8	17.18 ± 1.75	69.36
	2	8	18.07 ± 1.26	37.74
	3	8	17.62 ± 2.62	51.37
	4	8	16.71 ± 2.63	96.27
	5	8	18.05 ± 1.56	38.15
Leaf	1	0	> C_q LOD	n.d.
	2	0	> C_q LOD	n.d.
	3	0	> C_q LOD	n.d.
	4	0	> C_q LOD	n.d.
	5	0	> Cq LOD	n.d.

[a]**:** Number of samples with positive detection of *P. pannosa*. [b]**:** Cq, Quantification cycle, expressed as mean ± std. err. [c]: Cq LOD, Cq of sample greater than Cq determined for the limit of detection (LOD). [d]: n.d., not determined.

4.5.Discussion

A qPCR-based protocol was developed for the specific detection and quantification of *P. pannosa* in biological samples. A specific primer pair, named PpanITS1-F/PpanITS1-R, was designed and successfully validated using both artificially-prepared (e.g. conidia suspensions) and environmental samples (e.g. spore-trapping tapes from a volumetric air sampler, and different plant tissues). To the best of our knowledge, this is the first time that a molecular qPCR-based tool for the detection

and quantification of *P. pannosa* was developed. The primer pair targeting the ITS region designed in this study proved to be highly specific, as indicated by the positive detection of *P. pannosa* DNA and the negative amplification of DNA from other *Podosphaera* species, either from Rosaceae hosts (*P. aphanis*, *P. clandestina*, *P. leucotricha*, and *P. tridactyla*) or non-Rosaceae hosts (*P. fusca*, and *P. macularis*). The ITS region has been shown to be appropriate for studying genetic variation at species level in powdery mildew fungi belonging to the genus *Podosphaera* (Ito and Takamatsu, 2010). Thus, few nucleotide differences in the ITS sequences could be associated with *Prunus* specialization within the *Podosphaera tridactyla* complex (Cunnington et al., 2005). Moreover, Leus et al. (2006) showed that one single nucleotide difference in the ITS sequences of *P. pannosa* isolates distinguished different host-specific groups on *Rosa* and *Prunus* species.

Regarding the detection thresholds obtained in this study, they were set at 2.81 ± 0.49 pg DNA $\mu L^{-1}$and 6 ± 2 conidia mL^{-1}. Previous studies on the detection threshold for other powdery mildew species have been reported elsewhere. Thus, Falacy et al. (2007) reported 10 conidia as the detection threshold for the grapevine powdery mildew, *Erysiphe necator*, in a single PCR reaction mixture. In addition, Sholberg et al. (2005) reported that 20 to 30 conidia of *P. leucotricha*, the apple powdery mildew, could be detected using a DNA macroarray. The results obtained in this study are therefore comparable to those of previous studies based on different analytical techniques.

The detection and quantification of airborne *P. pannosa* conidia using a volumetric air sampler coupled with the qPCR method was successfully performed. When compared with the microscopical observation of trapped conidia on plastic tapes, the molecular technique was able to determine the period when *P. pannosa* conidia are present in the air, as similarly done with microscope examination. In addition, the qPCR method was successfully used to obtain a reliable quantification of airborne conidia, as shown by the high correlation found between the quantifications conducted through microscope and molecular approaches. Furthermore, molecular detection using specific primers allowed us to overcome some important limitations which are not uncommon in the microscope examination of aerobiological samples: *i*) the required time of handling and posterior microscope observation of samples (Dung et al., 2018), *ii*) the morphological similarity of conidia from different powdery mildew species (Braun,

1987), which makes difficult species differentiation and therefore demands trained skills to analysts, and *iii)* the inaccurate identification due to co-location of overlapping structures that can disfigure spore morphology (Mahaffee and Stoll, 2016). Thus, the present study reports on a rapid and reliable detection and quantification method for PPM airborne propagules. We additionally hypothesize that low quantifications based on visual identifications, as compared to molecular quantifications, may be due to i) the occassional large amounts of particles (dust, pollens, other fungal spores…) present in the trapping tape which could have interfered with the microscopic identification of P. pannosa conidia, and ii) an eventual degradation of P. pannosa conidia, thus making difficult the morphological identification of the species.

The detection and quantification of pathogen overwintering structures in different plant tissues was also studied. Chasmothecia of *Podosphaera* species perennate in winter as fruiting bodies immersed in the mycelium attached to the host (Jarvis et al., 2002). In *P. clandestina*, on sweet cherry, chasmothecia survive on senescent leaves, on fallen leaves on the orchard floor and in tree bark crevices (Grove, 1991). In the case of *P. pannosa*, Ogawa and English (1991) reported the formation of chasmothecia on twigs and stems, most frequently around the thorns on rose. In the case of peach infections, several authors suggested that the fungus overwinters as mycelium deep within the buds, from where infected shoots arise after the spring budburst (Weinhold, 1961; Yarwood, 1957). However, to date, no molecular detection of PPM in overwintering structures had been described. In our study, the use of the specific primer pair PpanITS1-F/PpanITS1-R confirmed that the pathogen is mostly present on the surface of twigs, where mycelium and chasmothecia were also clearly detected by visual examinations. Besides twigs, *P. pannosa* was detected in lower concentrations in foliar bud tissues, where the pathogen mycelium was previously detected using a stereomicroscope (Weinhold, 1961). Conversely to what we expected, no positive detection of *P. pannosa* from autumn leaves was confirmed. In that scenario, first spring infections could be developed either from airborne ascospores released from chasmothecia present on twigs and shoots, or from latent mycelium inside bud tissues.

In recent years, the study of epidemiology of air-borne pathogens has increasingly been based on the pathogen detection and quantification by molecular-based techniques, which helped to answer complex questions regarding the biology of tree fruit pathogens

(Michailides et al., 2005). The methodology developed in our study can be applied in the study of the PPM epidemiology, and therefore it can help in improving the management of this disease through the early detection and quantification of the pathogen.

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5. Fine mapping and identification of candidate genes for the peach powdery mildew resistance gene *Vr3*

5. Fine mapping and identification of candidate genes for the peach powdery mildew resistance gene *Vr3*

5.1. Abstract

Powdery mildew is one of the major diseases of peach (*Prunus persica)*, caused by the ascomycete *Podosphaera pannosa*. Currently, it is controlled through calendar-based fungicide treatments starting at petal fall, but an alternative is to develop peach resistant varieties. Previous studies mapped a resistance gene (*Vr3*) in interspecific populations between almond ('Texas') and peach ('Earlygold'). In this study Vr3 has been fine mapped to a genomic region of 270 kb with 27 candidate genes. To find evidence supporting one of these positional candidate genes as being responsible of *Vr3*, we analyzed the polymorphisms of the resequences of both parents and used near-isogenic lines (NILs) for expression analysis of the positional candidate genes in symptomatic or asymptomatic leaves. Genes differentially expressed between resistant and susceptible individuals were annotated as a Disease Resistance Protein RGA2 (*Prupe2G111700*) or an Eceriferum 1 protein involved in epicuticular wax biosynthesis (*Prupe2G112800*). Only *Prupe2G111700* contained a variant predicted to have a disruptive effect on the encoded protein and was overexpressed in both heterozygous and homozygous individuals containing the *Vr3* almond allele, compared with susceptible individuals. This information was also useful to identify and validate molecular markers tightly linked and flanking *Vr3*. Additionally, the NILs used in this work will facilitate the introgression of this gene into peach elite materials, alone or pyramided with other known resistance genes such as peach powdery mildew resistance gene *Vr2*.

5.2. Introduction

Peach [*Prunus persica* (L.) Batsch] is an important stone fruit crop in temperate regions: more than 24 million tons of peaches, nectarines and flat fruits produced worldwide in 2018 (FAOSTAT, 2020). Most commercial peach cultivars are susceptible to different pests and diseases. One of the most important being peach powdery mildew (PPM) (Pascal et al., 2010, 2017), caused by the ascomycete *Podosphaera pannosa* (Dirlewanger et al., 1996). To our knowledge, all peach commercial cultivars are susceptible PPM to a variable degree. The pathogen infects the fruits, leaves, buds and shoots, where mycelium develops as white-grayish spots on the surface, and heavy infections on fruit and leaves may induce their premature fall (Dirlewanger et al., 1996;

Foulongne et al., 2003). PPM can be controlled effectively through foliar fungicide applications, applied regularly every 7 to 14 days during the year (Grove, 1995) from prebloom to the end of harvest (Pascal et al., 2010). Recently, a predictive model for disease progress has been described (Marimon et al., 2020), which included a threshold alert to initiate fungicide programs at early infection set.

An environmentally safe alternative to fungicide applications is the development of resistant varieties through plant breeding (Pascal et al., 2017). Little information is currently available on breeding for resistance to pests and pathogens in stone fruit crops (Aranzana et al., 2019), probably due to the length of time required to introduce genes from exotic sources in perennial plants. Two descriptions of PPM major resistance genes have been published. Pascal et al. (2010, 2017) described a monogenic dominant locus in linkage group 8 (G8), named *Vr2*, from the peach rootstock cultivar 'Pamirskij 5'. In the peach cross-compatible *Prunus* species almond (*P. dulcis*), Donoso et al. (Donoso et al., 2016) mapped a monogenic powdery mildew resistance gene in G2 in two interspecific populations between almond 'Texas' and peach 'Earlygold', with the dominant resistance allele from 'Texas'. The gene, named *Vr3*, was located in a genomic region of 2.7cM, where 187 genes were annotated in the peach reference genome. Other resistance sources are quantitative trait loci (QTLs) controlling PPM tolerance. Pacheco-Cruz et al. (2009) described a source of tolerance from peach 'OroA' in G7 that could explain up to 8% of the phenotypic variation, and several QTLs have been identified in *P. davidiana* (Dirlewanger et al., 1996; Foulongne et al., 2003). Furthermore, Dabov (1983) found that in *P. ferganensis*, *Vr* and *Sr* alleles conferred high and low resistance, respectively. PPM resistance has also been associated with leaf glands, being linked to the E/e locus controlling the presence and shape of leaf glands in peach (Saunier, 1973)..

Peach is one of the best genetically characterized species among Rosaceae, with many major genes and QTLs described for different agronomic traits (Arús et al., 2012). After release of the *Prunus* reference genome (Verde et al., 2013) and with the arrival of high-throughput sequence technologies it became easier to characterize genomic regions identified in fine mapping projects and so reduce the number of candidate genes for many traits (Aranzana et al., 2019). Functional validation of these genes is the main bottleneck in *Prunus* due to its recalcitrant regeneration behavior 'in vitro' (Zong et al., 2019).

Our objectives were to fine map the *Vr3* gene responsible for PPM resistance to obtain a reduced number of candidate genes and to characterize them by analyzing the polymorphisms of parent resequences and with expression analysis. The outcome of this study would provide valuable information on the *Vr3* candidate genes and the resistance mechanism, and provide better markers for marker-assisted selection (MAS) in peach breeding programs.

