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Brown rot resistance in peach:

a genomics approach.

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III. Summary

Brown Rot disease (BR) caused by the necrotrophic fungus *Monilinia* spp. is a major problem for the peach fruit market, causing significant losses at post-harvest level. Previous work demonstrated the possibility of discriminating between susceptible and tolerant peach genotypes, suggesting a quantitative nature of the BR resistance. In order to uncover genomic regions associated with this trait and identify molecular markers for marker assisted selection (MAS), an F1 segregating population from the intra-specific Contender (tolerant cultivar) x Elegant Lady (susceptible cultivar) peach cross has been chosen for QTL analysis. Phenotypic analysis was performed over two harvest seasons, using an artificial infection procedure that measured skin and flesh resistance to a *M. fructigena* field isolate. Significant correlations were found between the data obtained in the two years and between the two traits. Maturity date (MD) was also highly correlated with resistance traits. Genotyping 110 CxEL individuals allowed the construction of a linkage map (CxEL map), containing 78 SSR, covering a total genetic distance of 317.7 cM, and having an average marker density of 4.7 cM/marker. Both parametric (interval mapping) and non-parametric (Kruskal-Wallis analysis) QTL analysis using genotypic and phenotypic data from CxEL revealed two QTL clusters: a QTL underlying skin resistance located on LG CxEL-2 (explaining a 15% - 22% of the total phenotypic variability), and a QTL associated with flesh resistance (explaining a 30% - 35.2% of the total phenotypic variability), collocating with a major MD QTL on LG CxEL-4. These results suggest that resistance to BR has at least two main components: the first related to avoidance of fungal penetration, and a second earliness-associated factor associated with fungal spread after penetration. Furthermore, markers M1a (CxEL-2) and UDAp-439 (CxEL-4) may provide useful tools for MAS for BR-resistance breeding programmes.

1. Introduction

Peach is the name given to a deciduous tree species (*Prunus persica* L. Batsch) whose edible fruit is very appreciated for its fleshiness, sweetness and external appearance. Attractive organoleptic and nutritive features, coupled with agronomic progress and genetic improvement leading to increased production and yield around the second half of the twentieth century, have made this fruit an economically important commodity. Peach is consumed in a variety of forms: fresh or processed (dried, as juice, as jam, or canned).

In 2009, peach production reached 18,6 million of tons, corresponding to 3.34% of the total fruit production worldwide. The major producer country is China with 45.9% of world production, followed by Italy, Spain and the United States (8.8, 6.6 and 6.4%, respectively; FAOSTAT, 2010). For Italy (the second producer since 1993, when China reached the first place) peach production in 2009 resulted in a gross profit of € 568 millions (FAOSTAT, 2010).

Increasing consumer demand and expectations require continuing improvements in yield and quality of peach production. However, peach growers are constantly meeting with production problems impacting yield and quality, for instance the rise of pathogens resistant to pesticides. In order to overcome these drawbacks and achieve higher standards in peach production, good management of the existing resources (pest management, grower's know-how, harvesting techniques, storage conditions, etc.) needs to be coupled with development of new cultivars, allowing better use of these resources by the farmers. Breeders have generated thousands of peach varieties, exhibiting wide diversity for agronomic and quality traits, including tree size and growth habit, leaf form and colour, flower size and colour, chill hour requirement, blooming and ripening date, fruit size and shape, fruit skin pubescence and colour, fruit flesh texture, colour and acidity; pit adherence to flesh; kernel sweet/bitterness, etc. (Bassi and Monet, 2008). The available germplasm also shows ample variation with respect to resistance to adverse environments and multiple pests and diseases. Despite this diversity, the genetic basis of modern peach cultivars has been reported to be quite narrow (Scorza *et al.*, 1985). The reasons for this situation lay in the origin and history of peach cultivation, which is summarized in the following section.

1. 1. Peach history: a brief summary

Peach domestication, according to Li (1983), started in China, along with pear and apricot before the Zhou archaeological period (3300-2500 B.C.), but one of the first ancient books in which peach is mentioned was written around 1000 B.C.; peach is strongly twined in the Chinese folklore and mythology, appearing as the tree of immortality (or longevity) in many paintings and popular tales and legends (Faust and Timon, 1995).