5.3.Materials and methods

5.3.1. Plant material

From 2013 to 2018, several progenies of different generations derived from 'Texas' and 'Earlygold' crosses were screened for a PPM resistance fine mapping approach: F2 (named T×E, with 111 individuals), BC1 with 'Earlygold' as the recurrent parent (named T1E, with 189 individuals) and BC2 also with 'Earlygold' (with 51 screened individuals, from E2T-031, E2T-092 and 11P15 families). Other individuals used in the fine mapping approach were obtained from the open pollination of different individuals and families: 218 individuals from 'MB1.37' (the 'Texas' × 'Earlygold' F1 individual used for the construction of the T×E population), 329 individuals from T1E progeny families (named 72P18, 74P18, 84P18 and 93P18), and 217 individuals from BC2 progeny families (including 15P15, 19P15 and 25P15 families). Additionally, some recombinant individuals were obtained from crosses with several peach commercial cultivars and individuals from different 'Texas' × 'Earlygold' generations. This included 81 individuals derived from 'Nectatop' crossed with different BC1 and BC2 individuals. All the trees described (Table 5.1) were planted at IRTA facilities located in Cabrils (41º31'7"N, 2°22'34"E), Caldes de Montbui (41º36'47"N, 2º10'12"E), Gimenells (41º39'22"N, 0º23'26"E) and Mollerussa (41º37'07"N, 0º51'60"E). Orchards were not treated with fungicides to allow natural pathogen infections.

Regarding the gene expression analysis, three groups of four individuals from near-isogenic lines coming from open pollination of a BC2 individual were used. A first group contained only one introgression from 'Texas' almond in homozygosis in the *Vr3* genomic region, another with one introgression in heterozygosis in the same region, and

Table 5.1. Individuals used in fine mapping of the *Vr3* PPM resistance gene. *OP: Open Pollination

Year	Population type	Family code	Female parent	Male parent	Individuals	Recombinant individuals	Location
-	F1	MB1.37	'Texas'	'Earlygold'	1	-	Caldes de Montbui
-	F2	TxE	'MB1.37'	'MB1.37'	111	3	Cabrils / Gimenells
-	BC1	T1E	'MB1.37'	'Earlygold'	189	3	Cabrils / Gimenells
2014	BC2	E2T-031	'Earlygold'	T1E-031	26	2	Caldes de Montbui/Mollerussa
2015	F2	TxE	'MB1.37'	'MB1.37'	150	5	Caldes de Montbui
2015	BC2	11P15	'Earlygold'	T1E-031	14	1	Caldes de Montbui
2015	BC3	15P15	E2T-031-005	OP	26	11	Caldes de Montbui
2015	BC3	19P15	E2T-092-002	OP	127	3	Caldes de Montbui
2015	BC3	25P15	E2T-092-021	OP	64	1	Caldes de Montbui
2015	BC2	-	T1E-042	'Nectatop'	4	1	Caldes de Montbui
2015	BC2	-	'Nectatop'	T1E-03	1	1	Caldes de Montbui
2015	BC2	-	'Sweetlove'	T1E-03	2	1	Caldes de Montbui
2016	BC2	E2T-092	'Earlygold'	T1E-092	11	1	Caldes de Montbui / Mollerussa
2016	BC3	14P16	'Nectatop'	E2T-092-025	22	6	Mollerussa
2016	BC3	1114	P01F002A054	E2T-092-025	28	11	Mollerussa
2017	BC3	44P17	'Nectatop'	E2T-092-025	54	2	Gimenells
2017	BC2	51P17	'MB1.37'	OP	218	6	Caldes de Montbui
2018	BC2	72P18	T1E-021	OP	33	2	Caldes de Montbui
2018	BC2	74P18	T1E-024	OP	12	1	Caldes de Montbui
2018	BC2	84P18	T1E-040	OP	25	3	Caldes de Montbui
2018	BC2	93P18	T1E-064	OP	259	2	Caldes de Montbui
-	BC1	T1BT	'MB1.37'	'Big Top'	21	1	Mollerussa

the final one with no almond introgression in the *Vr3* genomic region but including an almond introgression in G3. The four individuals of each group were considered as independent biological replicates for each case, and two technical replicates of three young leaves measuring 3-4 cm were sampled from sight-heighted and sun-exposed branches. The samples collected were symptomatic and visually asymptomatic leaves. In addition, presence of the pathogen in the field was assessed through detection of airborne *P. pannosa* propagules captured with a volumetric spore sampler VPPS 2000 (Lanzoni, Bologna, Italy) and using a specific qPCR-based protocol developed in a previous study (Chapter 4, this thesis).

5.3.2. Phenotypic evaluation

All recombinant individuals used in this study were phenotyped for PPM susceptibility every year between 2016 and 2019. Each year, PPM was phenotyped twice, first in May or June (corresponding with the developing stage of infection) and then in September (corresponding with the end of infection but with symptoms still noticeable). Young leaves from a minimum of four differently oriented branches were examined for PPM symptoms. A given individual was scored as resistant when total absence of PPM symptoms on leaves was confirmed throughout the monitoring period. In contrast, trees showing PPM symptoms in at least one year were considered susceptible. Trees for all the experimental orchards evaluated for PPM resistance were not treated, to ensure infection and serve as positive controls.

5.3.3. Vr3 *fine mapping*

Genomic DNA from the individuals described in Table 5.1 was extracted from young leaves using a modification of the CTAB protocol (Doyle and Doyle, 1990), omitting the final RNAse step. DNA quality and concentration were checked and quantified using a DNA spectrophotometer (Nanodrop Technologies, Wilmington, USA).

New markers (Table 5.2 and 5.3), including SSRs (Simple-Sequence Repeat Markers), Indels (Introgression and Deletion markers) and SNPs (Single Nucleotide Polymorphism) were designed using resequencing data of 'Texas', 'Earlygold' and 'MB1.37'. Library preparation and 2 x 100 bp pair-end genome sequencing data was obtained by Serra (2017) using HiSeq2000 sequencer (Illumina Inc.). High quality 220-480 bp size fragmented DNA was ligated to Illumina paired-end adaptors. Adapter removal was done using AdapterRemoval v1.5.2 (Lindgreen, 2012). Only reads with a minimum size of 35 bp and a mean quality of 25 were kept. High quality reads were mapped to the peach reference genome using BWA v0.7.5 (Li and Durbin, 2009) with default parameters. The SAM file obtained was converted to BAM using BAMTools v0.1.19 (Li et al., 2009) and reads mapping to more than one position or reads from PCR duplication events were excluded from the alignment. Raw Illumina data for 'Texas',

'Earlygold' and 'MB1.37' are available at the European Nucleotide Archive (ENA) under the accession numbers ERS4540423, ERS3508161 and ERS4540424, respectively.

Table 5.2. Sequence information of Indel and SSR primers designed and used in the fine mapping of *Vr3*. For each marker, the location is identified from the P. persica v.2.0 reference genome (Verde et al., 2017). [a] Indel marker. [b] SSR marker.

Marker name	Forward 5'-3' sequence	Reverse 5'-3' sequence	Marker location (bp)
CPP08188 [b]	AAAAGGGGTTTCGGAAGATG	ATGGCATCTCGTCACACTTG	14,103,569
Indel14417 [a]	TTTCAATTTGGGTGGTTTGC	CCCAACTCCGAAAAATTCAA	14,417,443
Indel15174 [a]	ATTCACCTTCATTGGCTTGG	AGGAGATTGTGGTTGGTTCG	15,174,881
Indel16614 [a]	TTTAACAGGTTGAGATGGTGGT	TGGGGCAGAATCTTTATCCA	16,614,476
Indel16724 [a]	CCACCAGTGAGCCATCAAC	GGCGTTGACTCCATACGAAA	16,724,717
Indel16748 [a]	AAGGCTCCCACTGAATGATG	CCTGCAATGTGGTTGACAAT	16,748,613
CPP08472 [b]	GTCATGCAGACCTCCAATCC	TTGCAGGCTAGGCTAGAGAAA	16,484,677
Indel16912 [a]	AAGTCTAGTTCCAGCACACC	ACAAGTAAGGGTGTTCATCCAT	16,912,809
Indel16949 [a]	ACTGTTTATTGTCCTGGATGCA	CTTCAAGCCCGTGACTAGAGT	16,948,818
Indel17030 [a]	TGACTCTACAGCAGGAAAAGGA	CGATGCTAAAGGTATGGCGG	17,030,526
Indel17032 [a]	CTCCACAAGTGCAGCCTACA	TGAGAACCACCCTATGATTTTGT	17,032,695
Indel17050 [a]	GTGCAGGACATCACGGAGAA	TGCGACACACCTGAACGTTA	17,050,734
Indel17053 [a]	AGACAAGGCACATGACAGCT	GTTGGTTGTTGCTTGAGGACC	17,053,061
Indel17061 [a]	GGCTGTACTCGCGGATATGA	CAAGAGGAGTCCATGGCCAG	17,061,201
Indel17186 [a]	AAGGGGGTGTCAATGTCAAG	TGTGGGATACAAATTCCACAAG	17,186,620
Indel17181 [a]	TGTTTTGATGAAGGCGATCGA	TGGAAGGTTGGAAGGAGCAT	17,181,256
CPP17182 [b]	TCTCTACTCTTACAGGCGAGC	GGGTTGTGGATGGAAGTAGC	17,182,435
CPP17184 [b]	GTAGGTTGCAGTTCGACACG	GACACCACAGTACCCACCTT	17,184,920

Table 5.3. Sequence information of SNPs used in the fine mapping of *Vr3*. For each SNP, the location is identified from the P. persica v.2.0 reference genome (Verde et al., 2017).

	KASPar forward primers (without tail sequences)		Common reverse primer	Location (bp)
SNP16932	AGTGATTCTGCAAAGTGGTGGAGA	GTGATTCTGCAAAGTGGTGGAGG	TGGCACGGCTATCAGGCATAGAAA	16,932,290
SNP16940	TGATCGAGAATAAATTCAGATGTTAAAGAATT	ATGATCGAGAATAAATTCAGATGTTAAAGAATA	TGTTGACTTATTGCTCCTTCAGTCTACTT	16,940,264
SNP17180	CTCACCAATTTATAAGAGTTTGGTATGTT	CTCACCAATTTATAAGAGTTTGGTATGTC	CGGATGTCCTGCTCCCTTATTGAAT	17,180,556
SNP17184	CATATACATCCCAGAGGCCCATATA	ATATACATCCCAGAGGCCCATATG	ACCTCACCACATACTTCCATTGTTTTCTT	17,184,692

Polymorphisms in these resequencing data were detected using Integrative Genomics Viewer software (Robinson et al., 2011). SSRs and Indels (Table 5.2.) were designed from the flanking sequences of the polymorphisms using Primer 3 (http://primer3.ut.ee, v4.1.0 (Untergasser et al., 2012) with the default parameters. PCR reactions were in a final volume of 10 µL containing 200 ng of genomic DNA, 1 µL 10× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (10mM), 0.2 µM of each marker and 1 U of BIOTaq (Bioline, London, UK) and HPLC H_2O to reach the final volume. PCRs were performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, CA, USA), with the following conditions: initial denaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 15 s, primer annealing at specific temperature for each primer for 15 s, extension at 72 °C for 30 s or 1 minute if product expected size was higher than 500 bp, and a final extension at 72°C for 5 min, and a final extension at 72°C for 5 min. For Indels less than 40 bp and for SSRs, forward primers were designed with a generic fluorochrome sequence at the 5' ends (FAM, VIC, NED or PET), named 'tag primers' (Hayden et al., 2008). PCR reaction conditions for these 'tag primers' were the same as described above, with the following modifications: 0.4 µM of each marker and 0.20 µM for each 'tag' primer pair. PCR amplifications were with an initial denaturation at 94°C for 1 min, followed by a total of 60 cycles with the profile: 20 cycles for 15 s at 94°C, 15 s at 63°C, and 30 s at 72°C, followed by 40 cycles for 15 s at 94°C, 15 s at 54°C, and 30 s at 72°C, followed by a final extension step of 5 min at 72°C. PCR products were added to 12 µL of deionized formamide containing 0.35 µL of GeneScan500 LIZ size standard (Applied Biosystems). The mixture was heated at 94°C for 3 min and capillary electrophoresed using an ABI Prism 3130xl automated sequencer (Applied Biosystems). GeneMapper v5.0 software (Applied Biosystems) was used for SSR allele sizing. For Indels larger than 40 bp, standard primers were designed flanking the polymorphism and results were observed in ethidium bromide-stained agarose gels (1.8%) under UV light. From all the SSRs and Indels designed, only those showing clear segregation among the parents were kept for the fine mapping approach, avoiding those with preferential amplification for peach alleles or that did not amplify. Otherwise, primers for SNPs detection (Table 5.3) were designed using the Primer Picker Lite tool from KASPar SNP Genotyping System (Kbiosciences, Herts, UK). SNP genotyping was performed by qPCR through a LightCycler 480 device (Roche Diagnostics, Spain) using universal KASPar MasterMix (LGC, Teddington, UK) following the supplier's technical instructions.

5.3.4. Prediction of variants effect of candidate genes sequences

Almond and peach resequences of candidate genes defining the *Vr3* region, located between Pp02:16,912,811 and Pp02:17,184,692 physical positions of the *P. persica* v.2.0 reference genome (Verde et al., 2017), were compared to predict the variants in the region. Their effect on annotated genes of the region was determined using SnpEff v4.3p software (Cingolani et al., 2012). Variant effect was defined by the impact on the protein in three categories: (i) high impact, by impairing protein function, i.e. affecting splice-sites or start and stop codons, (ii) moderate impact including non-disruptive variants, and (iii) low impact including synonymous variants.

5.3.5. Gene expression analysis

RNA isolation and cDNA synthesis. The sampled leaves were immediately frozen in liquid nitrogen after collection and RNA was isolated with the Spectrum Plant Total RNA kit (Sigma Aldrich, Munich, Germany), according to the manufacturer's instructions. RNA concentration and purity were checked with a Nanodrop ND-1000 spectrophotometer. Samples were only further processed for cDNA synthesis if the 260/280 ratio was between 1.9 and 2.1, and the 260/230 ratio > 2.0 (Román et al., 2019). cDNA was synthetized from 1 µg of total RNA for each sample using the PrimeScript RT-PCR Kit (Takara, Otsu-shi, Japan) according to the manufacturer's instructions.

Primer design of candidate genes. Primer pairs were designed for the 27 candidate genes defined in the region of interest after *Vr3* fine mapping. Coding DNA sequences of candidate genes were obtained from the Genome Database for Rosaceae (Jung et al., 2019). Sequences were analyzed by BLAST alignment for specificity checking (Altschul et al., 1990), primer pairs suitability (GC content, self-complementarity and dimer formation) was checked using Oligoanalyzer 3.1 (Integrated DNA Technologies, URL: https://eu.idtdna.com), and *mfold* (Zuker, 2003) (URL: http://unafold.rna.albany.edu) was used to predict secondary structure formation.

qPCR expression analysis. qPCR assays were performed using a Fluidigm 48.48 dynamic array chip on the BioMark HD System Real-Time PCR (Fluidigm, CA, USA).

Prior to the high-throughput qPCR, a pre-amplification of the cDNA samples was performed. Diluted (1:3) pre-amplified cDNA samples were loaded according to Fluidigm's EvaGreen DNA binding dye protocols. Negative controls were used in the assay to detect possible DNA contamination. Four reference genes used in previous expression analysis were evaluated: actin (*Act*), expansin (*Exp1*) (Rubio et al., 2015), pre-mRNA splicing factor 7 (*SLU7*) (Zúñiga et al., 2019), and translation elongation factor 2 (*TEF2*) (Tong et al., 2009). The stability of each reference gene was defined with the SATqPCR statistical analysis tool (Rancurel et al., 2019) based on the geNorm method (Vandesompele et al., 2002), considering the lowest gene variability. Considering the use of at least two reference genes, as described in MIQE rules (Bustin et al., 2009), *Act*, *Exp1* and *SLU7* were finally chosen for normalizing relative quantities for each candidate gene.

The effects of two factors in the relative expression of candidate genes were considered: (i) disease status, i.e. symptomatic or asymptomatic leaves, and (ii) presence of the *Vr3* almond alleles, either in homozygosis or heterozygosis. Considering normal distributions and independence of the observations, two-way analysis of variance (ANOVA) was used to assess the independent effect of each of these two factors in normalized expression of all candidate genes. ANOVA tests were performed using the 'RqPCRAnalysis' R-package (R Core Team, 2020) included in the SATqPCR statistical analysis tool (Vandesompele et al., 2002). Orthogonal contrasts were used to detect differences in different levels of the factor describing the disease status. The first contrast was among individuals with the *Vr3* introgression (including heterozygous and homozygous individuals) and individuals without the introgression from 'Texas', and the second among heterozygous and homozygous individuals. Statistical significance of these tests was set at $\propto < 0.01$.

5.4. Results

5.4.1. Fine mapping and identification of Vr3 *candidate genes*

A total of 729 descendants derived from individuals carrying *Vr3* in heterozygosis were genotyped using two SSR markers (CPDCT044 and BPPCT004) known to include

Vr3 (Donoso et al., 2016). These were from nine populations shown in Table 5.1 (T×E, T1E, E2T-031, 11P15, 15P15, 19P15, 25P15 and T1BT).

The recombination between the two markers in 30 of these 729 individuals was observed, with 16% phenotyped as resistant and 84% as susceptible. Recombinant individuals (Table 5.1) were genotyped using markers previously described (Table 5.2 and Table 5.3) to narrow down the genomic region where *Vr3* was located. After phenotyping the recombinant individuals, and using the new genotyping information, we located *Vr3* in the region between markers Indel16912 and SNP_17184692 (Table 5.4), corresponding to physical positions 16,912,811 and 17,184,692, respectively.

Table 5.4. Phenotypes and genotypes of individuals with a recombinant breakpoint (dashed lines) near *Vr3*. b, allele from the susceptible parent 'Earlygold'. h, allele from heterozygote individuals. R, resistant. S, susceptible. N, Number of recombinant individuals.

Marker	**Position**	**Genotype**										
CPDCT044	16,847,924	b	b	b	b	b	b	b	h	h	h	h
Indel16883	16,883,671	b	b	b	b	b	b	b	h	h	h	h
Indel16912	16,912,811	b	b	b	b	b	b	b	h	h	h	h
SNP_16932290	16,932,290	b	b	b	b	b	b	h	h	h	h	b
SNP_16940264	16,940,264	b	b	b	b	b	b	h	h	h	h	b
Indel16949	16,948,818	b	b	b	b	b	b	h	h	h	h	b
Indel17019	17,019,668	b	b	b	b	b	b	h	h	h	h	b
Indel17048	17,048,260	b	b	b	b	b	b	h	h	h	h	b
Indel17050	17,050,734	b	b	b	b	b	b	h	h	h	h	b
Indel17061	17,061,201	b	b	b	b	b	b	h	h	h	h	b
SNP_17180556	17,180,556	b	b	b	b	b	b	h	h	h	h	b
SSR_17181256	17,181,256	b	b	b	b	b	b	h	h	h	h	b
SSR_17182435	17,182,435	b	b	b	b	b	b	h	h	h	h	b
SNP_17184692	17,184,692	b	b	b	b	b	b	h	h	h	b	b
SSR_17184920	17,184,920	b	b	b	b	b	b	h	h	h	b	b
6620	17,166,620	b	b	b	b	b	h	h	h	h	b	b
Indel17186	17,186,620	b	b	b	b	b	h	h	h	h	b	b
indel17229	17,229,285	b	b	b	b	h	h	h	h	h	b	b
Indel17242	17,242,814	b	b	b	b	h	h	h	h	h	b	b
2031	17,262,031	b	b	b	b	h	h	h	h	h	b	b
Indel17272	17,272,322	b	b	b	b	h	h	h	h	h	b	b
Indel17479	17,479,459	b	b	b	h	h	h	h	h	h	b	b
Indel17909	17,909,204	b	b	h	h	h	h	h	h	b	b	b
Indel18610	18,610,981	b	h	h	h	h	h	h	b	b	b	b
BPPCT004	18,641,408	h	h	h	h	h	h	h	b	b	b	b
	Phenotype	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**S**
	N	3	4	13	2	3	1	2	3	1	2	1

Five individuals had the nearest recombination to the *Vr3* gene, so determining the *Vr3* region. Two resistant individuals from T×E and 44P17 families and one susceptible individual from 14P16 family showed a recombination between Indel16912 and SNP_16932290, defining the lower limit of the *Vr3* region. Two resistant individuals with a recombination between SSR_17182435 and SNP_17184692, corresponding to families E2T-031-06 and 51P17 defined the upper limit. In this region, spanning approximately 270 kb, 27 annotated genes (Table 5.5) were found in the *P. persica* Genome Annotation v2.1 (Verde et al., 2017) retrieved from the Genome Database for Rosaceae (www.rosaceae.org/species/prunus_persica/genome_v2.0.a1).

Among the 27 candidate genes, five were annotated as involved in plant defense, an additional five encoding for structural function, ten genes were predicted to be involved in plant metabolism, and seven were annotated as unknown (Table 5.5). Among the five candidate genes described as involved in plant defense, *Prupe.2G110900* was predicted to function as a germin-like protein. The other four (*Prupe.2G111700*, *Prupe.2G111800*, *Prupe.2G112700*, and *Prupe.2G113200*) were predicted to be plant resistance genes (R genes). Moreover, *Prupe.2G112700* and *Prupe.2G113200* were specifically included in the TIR-NBS-LRR class of plant R genes. Five genes (*Prupe.2G112600*, *Prupe.2G112800*, *Prupe.2G112900*, *Prupe.2G113000*, and *Prupe.2G113500*) were predicted to encode protein Eceriferum 1, involved in epicuticular wax biosynthesis. Finally, of the ten genes predicted to be involved in plant metabolism, three were annotated with hydrolase function (*Prupe.2G111900*, *Prupe.2G112000*, and *Prupe.2G112100*), three related to DNA binding (*Prupe.2G110900*, *Prupe.2G112300*, and *Prupe.2G113400*), three ATP-related genes (*Prupe.2G111300*, *Prupe.2G111400*, and *Prupe.2G111500*) and one predicted as a multifunctional enzyme (*Prupe.2G112200*).

Table 5.5. *Vr3* resistance candidate genes to peach powdery mildew.