Peach is supposed to have spread from China to Persia following the Silk Routes, during the second century B.C. Later, peach was introduced in the Mediterranean region, probably during the Roman occupation of Syria (70 B.C.). One of the first Roman writers that refer to peach is Virgil (70-19 B.C.). Other evidence of the importation of this fruit to Italy dates around the first century B.C. (Faust and Timon, 1995). Following arrival in the Mediterranean, peach cultivation spread from France and Italy to Eastern and Western Europe.

Soon after Spaniards arrived to Americas, peach cultivation was already quite common in Mexico (evidence dating 1571), from where it spread to New Mexico, Arizona and California (evidences dating 1799; Hendrick, 1917). Also, different introductions were documented in this period: by Spaniards in Florida (1565), after the French settling in Louisiana (1698) and later in 1714 in the Carolinas (Hendrick, 1917; Faust and Timon, 1995). The Spaniards also brought peach cultivation to South America, on both the Atlantic and Pacific coast (Faust and Timon, 1995).

A turning point for the origin of modern peach cultivars was the arrival of Chinese peach varieties to the United States from London between 1850 and 1875. A seedling from cultivar now known as 'Chinese Cling' was pollinated with a local cultivar (believed to be 'Early Crawford'; Myers *et al.* 1989) generating 'Elberta', worldwide the most important peach variety of its times. Another important cultivar deriving from 'Chinese Cling' was 'J. H. Hale' (registered in 1912), a chance seedling probably generated from an 'Elberta' seed, but that ripened a week earlier giving larger and less pubescent fruit (Faust and Timon, 1995). Later, these two varieties became the base cultivars used in peach breeding, raising concerns on the narrow genetic basis of modern peach germplasm (Scorza el al., 1985).

During the period 1850-1900, several cultivars were developed including 'Late Crawford' (1815), 'Early Crawford' (1820), 'Oldmixon Free' (1835), 'Belle of Georgia' (1870), 'Carman' (1889), 'Halford' (1921) and 'Mayflower' (1937). As of 1917, 2181

peach cultivars had been registered (Hendrick, 1917), rising to 6000 by 1985 (Okie, 1985), and over 8000 in 1998 (*Handbook of Peach and Nectarine varieties*; Okie, 1998).

In the second half of the twentieth century, varieties generated in the United States have been tested in Italy, France, Spain, Hungary and Chile. Their importance in the modern peach market is such that, for instance, around a 60% of the Western Europe peach production is based on American cultivars (Faust and Timon, 1995). Thus, with the exception of Asia, the world peach and nectarine production is largely based on North American cultivars, which have been generated starting from a few Chinese seedlings.

1.2. Peach Botany

1.2.1. Systematics

Classified in the order *Rosales*, family *Rosaceae*, sub-family *Prunoidae*, genus *Prunus*, the species *Prunus persica* L. Batsch (peach) together with *Prunus dulcis* (Mill) D. A. Webb (almond) form the subgenus *Amygdalus*, distinguished from the other subgenera by the presence of a deeply-rough stone. Peach differs from almond because the mesocarp of the latter becomes dry, splitting at maturity, while the leaves are serrulate (Bassi and Monet, 2008).

Being a tree species, peach shows a long generation time, taking 3-4 years between seed germination and the first reproductive season. This explains the difficulty of generating inbred (homozygous) lines by selfing. In order to maintain homogeneity, commercial cultivars are propagated by grafting, activity useful also to protect the cultivar against soil borne pests and water lodging.

In general, *Prunus persica* L. Batsch, as all members of the *Prunus* genus, is a diploid species (2n=16), although some haploid cultivars (n=8) have been developed (Toyama, 1974).

The tree has a medium size, reaching a maximum height of 8 m. Leaves are lanceolate, glabrous and serrulate; flowers can be from red to white (mostly pink), and autogamous. The fruit is a fleshy drupe, pubescent (also glabrous, *e.g.* nectarine), that does not split at ripening. The endocarp is lignified and deeply-rough.

While these features are generally stable across the existing peach cultivars, other attributes display wide variability and will be described in section 1.2.3.

1.2.2. Peach relative species

In wild relatives of peach, fruit quality is mostly not suitable for consumption, but interesting features – *e.g.* disease resistance or capacity to grow in recalcitrant soils - make them good candidates to be used as rootstocks or pest and disease tolerance donors in commercial peach breeding. Among these species, the following are considered of special interest:

• *Prunus davidiana* (Carr.) Franch. Highly tolerant to drought, sensitive to nematodes. Fruit quality is poor due to the high content of malic acid, neochlorogenic and cryptochlorogenic acid and sucrose concentrations lower than peach (see section 1.2.3.4-c.3). The tree is taller than peach (10m), with larger and more ovate leaves, with a smaller fruit and pit, and freestone flesh. It has been hybridized with peach, yielding seedlings with improved resistance to plum pox virus, powdery mildew and leaf curl, and sugar content comparable to peach (Moing *et al.* 2003; Foulogne *et al.*, 2003; Quilot *et al.* 2004).