Gene	Position	Predicted function	Function classification
Prupe.2G110900	Pp02:16913576..16914676	Agamous-like MADS-box protein (*Arabidopsis thaliana*)	Metabolism
Prupe.2G111000	Pp02:16920605..16921195	Germin-like protein (*Oryza sativa* subsp. Japonica)	Plant defense
Prupe.2G111100	Pp02:16922483..16922915	n/a	Unknown
Prupe.2G111200	Pp02:16923107..16925825	n/a	Unknown
Prupe.2G111300	Pp02:16926230..16929679	26S protease (*Arabidopsis thaliana*)	Metabolism
Prupe.2G111400	Pp02:16930138..16934333	ABC transporter (*Arabidopsis thaliana*)	Metabolism
Prupe.2G111500	Pp02:16936869..16945304	ABC transporter (*Arabidopsis thaliana*)	Metabolism
Prupe.2G111600	Pp02:16993968..16994329	n/a	Unknown
Prupe.2G111700	Pp02:16996435..17001837	Disease resistance protein RGA2 (*Solanum bulbocastanum*)	Plant defense
Prupe.2G111800	Pp02:17003896..17010678	Putative disease resistance protein RGA3 (*Solanum bulbocastanum*)	Plant defense
Prupe.2G111900	Pp02:17011424..17014545	Hydrolase domain-containing protein Sgpp (*Arabidopsis thaliana*)	Metabolism
Prupe.2G112000	Pp02:17015308..17018250	Endoglucanase 12 (*Arabidopsis thaliana*)	Metabolism
Prupe.2G112100	Pp02:17020262..17023645	Riboflavin biosynthesis protein PYRD (*Arabidopsis thaliana*)	Metabolism
Prupe.2G112200	Pp02:17024102..17036171	DNA replication helicase (*Arabidopsis thaliana*)	Metabolism
Prupe.2G112300	Pp02:17039404..17042658	Zinc ion binding (*Arabidopsis thaliana*)	Metabolism
Prupe.2G112400	Pp02:17049419..17050149	n/a	Unknown
Prupe.2G112500	Pp02:17050202..17050814	n/a	Unknown
Prupe.2G112600	Pp02:17061213..17068962	Protein ECERIFERUM 1 (*Arabidopsis thaliana*)	Structural
Prupe.2G112700	Pp02:17073807..17075686	TMV resistance protein N (*Nicotiana glutinosa*)	Plant defense
Prupe.2G112800	Pp02:17099320..17103427	Protein ECERIFERUM 1 (*Arabidopsis thaliana*)	Structural
Prupe.2G112900	Pp02:17113525..17117895	Protein ECERIFERUM 1 (*Arabidopsis thaliana*)	Structural
Prupe.2G113000	Pp02:17138061..17139410	Protein ECERIFERUM 1 (*Arabidopsis thaliana*)	Structural
Prupe.2G113100	Pp02:17141136..17142354	n/a	Unknown
Prupe.2G113200	Pp02:17142564..17145425	TMV resistance protein N (*Nicotiana glutinosa*)	Plant defense
Prupe.2G113300	Pp02:17151739..17152569	n/a	Unknown
Prupe.2G113400	Pp02:17166049..17166711	RING-H2 finger protein ATL3 (*Arabidopsis thaliana*)	Metabolism
Prupe.2G113500	Pp02:17168568..17172597	Protein ECERIFERUM 1 (*Arabidopsis thaliana*)	Structural

5.4.2. Variant calling and effect prediction of polymorphisms

A total of 3,510 variants including 3,073 SNPs, 222 insertions and 215 deletions were identified (details shown in Table 5.6). These variants were predicted to cause 11,958 effects on the sequences (Table 5.6). Most of them (93.7%) were considered non-coding variants or variants affecting non-coding genes, while 13 (0.11%) were predicted as high-impact variants, 350 (2.9%) as moderate, and 392 (3.28%) as low-impact variants. The 13 high impact variants producing a disruptive effect on the coded protein, were

Table 5.6. Description of the effects of all the variants detected in the *Vr3* region, as predicted by SnpEff (Cingolani et al., 2012).

Effect	Count	Percent	Impact
frameshift_variant	7	0.06%	High
splice_acceptor_variant	1	0.01%	High
splice_donor_variant	2	0.02%	High
start_lost	1	0.01%	High
stop_gained	2	0.02%	High
stop_lost	1	0.01%	High
inframe_deletion	6	0.05%	Moderate
inframe_insertion	2	0.02%	Moderate
missense_variant	342	2.85%	Moderate
splice_region_variant	61	0.51%	Low
synonymous_variant	305	2.54%	Low
3_prime_UTR_variant	207	1.72%	Modifier
5_prime_UTR_premature_start_codon_gain_variant	34	0.28%	Modifier
5_prime_UTR_variant	222	1.85%	Modifier
downstream_gene_variant	3,805	31.65%	Modifier
intergenic_region	1,934	16.09%	Modifier
intron_variant	1,422	11.83%	Modifier
upstream_gene_variant	3,667	30.51%	Modifier

detected in six candidate genes (Table 5.7). Genes annotated as RGA2 resistance protein (*Prupe.2G111700)*, RGA3 resistance protein (*Prupe.2G111800)*, DNA replication helicase (*Prupe.2G112200)*, and three genes with non-available annotation (*Prupe.2G111100, Prupe.2G112400* and *Prupe.2G112500*) presented one high impact variant each, whereas genes annotated as Eceriferum 1 (*Prupe.2G113500)* and another with non-available annotation presented three and four high impact variants respectively.

5.4.3. Expression analysis of candidate genes

Relative normalized expression profiles of the 27 candidate genes (Table 5.8) annotated in the *Vr3* region in this study were analyzed to describe the effect of the infection status and the presence of *Vr3* introgression. One gene, *Prupe.2G111600*, was excluded from the analysis because we could not obtain a regular amplification signal and it was considered inappropriate for qPCR expression analysis. No variants with high or medium impact were detected in this gene.

Table 5.7. Nucleotide changes of high impact variants detected in the *Vr3* region.

Position	Gene	Effect	Variants	Peach gneome	Almond genome
Pp02: 16,922,914	Prupe.2G111100	Stop lost	1	A	C
Pp02: 16,997,443	Prupe.2G111700	Stop gained	1	A	C
Pp02: 17,004,316	Prupe.2G111800	Splice acceptor variant	1	T	C
Pp02: 17,030,265	Prupe.2G112200	Stop gained	1	A	C
Pp02: 17,049,441	Prupe.2G112400	Frameshift variant	1	CT	CTT
Pp02: 17,049,771	Prupe.2G112400	Frameshift variant	1	GTA	G
Pp02: 17,049,772	Prupe.2G112400	Frameshift variant	1	TAC	T
Pp02: 17,050,147	Prupe.2G112400	Frameshift variant and start loss	2	CA	C
Pp02: 17,050,533	Prupe.2G112500	Frameshift variant	1	CT	CTT
Pp02: 17,181,256	Prupe.2G113500	Frameshift variant	1	ATTTT	ATTTTT
Pp02: 17,182,435	Prupe.2G113500	Splice donor	1	CATATATATATATATA AAGAGAGAGAGAGAG	CATATATATATA AAGAGAGAGAGAGAG
Pp02: 17,184,920	Prupe.2G113500	Splice donor variant	1	AGAGAGAGAGA	AGAGAGAGAGAGAGA

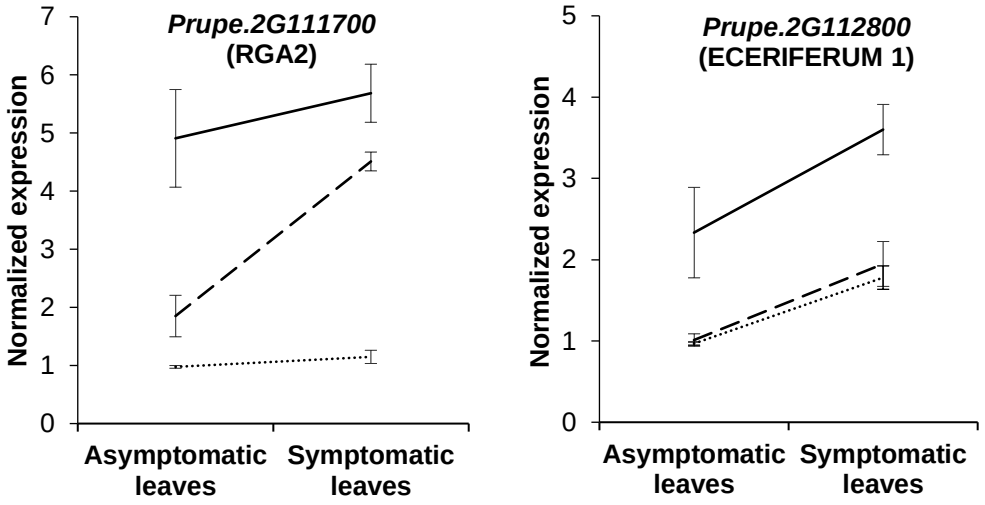
Table 5.8. Primer sequences used in qPCR to amplify candidate genes in the delimited region containing *Vr3*.

Primer	Swissprot description	Gene location	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
Prupe.2G110900	Agamous-like MADS-box protein AGL36	Pp02: 16,913,576..16,914,676	TCCGAGTTCGATGGCAATGT	CCCGCCCTGTACTGAAGATT	72
Prupe.2G111000	Germin-like protein 9-3	Pp02: 16,920,605..16,921,195	GAGTTCCCTGCCCTTACTGG	CGGGTGTGAGGTGGGAATAG	83
Prupe.2G111100	n/a	Pp02: 16,922,483..16,922,915	AACACACTGTTCCCACCTCC	CCAAAGAACCATCAACAAGGAACA	70
Prupe.2G111200	n/a	Pp02: 16,923,107..16,925,825	GCAGGACTTGTGGTGGAAGT	GTACGGCGGTCTTGATTCCT	154
Prupe.2G111300	26S protease regulatory subunit 8 homolog A	Pp02: 16,926,230..16,929,679	CCGAAGACGCAGCTATACCA	GCTCATCAAGACCACCAATCA	122
Prupe.2G111400	ABC transporter C family member 9	Pp02: 16,930,138..16,934,333	TCATTCCACAGGACCCAACA	GCCTCCCAAACATCACTATCG	91
Prupe.2G111500	ABC transporter C family member 9	Pp02: 16,936,869..16,945,304	ACTCACAGCAGGCAGAGTTC	GCGGAGAGCAAGTCAGGTAG	87
Prupe.2G111600	Probable geranylgeranyl transferase type-2 subunit beta	Pp02: 16,993,968..16,994,329	GATGACTCAGGTGGGTTTGG	AGGCTGGTGTGTGGTATAACTC	144
Prupe.2G111700	Disease resistance protein RGA2	Pp02: 16,996,435..17,001,837	TGTGGAAGATTGCGGTAGTCTAG	ACAACTCCTGACAGCCTGTAA	92
Prupe.2G111800	Putative disease resistance protein RGA3	Pp02: 17,003,896..17,010,678	ACAGGCTGTCAGGAGTTGTC	TCTGGACTCATGCTGCAACC	161
Prupe.2G111900	Haloacid dehalogenase-like hydrolase domain-containing protein	Pp02: 17,011,424..17,014,545	TTGTAGTATCCTCTTTCCTGAGTGG	CGAAACATGGCTTCCTTATCTTC	75
Prupe.2G112000	Endoglucanase 12	Pp02: 17,015,308..17,018,250	ATTCGGGAGCAAACTTGAAGC	ATGTCTTCGGGTCTCTGCCA	165
Prupe.2G112100	Riboflavin biosynthesis protein PYRD, chloroplastic	Pp02: 17,020,262..17,023,645	CGCCTCTATTCCAGTATCCCAAG	CACCGTTTCAATACCTGTGTGAG	187
Prupe.2G112200	DNA replication ATP-dependent helicase/nuclease DNA2	Pp02: 17,024,102..17,036,171	TTTGAGGGCAGTCGGAGTTG	TCCTGGCGAGAACTTGAAGC	106
Prupe.2G112300	Sister chromatid cohesion protein DCC1	Pp02: 17,039,535..17,041,908	AAGGTGATTGAGGGTGTGAGC	AGTCCTCCATGAACCTCTCC	124
Prupe.2G112400	n/a	Pp02: 17,049,419..17,050,149	GTTGAGGGTGTGAGCACAGTAG	AAGAGGACGGAGAATCGCTTTG	179
Prupe.2G112500	n/a	Pp02: 17,050,202..17,050,814	CCACCATCTCCAGACTACAACG	AGATCATCGTGAGGACCAAAGG	155
Prupe.2G112600	Protein ECERIFERUM 1	Pp02: 17,061,213..17,068,962	TGGCCGAATCGTTGACAAACC	AGATTTTGAGCCCCAGGCAT	129
Prupe.2G112700	TMV resistance protein N	Pp02: 17,073,807..17,075,686	CCAAGAGAAGGTGCCTGTGT	ACTTGCATCAGCCAAAGGTG	80
Prupe.2G112800	Protein ECERIFERUM 1	Pp02: 17,099,320..17,103,427	CAAGCCAAGCCAGTCCCTAA	ACTCACCATGTCCACCTACT	193
Prupe.2G112900	Protein ECERIFERUM 1	Pp02: 17,113,525..17,117,895	TCCTTGTCCTCTAAGCCCCA	AGTTCGTGATGGTCTTACTTGC	189
Prupe.2G113000	Protein ECERIFERUM 1	Pp02: 17,138,061..17,139,410	TGCTATACCAATAGCTGTAACAAGT	CTGTTCAGCTCCCTCACCCTG	198
Prupe.2G113100	n/a	Pp02: 17,141,136..17,142,354	CCAAGAGAAGGTGCCTGTGT	CTGTCAGGGTAAGCGTCAGA	158
Prupe.2G113200	TMV resistance protein N	Pp02: 17,142,564..17,145,425	TCACACGTACAAGGAAGAACA	AAAAGGTGAAGCGCTGGAGA	191
Prupe.2G113300	n/a	Pp02: 17,151,739..17,152,569	GCCTCTGCCTTTCTTCCTTCA	TGGTGGTTTGGGTTGTAGACT	195
Prupe.2G113400	RING-H2 finger protein ATL3	Pp02: 17,166,049..17,166,711	GGTGTGCCTCCAAGAAGTGA	TGCGTATGAACAAACGATGCC	153
Prupe.2G113500	Protein ECERIFERUM 1	Pp02: 17,168,568..17,172,597	GCTCTTGGAAGCCTCAAGGT	ACGTAGCTTGCAGTTCGACA	89