• *Prunus ferganensis* (Kost. and Rjab) Kov. and Kost. Often classified as a subspecies of *P. persica*, it shows high variability in fruit types (yellow/white flesh, pubescent/glabrous skin). Examples of mendelian traits include leaves with parallel veins and parallel grooves in the stone (Okie and Rieger, 2003). It has been hybridized with peach commercial cultivars as a donor of resistance to powdery mildew by *Sphaeroteca pannosa* (Dettori *et al.* 2001, Verde *et al.* 2005).

• *Prunus kansunensis* Redh. Bushy tree that shows early sprouting and flowering, suggesting it may be a frost-resistance species. However, the fruit shows very poor quality because of its astringency. It has been recently used to develop crosses with peach, resulting in progeny with decreased branching and the consequent cost reduction by means of pruning (Carrillo-Mendoza *et al.* 2010).

1.2.3. Phenotypic diversity among peach cultivars

Commercial peach varieties, are often classified distinguishing three main groups based on fruit characteristics, *i.e.* pubescent/glabrous fruit skin (peach/ nectarine) and fruit flesh tex   ture (melting/non melting). However, variation

across peach germplasm exists for a number of other traits as described in the following section.

1.2.3.1. Tree

Peach trees have mainly two general uses: for fruit production and ornamental. The main traits, or elements of the tree morphology that vary among peach accessions are:

a. Internode length: standard internode length in peach is between 15 and 25 cm. This trait has been largely described to be influenced by both qualitative (mendelian or monogenic) and quantitative (polygenic and environment-influenced) loci. Dwarf phenotypes show a reduced internode distance (less than 10 cm), and various genes have been mapped for this trait, most of them monogenic recessive (Monet and Salasses, 1998; Gradziel and Beres, 1993);

b. Tree growth habit (TGH): defined as "tree form" or "overall appearance of a tree's canopy" (Bassi and Monet, 2008). This trait, depending on the genetic background of the cultivar, appears to be conditioned by both quantitative and mendelian loci. The more recurrent types of TGH in peach germplasm are arching, columnar, compact, open, spreading, spur, standard, twister, upright and weeping.

1.2.3.2. Leaf

a. Form. The leaf blade can be flat or wavy as determined by dominant or recessive alleles of the *Wa* locus, respectively (Scott and Cullinan, 1942). Narrow leaf phenotypes have also been associated to Mendelian loci, and are generally linked to dwarf genes (Chaparro *et al*, 1994).

b. Leaf glands. There are three types of leaf glands, that are the expression of a mendelian locus with incomplete dominance (*E/e*; Connors, 1920): reniform (*Ee/Ee*), globose (*Ee/ee*) and eglandular (*ee/ee*). This trait is strongly linked to the tolerance to powdery mildew caused by *Podosphaera pannosa,* with homozygous dominant plants (reniform gland) showing lowest susceptibility to the pathogen (Watkin and Brown, 1956; Saunier, 1973).

c. Leaf colour. Various phenotypes have been described resulting from expression of different monogenic loci on different red-coloured cultivars, as the "redleaf" monogenic traits. The *Anthocyanin deficiency* (*An/an*) and *anthocyaninless* (*W/w*) genes also affect the colour of leaf, flowers and fruit (Bassi and Monet, 2008).

1.2.3.3. Flowers

Peach has hermaphroditic and perigynous flowers with gamosepalous calyx (Bassi and Monet, 2008). The inner part of the calyx is yellow when the cultivar bears a yellow-fleshed fruit and white/green in genotypes producing white flesh peach.

a. Corolla type. Mainly, two types of corolla can be found: "showy" (rose shaped, large petals) and "non-showy" (bell shaped, small petals). The inheritance of this trait is monogenic, being non-showy the dominant (*Sh/sh*). In the showy phenotype the size of petals is also controlled by one gene (L/l), being the large-sized showy trait the dominant (Connors, 1920; Bailey and French, 1949).

b. Petal number, shape and colour. The simple, semi-double and double flower phenotypes show differences in petal number: simple flowers have typically 5 petals; in semi-double flowers, few stamens are transformed to petals resulting in flowers with 12-24 petals; in double phenotypes, the latter situation is amplified to almost all sepals transformed in petals.