From the 26 candidate genes that could be successively analyzed, Eceriferum 1 (*Prupe.2G112600*) was the only gene with significant interaction ($p < 0.01$) between infection status and the *Vr3* almond allele. Seven candidate genes were found to be significantly differentially expressed for one or both factors ($p < 0.01$). For the infection status factor, three differentially-expressed genes were identified, namely Eceriferum 1 (*Prupe.2G113000*), RING-H2 finger protein (*Prupe.2G113400*) and an unknown annotated gene (*Prupe.2G113100*). Their expression increased in all three cases when infection occurred regardless of the *Vr3* allele presence (data not shown). Regarding the allelic status, the genes *RGA2* (*Prupe.2G111700*) and *Eceriferum 1* (*Prupe.2G112800*) were overexpressed in individuals homozygous (*Vr3Vr3)* and heterozygous (*Vr3vr3)* for *Vr3* (Fig. 5.1).

In both symptomatic and asymptomatic leaves, the RGA2 annotated gene (*Prupe.2G111700*) had higher relative expression for *Vr3Vr3* individuals compared with *Vr3vr3* individuals: the normalized expression was 4.91 ± 0.84 (mean ± SE) and 1.85 ± 0.36 respectively in asymptomatic leaves, and 5.68 ± 0.50 and 4.51 ± 0.16, in symptomatic leaves. In addition, the normalized expression for susceptible individuals

Figure 5.1. Relative normalized expression of candidate genes with significant differences in symptomatic and asymptomatic leaves ($p < 0.01$). Solid and dashed lines correspond to homozygous (*Vr3Vr3*) and heterozygous (*Vr3vr3*) individuals for the *Vr3* allele from 'Texas', respectively. Dotted lines correspond to individuals with *Vr3* peach alleles. Bars indicate standard error of the mean.



with no *Vr3* almond introgression (1.15 ± 0.11) was significantly lower compared with resistant individuals ($p < 0.01$). Eceriferum 1 (*Prupe.2G112800*) was upregulated for individuals containing the *Vr3* allele, and again gene expression in *Vr3Vr3* differed significantly from heterozygous individuals. Nevertheless, no significant differences were detected among *Vr3vr3* individuals and susceptible individuals not carrying the *Vr3* allele. Finally, genes encoding for Agamous-like MADS-box (*Prupe.2G110900*) and Germin-like protein (*Prupe.2G111000*) were significantly underexpressed in individuals containing the Vr3 allele ($p < 0.05$), and no interaction between factors was detected.

5.5. Discussion

The PPM resistance gene *Vr3* was located in a 1.8 cM genomic region of chromosome 2 where 187 genes were annotated in the peach reference genome (Donoso et al., 2016). In our study, through a fine mapping approach, we narrowed the region down to 270-kb (between Pp02:16,912,811 and Pp02:17,184,692), with twenty-seven genes annotated that were considered as a first set of *Vr3* positional candidate genes. Additional evidence in support of some of these genes being responsible for PPM resistance was gathered through expression analysis and prediction of the effect of variants in the coding sequences of the candidate genes Among the variants detected in the region, only those predicted to have a high or moderate impact on the protein encoded were considered candidates for the *Vr3* resistance gene. As defined by SnpEff software, high impact predicted variants were assumed to have disruptive impact in the protein, causing protein truncation or loss of function; and moderate predicted variants might alter protein effectiveness (Cingolani et al., 2012). In the current study, 23 candidate genes were predicted to have a moderate effect on the protein, and six variants also had a high impact effect.

From the two candidate genes that were differentially overexpressed in resistant individuals, encoding for RGA2 (*Prupe.2G111700*) and Eceriferum 1 (*Prupe.2G112800*), only RGA2 (*Prupe.2G111700*) had a high impact variant that is producing a stop codon. This gene also presented 35 moderate variants. Regarding gene expression, RGA2 (*Prupe.2G111700*) was the only gene significantly overexpressed in resistant *Vr3Vr3* and *Vr3vr3* individuals compared to susceptible individuals (*vr3vr3*)

independently of the infection status. Therefore, RGA2 (*Prupe2.G111700*), was considered our strongest candidate gene for *Vr3*. Moreover, as the expression of RGA2 (*Prupe.2G111700)* did not differ significantly with respect to the infection status, it is assumed to be constitutively expressed, as previously reported for RGA genes involved in fungal resistance in *Rosaceae*, such as for crown rot in octoploid strawberry (Chen et al., 2016) and powdery mildew in apple (Calenge and Durel, 2006). RGA genes are involved in the recognition and prevention of plant pathogens (Kim et al., 2012), with highly conserved amino acid domains that are already known in *P. persica* (Lalli et al., 2005). *Vr3* has been described as a monogenic resistance gene (Donoso et al., 2016), and is thought to show completely dominant gene action, being the heterozygous and homozygous plants equally resistant. When comparing the allelic status on the expression of RGA2, homozygous individuals significantly overexpressed *Vr3* as compared to heterozygous individuals despite of the infection status. Conversely, when in heterozygosity, gene expression differed significantly between asymptomatic and symptomatic individuals. This could have implications in the resistance mechanisms and should be borne in mind if this gene is used for future breeding purposes.

Another candidate gene differentially overexpressed in resistant individuals was Eceriferum 1 (*Prupe.2G112800*), an ortholog of an *Arabidopsis thaliana* gene related to fungal recognition, based on cuticle wax components (Dhanyalakshmi et al., 2019). This gene had variants with moderate effect, and was overexpressed only in homozygous individuals containing the *Vr3* almond allele as compared to heterozygous and susceptible individuals. As no significant differences in expression were detected between the susceptible and the *Vr3vr3* individuals, phenotyped as resistant, *Prupe.2G112800* was not considered as a candidate gene for *Vr3*.

Results obtained in this study provide important information to identify a limited number of genes as *Vr3* candidates, responsible for PPM resistance. A validation process through genetic transformation is required, but this is currently difficult due to the recalcitrant character of peach (Zong et al., 2019). Another possibility could be the use of a heterologous system such as plum for which an efficient transformation approach has been described (Petri et al., 2012), although a limitation of this approach is that species causing powdery mildew in peach differ from that in plum (*P. tridactyla*). This would only be successful if our RGA2 candidate gene conferred broad-spectrum resistance to

powdery mildew, as it has been described for the *Pm21* RGA gene in wheat (Perazzolli et al., 2014).

Until efficient peach transformation strategies are available, a feasible alternative to integrate the *Vr3* gene in peach breeding programs could be marker-assisted introgression (MAI) (Serra et al., 2016). For that, a near isogenic line carrying a unique introgression from almond containing the *Vr3* gene needs to be developed to cross with the parentals from a specific breeding program and then resistant individuals can be selected using the molecular markers described in this work. This strategy is currently in progress in our laboratory to introgress *Vr3* resistant alleles from 'Texas' almond into high quality peach commercial cultivars. Finally, we propose to pyramid these lines with other PPM resistance genes such as Vr23 to increase PPM resistance durability, and with other peach biotic resistance genes to increase crop sustainability.

5.6. References

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6. Marker assisted introgression of the *Vr3* resistance gene into peach elite cultivars

6. Marker-assisted introgression of the *Vr3* resistance gene into peach elite cultivars

6.1. Summary

Powdery mildew is one of the major foliar diseases of peach (*Prunus persica* (L.) Batsch). Previous studies identified a resistance gene (*Vr3*) in interspecific populations between the resistant 'Texas' almond and the susceptible 'Earlygold' peach cultivars. In a previous work, using the marker-assisted introgression strategy, several introgression lines containing one or two introgressions from almond including the *Vr3* resistant almond allele were obtained. One of the main drawbacks of these lines is depending on the low fruit quality and very short postharvest life of 'Earlygold' peach. In this study we introgressed the *Vr3* resistant allele from almond into peach elite cultivars using the introgression lines and selecting the resistant individuals using marker-assisted selection. Since 2016, 132 individuals containing the *Vr3* gene have been obtained. Molecular markers and tips to improve future projects on marker-assisted selection have been discussed. As individuals carrying the *Vr3* single gene might show a compromised durability in resistance, a pyramidization approach of several resistance genes has been proposed to increase resistance durability.

6.2. Introduction

Peach breeding is currently driven by the improvement of commercial characteristics related to fruit quality traits, to the adaptation to changing environmental conditions and to the improvement of disease and pest resistance (Monet and Bassi, 2008). Until the end of XXth century, breeding strategies to include those characteristics into new cultivars were conducted by breeding programs mainly located in USA, Italy and France (Iglesias, 2017). The adaptation of those new released peach cultivars in Spain was challenging due to the highly diverse climatic conditions in Spanish orchards. Over ten Spanish peach breeding programs were started in the past two decades to obtain better locally-adapted cultivars. These breeding programs have released more than 100 peach commercial cultivars and represented more than 30% of new peach cultivars introduced in the market within the period 2010-2015 (Iglesias, 2016). Therefore, Spain has gradually decreased its dependence on foreign cultivars and focused on fruit quality traits and cultivar adaptation to specific environmental cropping conditions.

In Catalonia, the IRTA-ASF-FruitFutur peach breeding program, which includes a public research institute (IRTA), a private breeding company (ASF), and an organization of peach producers (FruitFutur), has released 26 new cultivars including peach, nectarine and flat peaches from 2004. The regular activities carried by IRTA-ASF-FruitFutur breeding program, comparable to other peach breeding programs, have been based on cycles of crosses using parents previously selected regarding specific objectives. Since 2008, molecular markers were introduced in this breeding program in order to develop breeding activities more efficiently, such as the optimization of crosses, the characterization of parents, and mainly for its application in marker-assisted selection (MAS) (Eduardo et al., 2015). MAS is based on an early selection, using molecular markers, of individuals carrying an allele linked to a trait of interest. MAS was applied for first time at the IRTA-ASF-FruitFutur program to select for subacid taste and flat shape fruit characters. Other available markers for peach MAS include peach/nectarine fruit type (Picañol et al., 2013; Vendramin et al., 2014), fruit flesh color (Adami et al., 2013) and acidity (Eduardo et al., 2014) (Meneses et al., 2016), among others. There are currently few molecular markers described for traits related to peach resistance to pests and diseases. These include *Vr3* (Donoso et al., 2016; this thesis) and *Vr2* (Pascal et al., 2017) for peach powdery mildew (PPM), and *Rm1* (Pascal et al., 2017) and *Rm2* (Lambert and Pascal, 2011) for aphids. Other disease and pest resistance sources have been described in Table 1.2. (Chapter 1). These resistance genes have been mostly obtained from other peach cultivars or exotic germplasm, with very low fruit quality. The introgression of biotic resistances from this exotic germplasm needs several generations to restore commercial fruit traits. Therefore, strategies to introgress these biotic resistance genes are needed.