Petal colour can vary from pure white to dark red and variegated. Also in some ornamental cultivars chrysanthemum-like petal has been described (Yoshida *et al.* 2000).

d. Pollen sterility. Although this trait is typically eliminated from breeding, some male-sterile cultivars exist. This trait is conditioned by two recessive loci: *ps* (homozygous in 'J. H. Hale'; Bailey and French, 1949) and *ps2* (found in cv. 'White Glory'; Werner and Creller, 1997).

1.2.3.4. Fruit

a. Shape and size.

Fruit weight of commercial varieties varies from 180 to 230 g at harvest time. This trait is clearly quantitative and likely controlled by multiple genes. Recent genetic analyses showed a significant genetic component (Quilot *et al.* 2005, Etienne *et al.* 2002), being also affected by the pleiotropic action of some major genes (Eduardo *et al.*, in press).

Popular "flat" peaches (also "saucer", "pan-tao", or "peento"), are flattened at opposite poles. The saucer trait is monogenic dominant (*S*) over round peach (*s*), and the homozygous is lethal (Lesley, 1940). In these genotypes, also the seed is flattened, with a minor germination rate or viability.

b. Skin. Two important aspects of the skin have great importance for the peach market.

b.1. Surface differs in standard (pubescent, fuzzy) peaches and nectarines (glabrous, smooth skin). The nectarine phenotype is monogenic recessive (*g*) and may have originated from a mutation, probably from the north west of China (Faust and Timon, 1995). The nectarine character makes fruit more susceptible to mechanical bruising and pest damage.

b.2. Skin colour (similar to flesh colour, see the section below) is determined by two main pigments, with distinct sub-cellular localizations: carotenes and xanthophylls, that give the orange and yellow ground colour, respectively, and are located in the chromoplastides; and the anthocyanins, responsible of red/blue over-colour, that accumulate in the vacuole. In the skin, the red over-colour is a quantitative character, probably influenced by light exposure, and ripening-dependent. Two *loci* that affect the fruit red colour have been identified: the "redleaf" gene, expressed also in leaves; and *Rf/rf*, expressed in fruit skin only (Beckman and Sherman, 2003). A third mendelian locus controlling the trait is "highlighter" (*H/h*), which is recessive and suppresses the presence of skin over-colour (Beckman *et al.* 2005).

c. Flesh. Fruit endocarp corresponds to the major part of the fruit that we consume. Hence, traits relating to this tissue are major determinants of fruit quality.

c.1. Flesh colour. One of the most commercially important traits in peach fruit with cultivars being classified into two main groups: white and yellow peaches. In contrast to yellow peaches, white peaches show reduced or absent carotenes and xanthophylls content. Although white peaches have a distinct flavour compared to yellow peaches, the latter are often preferred by consumers, possibly because of their higher concentration in orange carotenoids that could mask flesh oxidation caused by blemishes. White flesh ground colour (monogenic trait) has been reported to be dominant over yellow flesh (*Y/y*). In contrast, anthocyanin distribution in the mesocarp is a

quantitative character. However, "Red blood-flesh peaches" are characterized by a red stain in almost all the flesh, independent of the ground colour: the red blood trait has been described as dominant (Werner *et al.* 1998).

c.2. Texture. Affected by the cell-wall composition and metabolism this character determines the distinction of the two most known flesh textures of peach: melting flesh (M), undergoing a strong melting in the last stage of ripening; non melting flesh (NM) that maintains a firm texture until full ripening, and slowly softens towards senescence. The difference between these two flesh types lay in a lack on endopolygalacturonase (endoPG) activity, one of the enzymes responsible for cell wall disruption during the softening process, although the typical climacteric increase of ethylene is present in both flesh types (Mignani *et al.* 2006). This trait as been shown to be highly associated to the *endoPG* locus and the freestone/clingstone trait of pit adherence to the flesh, that is another commercially important criterion to classify peach cultivars (Morgutti *et al.* 2006; Peace *et al.* 2005).

c.3. Flavour components. Various organic components contribute to peach flavour: organic acids, sugar content, phenolics and volatile compounds (Bassi and Monet, 2008). Because of the multiplicity of biochemical pathways and compounds forming the different groups (see below), the genetic basis of these traits is mostly quantitative.