The first reported introgression in fruit tree crops from an exotic source was proposed by Serra et al. (2016), through a strategy named marker-assisted introgression (MAI). This method describes how to introduce new genetic information from an exotic species into the genomic background of a close-related species using molecular markers to increase the process efficacy. The proof of concept described by Serra (2017) involved the crossing between ‘Texas’ almond as the exotic species and ‘Earlygold’ peach as the recurrent. The strategy included the obtention of a collection of lines with a single introgression, named near isogenic lines (NILs), covering the whole genome of the exotic germplasm, or of just a NIL containing a trait of interest. These newly obtained NILs can

be considered as a valuable prebreeding material, as they only contain a single almond introgression. One of the traits segregating in the T×E and T1E populations was the PPM resistance gene *Vr3*, which was mapped by Donoso et al. (2016) and finely mapped and characterized in this PhD Thesis (Chapter 5). Despite the *Vr3* gene characterization is important to decipher the resistance mechanism, it can be rather introduced in breeding programs well before its full characterization. One limitation of the NILs resulted from the use of 'Earlygold' peach as the recurrent parent is concerning its old early-ripening and low fruit quality characters.

The main objective in this study was to introgress the *Vr3* resistance gene into peach elite cultivars using MAS and the NILs as prebreeding material. We also propose a strategy to pyramidize other PPM resistance genes into peach to increase its PPM resistance durability.

6.3.Materials and methods

6.3.1. Plant material

Three introgression lines derived from the T×E cross were used as pollen donors in subsequent crosses (Table 6.1). These individuals had one (19P15-15 and 19P15-57) or two (E2T-092-25) almond introgressions, respectively (Table 6.2). All three individuals had an introgression in G2 containing the genomic region where the *Vr3* resistance gene was mapped (Donoso et al., 2016). These individuals with one or two almond introgressions in G2 were heterozygous and placed at an orchard in Caldes de Montbui (41º36'47"N, 2º10'12"E). Other NILs placed at the same orchard with the G2 introgression in homozygosity were trees that produced few flowers and consequently few fruits. The homozygotic NILS failed to produce any fruit in 2018 and 2019 therefore, they were finally discarded as maternal parents. These individuals showed a low vigor so their trunk diameter at 0.2 m above soil level was measured and compared to other individuals used in the experiments.

Table 6.1. Crossings performed for the introgression of *Vr3* resistance gene. Fruit type: nectarine (N) / peach (P); white (W) / yellow (Y) flesh; flat (F) / round (R) shape.

Year	Maternal	*Vr3* donor	No. of introgressions	Fruit type	Pollinated flowers	Fruits	Seeds	Seedlings	MAS Markers	Resistant individuals
2016	P02F210A057	E2T-092-25	2	NWR	-	≥5	5	1	6,7	0
2016	P01F002A054	E2T-092-25	2	PWR	-	≥28	28	27	6,7	8
2016	'Nectatop'	E2T-092-25	2	NYR	3750	71	36	19	6,7	4
2017	'ASF 06-20'	E2T-092-25	2	NYR	1000	≥414	414	359	2,4	85
2017	'Nectatop'	E2T-092-25	2	NYR	5000	121	108	54	1,5	16
2017	'Nectatop'	E2T-092-25	2	NYR	1200	111	75	60	2,6	19
2018	'Nectatop'	19P15-15	1	NYR	400	2	1	0	2,3	0
2018	'Nectatop'	19P15-57	1	NYR	300	2	2	2	-	0
Totals					11650	754	669	522		132

Table 6.2. Number and length of introgressions from 'Texas' almond in individuals used for *Vr3* introgression.

***Vr3* donor**	**No. Introgressions**	**Introgression length (Mbp)**							
		G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**G8**
E2T-092-25	2		17.25				17.43		
19P15-15	1		11.07						
19P15-57	1		11.07						

Regarding maternal lines used for *Vr3* introgression, these included two commercial cultivars ('Nectatop', and 'ASF06-20') and two advanced selections (P01F002A054, and P02F210A057) from the IRTA-ASF-FruitFutur peach breeding program (Table 6.1). All the maternal lines were placed at Gimenells (41º39'22" N, 0º23'26" E), with the exception of 'Nectatop', placed at Mollerussa (41º37'07" N, 0º51'60" E).

6.3.2. Marker-assisted selection

The MAS methodology is schematized in Fig. 6.1. Pollen from donor individuals (Table 6.3) was collected in spring (March) as follows: petals were removed from selected flowers and anthers were collected using tweezers and kept in plastic vials (Fig. 6.1a). Anther samples were left to dry overnight at room temperature under a light bulb. Dried pollen was stored until the end of the pollination season (approximately one month) at room temperature in sealed plastic pots with Silica gel. When blooming of the pollen donor was later than that for the maternal parent, pollen was collected in the previous year and stored at -20ºC until use. Pollinations were performed from 2016 to 2018 as indicated in Table 6.3. The maternal flowers were emmasculated before blooming to avoid self or undesired cross pollinations (Bassi and Monet, 2008). The emasculation was performed at BBCH 61 phenological stage (Meier, 2001) using exclusively closed flowers, and consisted in the elimination of all anthers, sepals and petals in a flower (Fig. 6.1b). Finally, pollen of the selected donor parent was applied carefully on the stigma using a thin brush or the fingertips, washing them with alcohol (70%) between applications of different genotypes.

Figure 6.1. Graphical description of the marker-assisted selection process. a, b) Emasculation and pollination; c) Fruit collection; d, e) Seed extraction and stratification; f, g) Seed germination, h) Leaf sampling; i) DNA extraction and genotyping.

Fruits resulting from pollinations were collected when ripened (Fig. 6.1c), adequately labelled and kept at 4 ºC. Seeds were extracted from the fruit pit using pruning shears with one unsharpened blade to optimally crush the pit (Fig. 6.1d). The seeds were further surface-sterilized by soaking them 1 min in 10% bleach, rinsed three times with sterile distilled water, and finally soaked in a solution of captan (Merpan, 80% w/w, Adama, Spain) in distilled water (1 g/L) for a few seconds. Seeds were placed separately each other on a filter paper and dried overnight. Dried seeds (Fig. 6.1e) were placed in plastic trays filled with perlite that was previously saturated with an aqueous 1 g/L captan solution. Seeds were disposed in alternating layers with perlite (Fig. 6.2a). Plastic trays were stratified at 4 ºC for 12 to 14 weeks. The top layer of perlite was sprayed weekly with distilled water to keep seeds moistened.

After the stratification period, germinating seeds (Fig. 6.1f) were selected and sowed in plastic trays with a peat:perlite mixture (2:1, w:w) which was previously saturated with an insecticidal solution containing 2 g/L *Bacillus thuringiensis* (Bactur 2× WP, Comercial Química Massó, Spain). Once the seeds were sowed, a thin layer of vermiculite was spread over the surface of the seedbed (Fig. 6.2b). Thereafter, fungicides were applied weekly by alternating methyl thiophanate (Pelt 45 SC, 45% w/v, Bayer, Spain) and triadimenol (Bayfidan 312 SC, 31.2% w/v, Bayer, Spain) to keep seedlings (Fig. 6.1g) free from fungal infections.

Individuals carrying the *Vr3* resistance gene were selected and planted in an experimental orchard at Mollerussa. In addition, two individuals not carrying the *Vr3* gene were also selected and planted at the same orchard to be used as controls to detect natural PPM infections.

Figure 6.2. Seeds at the stratification tray (a) and sowed after germination (b).

6.3.3. Genotyping evaluation

Genomic DNA extraction and further PCR reactions were performed as previously described in section 5.3.3 of this thesis (Chapter 5). Markers used for the selection of *Vr3*-carrying individuals are described in Table 6.3.

6.3.4. Phenotypic evaluation

The individuals that were selected from 2016 to 2019 as resistant based on their genotypes were phenotyped for PPM resistance as previously described in section 5.3.2 (Chapter 5).

6.4. Results

About 11,650 flowers from eight different crosses were pollinated between 2016 and 2018 to obtain resistant individuals carrying the *Vr3* almond gene (Table 6.3). Only 754 of all pollinated flowers developed into a fruit, ranging from 0.5% ('Nectatop' × 19P15-15) to 41.4% ('ASF 06-20' × E2T-092-025) of attempted pollinations. In total, 669 seeds were extracted. Finally, 78% of these seeds germinated, thus obtaining 522 seedlings. These seedlings were genotyped with at least two markers surrounding the *Vr3* gene, which previously has been described in a region of 270-kb between Pp02:16,912,811 and Pp02:17,184,692 (Chapter 5). Markers used for the upper limit of the region containing *Vr3* were Indel16748, Indel16912, and UDP-098-025. For the lower limit of the region the markers Indel17019, Indel17186, Indel17242, and Indel18610 were used. The agarose gel profile of Indel118610 is shown in Fig. 6.3. Finally, 132 seedlings (25%) carrying an almond introgression including the *Vr3* gene were selected as resistant based on the genotyping. As we were using *Vr3* heterozygous individuals (*Vr3vr3*) as donors, we were expecting to obtain 50% of resistant individuals. Thus, the results obtained showed a segregation distortion, obatining half of the individuals expected.

Table 6.3. Markers used to select the individuals carrying the *Vr3* resistance gene.

PPM gene	Marker	Marker type	Marker name	Marker location	Product size	Forward 5'-3' sequence	Reverse 5'-3' sequence
Vr3	1	Indel	Indel16748	Pp02:16,748,613	57	AAGGCTCCCACTGAATGATG	CCTGCAATGTGGTTGACAAT
Vr3	2	Indel	Indel16912	Pp02:16,912,811	75	AAGTCTAGTTCCAGCACACC	ACAAGTAAGGGTGTTCATCCAT
Vr3	3	Indel	Indel17019	Pp02:17,019,709	184	GGATGAATGATTGTTTGGTTGCA	CTACACCACCCAGACACCTT
Vr3	4	Indel	Indel17186	Pp02:17,186,620	46	AAGGGGGTGTCAATGTCAAG	TGTGGGATACAAATTCCACAAG
Vr3	5	Indel	Indel17242	Pp02:17,242,814	427	AGCCCCTCTCACAACATCC	GGCTCTGCAACTTTTCTTGG
Vr3	6	Indel	Indel18610	Pp02:18,610,981	175	GTGGCCAGAGATAGGAGTCG	TTGCGAAAAACTCCCCATAC
Vr3	7	SSR	UDP-098-025	Pp02:13,651,450	20	GGGAGGTTACTATGCCATGAAG	CGCAGACATGTAGTAGGACCTC

Phenotyping data confirmed that all the individuals selected as resistant through genotyping did not show any PPM symptom during the evaluation period, whereas those not carrying *Vr3*, and kept as positive controls, showed PPM symptoms on leaves.

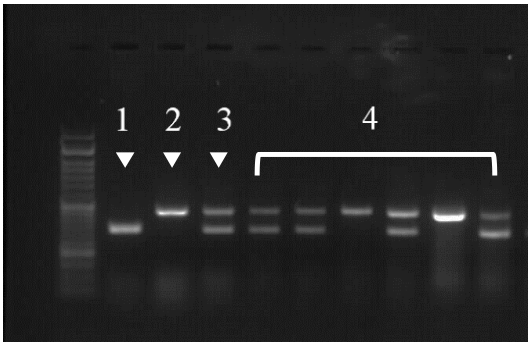

Figure 6.3. Agarose gel electrophoresis from Indel18610 used to genotype: 1) 'Texas', 2) 'Earlygold', 3) the hybrid 'Mb1.37' ('Texas' × 'Earlygold'), and 4) six additional genotype profiles obtained from samples screened during the MAS process.

Trunk diameter measurements were performed as a vigor estimation for *Vr3* homozygous NILs (Table 6.4). Mean trunk diameter of resistant homozygous individuals was 13.4 cm, whereas resistant heterozygous individuals measured 26.5 cm, and homozygous susceptible individuals 25.25 cm.

Table 6.4. Trunk diameter of indivuals from family lines used as *Vr3* donors.

Genotype	Individual	Trunk diameter (cm)
Resistant homozygous (Vr3/Vr3)	19P15-37	14.0
	19P15-50	12.0
	19P15-116	14.0
	19P15-120	13,5
Resistant heterozygous (Vr3/vr3)	19P15-12	26.0
	19P15-15	28.0
	19P15-44	26.0
	19P15-57	26.0
Susceptible (vr3/vr3)	19P15-25	23.0
	19P15-34	20.5
	19P15-46	27.0
	19P15-60	27.5
	19P15-77	25.5
	19P15-119	28.0

6.5. Discussion

In 2016, our group proposed a MAI breeding strategy for the introgression of new genomic information into the peach background (Serra et al. 2016). This previous research resulted in the obtention of a collection of NILs, i.e. lines that contain all the 'Earlygold' peach genome except for a single genomic fragment from the 'Texas' almond. In this study we used the NILs lines containing the *Vr3* resistance gene as prebreeding material to perform crosses with parental lines from the IRTA-ASF-FruitFutur peach breeding program. Although NILs with a single almond introgression and containing *Vr3* were already available, the peach cultivar 'Earlygold' used as the recurrent parent has a very low postharvest behavior and fruit quality. For this reason, these lines must be further improved for those traits. Therefore, T×E NILs with one or two almond introgressions and containing the *Vr3* resistant allele were later crossed with parental cultivars from the IRTA-ASF-FruitFutur peach breeding program to obtain lines carrying the *Vr3* PPM resistance gene with improved fruit quality and postharvest behavior traits. We finally obtained 132 lines that will be further evaluated for these traits to select potential cultivars and parents for the breeding program. Some of these lines come from parent E2T-092-025, which carries a second introgression from almond in G6. Therefore, they have to be genotyped to discard those including this second almond introgression to avoid the presence of unwanted linked traits. Furthermore, as these lines still carry a half of the genome from 'Earlygold', we think that an additional cross with a high-quality cultivar will be need to select individuals with the targeted traits, i.e. the PPM resistance and high fruit quality attributes.

In this study we used different markers (Table 6.3) surrounding *Vr3* as they have been developed during the time frame of this PhD and some of them were not available on the first years when MAS was performed. Here we propose markers Indel16912 and Indel17186 to be used in future MAS screenings as they closely ecompass *Vr3*. The main disadvantage of Indels markers is that genotyping through agarose gels is time-consuming when dealing with a high number of samples. A possible solution could be converting these markers into markers that can be scored in an automatic sequencer using labelled primers. A second solution could be the use SSRs markers that are close to the *Vr3* gene. In this case, the main disadvantage is that Indel17186 is further away from the *Vr3* gene and the probability of finding recombinants is a bit higher.

In the whole MAS process, we made several observations that have to be further discussed to improve future MAS screenings for *Vr3*. The first one is that variable percentages of fruit development from pollinated flowers were obtained, ranging from 0.5% to 41.4%. This could be explained by the different genotypes being crossed, and potential differences due to environmental conditions in the orchards among years. We should record additional data to hypothesize on possible causes for percentages of fruit development success.

Another worth-mentioning point is the distorted segregation observed from the individuals obtained for the introgression of *Vr3* gene. We propose two possible explanatory hypotheses that should be further studied. The first one is the existence of a lethal recessive allele, which in homozygous condition could contribute to the segregation distortion. An alternative hypothesis is based on eventual self- or cross pollinations with other peach cultivars present in the area. In this case we could not distinguish the alleles coming from other peach cultivars as we did not use specific markers for that.

Homozygous NILs for *Vr3*, conversely to heterozygous lines and those homozygous for peach alleles, were not producing fruits. Further, trunk diameter from homozygous NILs for *Vr3* resulted two times thinner than in other individuals. These observations are important for their eventual use in breeding programs dealing with the *Vr3* introgression, as homozygous individuals should be discarded if these observations are further confirmed in other populations carrying the *Vr3* gene in homozygosis.

Considering that *Vr3* is a monogenic trait, its durability could be limited by the appearance of new virulent strains of *P. pannosa* able to overcome the resistance (Parlevliet, 1993). First description of an introduced major resistance gene against powdery mildew was *M1g* in barley, which was overcame after ten years (Wolfe, 1984). Another example of monogenic resistance breakdown was described for the apple powdery mildew resistance gene *Pl2*, which appeared within six years after planting selected genotypes (Caffier and Laurens, 2005). One of the proposed strategies to achieve a durable resistance was based on the combination or pyramidization of several resistance genes into a single cultivar/line (Gautam et al., 2020). According to this idea, we propose a pyramidization approach using other monogenic genes for PPM resistance, such as *Vr1* and *Vr2* (Lambert, 2018; Pascal et al., 2010, 2017). Both *Vr1* and *Vr2* genes were described as major genes from the ‘Malo Konare’ canning peach and the ‘Pamirskij5’

peach rootstock, respectively. *Vr1* was located at the top region of G8 (Lambert, 2018), where *Vr2* was mapped (Pascal et al., 2017). A pyramidization approach was initiated in 2019 at IRTA in collaboration with Dr. Bénédicte Quilot-Turion (INRAE, Unité Génétique et Amélioration des Fruits et Légumes, Avignon, France). Some crosses were performed at INRAE and IRTA orchards aiming to obtain individuals carrying more than one monogenic PPM resistance gene. In addition, other resistance genes affecting other biotic stresses such as aphid resistance started to be pyramidized at INRAE. This could be a first step towards the identification of peach seedlings with pyramidized resistance genes to several biotic stresses as already described in apple (Laurens et al., 2018).

6.6.References

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7. General Discussion

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Peach powdery mildew (PPM) is one of the most important diseases affecting peach production worldwide. Currently, PPM is being controlled exclusively through fungicide applications following a calendar-based program (Grove, 1995), with fungicide applications every 7 to 14 days (Xu, 1999). The use of plant protection products could involve hazardous effects on human health and environment (Budzinski and Couderchet, 2018; EU, 2009; Krieger, 2010). In this thesis, some alternatives for the disease management of PPM have been studied. The first one was based on the study of PPM epidemiology and the development of a model to adequately initiate the preventive fungicide applications. The second approach was based on the characterization of a gene conferring resistance to PPM (*Vr3*) and the development of new resistant peach cultivars. This two-way approach, based on the prevention and avoidance of the disease risk infection, was developed to decipher new control strategies for PPM starting from different scientific disciplines such as Plant Pathology and Molecular Genetics. Different techniques from those fields of study have been used in combination to increase the knowledge about the peach-powdery mildew pathosystem and its control.

Peach powdery mildew is caused by the ascomycete *Podospaera pannosa*, that causes a reduction in yield across worldwide areas where peach is cultivated (Jarvis et al., 2002; Weinhold, 1961). To date, scarce information about the driving factors of pathogen epidemics and the evolution of specific disease stages in relation to environmental factors is available. The study of factors influencing the pathogen development are difficult to be performed through *in vitro* assays because of the biotrophic nature of the pathogen. Conversely to other *in vitro* assays performed on rose (Leus et al., 2006), we were not successful in maintaining *P. pannosa* alive on *in vitro* peach leaf discs or plantlets, neither inoculating it (Marimon, *unpublished*).

Although some epidemiological models have been described for powdery mildews affecting other *Rosaceae* crops such as rose (Xu, 1999) and cherry (Grove et al., 2000), no models have been yet described for PPM. This could be partially explained by the high effectiveness of current fungicide programs in PPM control, that is not favoring further research in alternative control methods. Nevertheless, currently sustainable use of

pesticides is mandatory, due to the implementation of regional and European regulations (EU, 2009); thus, the number of authorized active ingredients for disease control is decreasing in recent years. Therefore, the development of alternative strategies would be needed to reach a sustainable integrated control of the disease. An insight into the epidemiology of PPM, conducted in Chapter 3 and Chapter 4 of this thesis, is based on the study of factors influencing the development of the pathogen, and thus to explore solution strategies based on the prevention of the disease.

The first approach was based on modeling the disease progress of PPM. We have been able to determine a first component of an epidemiological model describing the relationship between the disease progress and some environmental variables (Marimon et al., 2020; Chapter 3). A beta-regression model fitted on disease incidence data showed a substantial contribution of temperature, expressed as accumulated degree-days (ADD) after 50% bloom, wetness duration, and ADD considering vapor pressure deficit and rain. When discarding the random effects of orchard and year in the dataset used to build up the model, the best predictor of PPM disease progress was the ADD variable, although model fitting was somewhat poor. Nevertheless, we think that more precise PPM epidemic drivers based on water and temperature can be obtained in future research. The epidemiological models currently available for powdery mildews in other *Rosaceae* species, such as those for rose and cherry (Grove et al., 2000; Xu, 1999), as well as other in non *Rosaceae* species such as cucurbits (Sapak et al., 2017), are based on the influence of different environmental factors such as temperature and wetness duration as drivers for the disease progress.

Otherwise, some previously described models focused only in one environmental factor to explain disease development. Carisse et al. (2009) defined a model based only in temperature, as expressed in ADD, to explain the initiation of primary infections of grapevine powdery mildew, as we similarly proposed for PPM in Chapter 3 of this thesis. From the relationship between ADD and the detection of early PPM infections, we established an operational threshold to initiate fungicide treatments at 220 ADD. This 220-ADD operational threshold, based only on one environmental variable –i.e., temperature– was thought to be a first approach to a predictive model for the onset of primary infections and the subsequent initiation of fungicide applications, which were later than predicted in current calendar-based fungicide programs. Thus, the commercial

validation of the model showed that this newly proposed strategy reached an overall reduction of 33% total fungicide applications. Furthermore, this threshold provided growers a reasonable period to mobilize application logistics before the onset of the risk period for PPM. In conclusion, an easily measurable environmental variable – temperature– can help growers to effectively predict the onset of infection. This could be a first approach for the implementation of an effective, rational, and sustainable integrated strategy to control peach powdery mildew. Similar reduction in the number of seasonal fungicide applications have been reported for cucurbits (Sapak et al., 2017) and grapevine powdery mildews (Carisse et al., 2009).

The epidemiological model described in this thesis is highly dependent on factors linked to each specific orchard and year conditions, such as the cultivar susceptibility to PPM, the orchard management practices, or the effectiveness of fungicides used to control PPM. Among other factors affecting disease progress, we hypothesise here that the primary inoculum present in each specific orchard and year combination, besides certain environmental conditions, could be highly influential on the disease progress and the final PPM incidence level. Therefore, we assumed that real-time detection and quantification of the pathogen would provide us with valuable information to predict more adequately the potential risk of the disease at each specific condition. To achieve this further objective, a specific qPCR-based protocol was designed and developed, which aimed at maximizing the specificity and sensitivity detection of *P. pannosa*. The protocol included the design of a primer pair, namely PpanITS1-F/PpanITS1-R, used to detect and quantify the pathogen in various biological samples. The protocol allowed us to identify and quantify the pathogen in the winter latent structures of the pathogen, for which no molecular detection tools have been described to date. The pathogen was detected as dormant mycelium on infected twigs and, to a lesser extent, in leaf buds. The presence of fungal resistance forms inside the leave buds had already been described from microscopical observations (Ogawa and English, 1991; Toma et al., 1998; Weinhold, 1961; Yarwood, 1957). *Podosphaera pannosa* was also detected and quantified from environmental samples, i.e. exposed plastic tapes with adhered propagules, taken with a volumetric air sampler. The specific detection through a molecular-based technique described in this study is essential to identify *P. pannosa*, as powdery mildews from other species show similar morphology of conidia (Braun, 1987). In addition, we proved that coupling spore traps with DNA-based assays is a faster and more reliable alternative to

the conventional detection of powdery mildew propagules through microscopical observation. Finally, we showed that a reliable quantification of airborne conidia was achieved, which allowed to describe the evolution of airborne propagules along the season. To the best of our knowledge, this is the first time that a qPCR-based method has been developed for the detection and quantification of *P. pannosa*. Therefore, the protocol described in this thesis (Chapter 4) represents an interesting tool for future research studies about the epidemiology of the pathogen through its molecular detection and quantification.

Regarding the molecular genetic basis of the host resistance to PPM, several sources of resistance have been described in *Prunus* species, mostly controlled by QTLs (Dirlewanger et al., 1996; Foulongne et al., 2003). Only a few cases of monogenic resistance genes to powdery mildew for *Prunus* have been described to date, i) the *Vr1* and *Vr2* gene, described as monogenic resistances in linkage group 8 that came from the rootstock cultivar 'Pamirskij 5' (Lambert, 2018; Pascal et al., 2010; Pascal et al., 2017), and ii) the *Vr3* gene, described in interspecific populations between 'Texas' almond and 'Earlygold' peach crosses (Donoso et al., 2016). As occurs with *Vr3*, for other powdery mildew species from *Podosphaera* genera, resistance is controlled by major genes with dominant effect. In melon, there are described 12 resistance genes conferring resistance to *Podosphaera xanthii*, despite only four of them are mapped (Fazza et al., 2013). Considering other *Rosaceae* species affected by powdery mildew, similarly there is described a monogenic resistance in sweet cherry (gene *PMR-1*) conferring resistance to *Podosphaera clandestina* (Olmstead and Lang, 2002), coming from a powdery mildew resistant sweet cherry selection. Infrequently the exact positions for the genes are described, and exceptionally, resistance gene is identified and characterized. Regarding the *Vr3* dominant gene, of which we discussed extensively in Chapter 5, was located in chromosome 2 and described in a genomic region spanning 3.7 Mbp and 1.3 Mbp in T×E and T1E populations, respectively (Donoso et al., 2016). As a dominant gene, it confers total resistance to peach powdery mildew despite the allelic combination, either for homozygous or heterozygous individuals. In this PhD thesis we have fine mapped the *Vr3* gene (Chapter 5) and developed a marker-assisted selection strategy to introduce this resistance gene in a peach breeding program (Chapter 6). The *Vr3* characterization, by identifying the most probable candidate gene, involved a fine mapping approach to narrow the region containing *Vr3*. Four SSR, 14 Indels and four SNPs markers were

designed in this thesis using the resequences of the parents to further narrow the region where *Vr3* was previously located (Donoso et al., 2016). The markers designed were used in different segregating populations to look for recombinant individuals. We were finally able to narrow the region to 270 kb containing 27 candidate genes to host the *Vr3* gene. After studying the polymorphisms in the resequences of both parents, a variation in sequence was predicted to have disruptive effect on the encoded RGA2 protein, which pointed *RGA2* as a potential candidate gene. Furthermore, an expression analysis of the 27 candidate genes including *RGA2* was performed in susceptible and resistant individuals, and only the gene encoding for RGA2 showed significant differential expression among resistant and susceptible individuals, being significantly overexpressed in homo- and heterozygous individuals while their phenotype were equally resistant to PPM. Resistant individuals were differentiated whether *Vr3* was host in homozygosis and heterozygosis, being higher expressed in homozygous individuals. Furthermore, overexpression of *RGA2* was independent of the infection status (Marimon et al., in preparation), which led us to think that *RGA2* was constitutively expressed, as previously reported for other RGA genes involved in fungal resistance (Calenge and Durel, 2006). These genes came from wild grapevine species which were cloned and transferred to susceptible commercial vineyard species. In this thesis, *RGA2* was finally proposed as the most probable candidate for the *Vr3* gene (Chapter 5), and this PhD thesis paves the way to a future functional validation to fully characterize the *Vr3* resistance gene.

As almond and peach genomes have a high degree of conservation, we were working on the polymorphisms existing among both sequences to identify *Vr3* gene. Despite being comparable sequences, recently it has been described that when compared, they showed a considerable number of variants of presence and absence, maybe attributable to transposable elements (TEs) (Alioto et al., 2019). A point which is not included in Chapter 5 is an analysis of synteny among peach and almond resequences of region containing *Vr3* gene. The aim was to detect possible regions that were present in almond sequence and non-mapping to peach genome. We identified a region in 'Texas' genome containing ten genes in the *Vr3* region that did not have a corresponding syntenic sequence on peach. After developing two markers from 'Texas' resequence of that region, we could not verify its presence in individuals from 'T×E' population because the amplified region did not cosegregate with *Vr3*. This approach could be repeated in this and in other populations to decipher which is the cause of these results, or also new

markers in 'Texas' region could be designed to perform again the genotyping of 'T×E' population.

As new molecular markers closely flanking the *Vr3* gene were described in this thesis, breeding strategies could be performed for its introgression into elite cultivars through a marker assisted selection (MAS) approach. The markers that were used for introgressing *Vr3* gene were mainly Indel16912 and Indel17186. Markers were developed since 2016 to narrow the region containing *Vr3*, thus the markers used for *Vr3* introgression were different depending when were used. Despite the closest markers to *Vr3* are Indel16912 and SNP17184692 (Chapter 5), at the introgression approach were used Indel markers because their agarose gel profiles are very clear and low number of samples were analysed. In the case of a large number of samples, working with Indels implies a lot of laboratory labour. If that is the case other markers that can be automatized, as the SSR CPDCT044 and the SNP SNP_17184692, could also be used. These newly developed markers could greatly aid in cloning and conducting marker-assisted selection of *Vr3* in peach breeding programs.

Another important issue to consider is that the sources of resistance identified earlier for peach have been described specifically for leaf tissue. Although it has not been verified that the leaf resistance is also applicable to fruits or other susceptible tissues, there are no individuals in whom infections have been found in the fruit but not in leaves, at least in 'Texas' and 'Earlygold' progenies.

Since 2016, several individuals containing a maximum of two almond genome introgressions into peach background including *Vr3* gene, have been crossed with high quality commercial peach/nectarine parentals of the IRTA-ASF-FruitFrutur peach breeding program and selected using MAS. To date, 132 individuals carrying an almond introgression with the resistant *Vr3* allele have been selected. In the near future, other crosses with elite peach cultivars would need to be performed to be able to obtain high quality fruit cultivars carrying PPM resistance. Thus, this has been an important approach to have valuable material for the obtention of peach elite cultivars with PPM resistance.

To avoid the resistance overcome in single resistance-depending genes, a MAS strategy was used to pile up several monogenic resistances into a single genotype, thus improving resistance durability (Muranty et al., 2014). In our case, a similar

pyramidization approach was proposed in Chapter 6 to include other previously described resistance genes (*Vr1* or *Vr2* besides *Vr3*¸ see Pascal et al. 2017) conferring PPM tolerance into peach. This study is the first one, to our knowledge, describing the introgression and pyramidization of biotic resistance genes in peach, which can significantly contribute to the obtention of new peach varieties resistant to powdery mildew.

Currently, social awareness about the care for environment and human health issues that are linked to pesticide usage is increasing (Budzinski and Couderchet, 2018). In this scenario, we attempted to combine several tools and solutions coming from related Plant Pathology and Molecular Plant Breeding subjects into an integrated PPM disease management which can be more respectful to both environment and human health. This has been done through the study of some biological key aspects involving the environment- and host-pathogen interactions in the PPM-peach pathosystem.

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8. Conclusions

8. Conclusions

1. In this thesis, innovative alternatives for the disease management of peach powdery mildew (*Podosphaera pannosa*) have been examined through the development of strategies based on the optimization of fungicide programs and the characterization of genetic resistance.

2. A beta-regression model was used to describe the progress of peach powdery mildew disease on fruit, expressed as proportion of affected fruit, which included the variables temperature (as accumulated degree-days, ADD), wetness duration, and ADD combined with vapor pressure deficit and rain, and a highly influential dependence on the random factors orchard and year.

3. When the random factors were removed from the model, disease progress was best predicted by the ADD variable alone.

4. Early primary infections of peach powdery mildew on fruit were detected at 240 ADD after 50% bloom, which allowed to establish an operating threshold to initiate fungicide applications at 220 ADD.

5. The 220-ADD alert spray program showed a statistically relevant reduction in disease incidence on peach fruit down to 7% as compared to the control disease incidence (24%).

6. The number of fungicide sprays in the 220-ADD alert spray program resulted in an overall reduction of 33% in the whole cropping season as compared to a standard calendar-based program.

7. A species-specific primer pair designed on the ITS region of the rDNA of *Podosphaera pannosa* enabled the identification and quantification of the fungus in different types of samples, such as fungal DNA suspensions (minimum: 2.81 pg of pathogen DNA), conidia suspensions either placed on plastic trapping tapes or not (six conidia), and different plant parts of peach trees.

8. Microscopical and molecular quantification techniques coincided in detecting peaks of airborne conidia of *Podosphaera pannosa* along the cropping season. Conidia amounts recorded through both quantification methods showed a good significant correlation ($r = 0.819$, $P < 0.001$).

9. The overwintering fungal inoculum of *Podosphaera pannosa* was detected mainly on the bark of affected twigs, and, to a lesser extent, in foliar buds.

10. Through a fine mapping approach, the genomic region containing the *Vr3* almond resistance gene was narrowed down from 6.7 Mb and 1.3 Mb in T×E and T1E populations where it was described to 270 kb (between Pp02:16,912,811 and Pp02:17,184,692), which included 27 annotated genes.

11. The combined analyses of resequence polymorphisms from 'Texas' and 'Earlygold' parents and expression analyses of the 27 candidate genes in symptomatic and asymptomatic leaves showed that the disease resistance protein RGA2 (*Prupe2G111700*) contained a variant predicted to have a disruptive effect on the encoded protein. RGA2 (*Prupe2G111700*) was overexpressed in both heterozygous and homozygous individuals containing the *Vr3* almond allele, as compared to susceptible individuals not containing the *Vr3* almond allele.

12. In the context of the IRTA-ASF-FruitFutur peach breeding program, T×E NILs containing *Vr3* had been crossed with high quality parents. Using MAS, 132 of the individuals obtained were selected carrying an almond introgression with the resistant *Vr3* allele.

NOTES

Towards an integrated control of peach powdery mildew *(Podosphaera pannosa)* through the application of molecular tools in epidemiological and genetic resistance studies.

Doctoral thesis

Neus Marimon de María

2020