In peach, the main organic acids are malic acid (often accounting for over 50% of the total acid content), quinic and succinic acids. The organic acid content is used as a classification criterion, allowing to divide peach cultivars in low- or sub-acid (LA, pH > 4.0) and acid (A). This trait is conditioned by a monogenic locus (*D/d,* Monet, 1979).

Sugar content determines the sweetness of the fruit and is measured as solid soluble content (SSC, measured by refractometry). In peach germplasm, SSC values range from 9 to 15% (Byrne *et al.* 1991; Crisosto *et al.* 1998), with fructose (the sweetest sugar in peaches) accounting for 40-80% of total sugars, followed by glucose, sorbitol, and in minor quantities, inositol, mannose, xylitol and xylose. The composition of sugars plays a significant role in the quality of peaches: Robertson *et al.* (1988) showed that low-quality

peaches may present 4-fold lower fructose content and a 3-fold higher of sorbitol in comparison with high-quality peaches.

Phenolic compounds are responsible for the astringency in the taste of foods. In good quality peaches, content of phenolics is moderate or low (Robertson *et al.*, 1988). Polyphenol content determines also the susceptibility to flesh browning after mechanical wounding (mediated also by the polyphenol oxidase, and peroxidase activities; Jimenez-Atienzar *et al.* 2007; Ogundiwin *et al.*, 2008). Finally, fruit phenolics content is positively associated with the tolerance to fungal pathogens (Lee and Bostock, 2007; Gradziel *et al.* 1997).

 Volatile compounds play a major role in determining the characteristic flavour of the different peach cultivars. An analysis of white and yellow flesh peaches indicated hexanal, trans-2-hexanal, linalool and g- and d-lactone as compounds that strongly contribute to the typical peach aroma (Robertson *et al.* 1990). Eduardo *et al.* (2010) compared aromatic profiles, identifying volatile compounds that varied among nine peach accessions.

The complex mechanisms of sensorial perception imply a difficulty in defining a high-quality- pattern of flavour components: the combination of volatile compounds, sugars, phenolics and acids defines the flavour and aroma of food (Eduardo *et al.* 2010). For instance, high levels of phenolics have been reported for Italian white peach cultivars (Bassi and Selli, 1990), suggesting that distinct aromatic compound profiles and/or sugar content may attenuate the effects of phenols in these cultivars (Bassi and Monet, 2008).

1.2.3.5. Seed

Peach endocarp is lignified, deeply furrowed and pitted. The level of lignification depends on the fruit development period (FDP) (Bassi and Rizzo,1995). Bitterness of the kernel is determined by its cyanidic glucoside content and is another trait that varies among cultivars. The bitter phenotype is dominant over the non-bitter (*Sk/sk*, Werner and Creller, 1997).

Seed germination requires a total amount of cold period. This requirement is also associated to the chill requirement of the mother tree (see below; Perez *et al.* 1993). Seed viability is reduced in some early-ripening genotypes (Perez *et al.* 1993).

1.2.4. Phenological diversity among peach cultivars

Peach is an autogamous species, with flowers self-pollinating in a high proportion of the fruit set (up to 90%). Cross-pollination rate with close trees in normal conditions is around 5% (Bassi and Monet, 2008). This results in the fact that normally, peach trees give place to a very high number of fruitless, situation that results in a final lose of quality of the fruit (mainly fruit size) and that is reverted by practices as thinning (crop reduction by fruit removal). Other traits of significant agricultural importance are listed below.

1.2.4.1. Blooming date and chilling requirement

Between the end of a harvest season and the start of the following reproductive phase (bloom), peach trees undergo a dormancy period, requiring certain duration of low temperature to initiate flowering. This chilling requirement is cultivar dependent, and determines the time in which the cultivar blooms. *Evergreen*, a non-dormant mutant, indicated that the non-dormancy trait is due to a recessive deletion (*Evg/evg*; Wang *et al.* 2002-a; Wang *et al.* 2003; Bielenberg *et al.* 2004). However, more genomic regions were shown to quantitatively affect chilling requirement (Fan *et al.* 2010).

1.2.4.2. Fruit development period (FDP)

After full blooming, and following the petal fall, fruit development is a complex process that is expressed in the accumulation of weight, increase in fruit size, and a shift in texture and biochemical composition. The time from fruit set until reaching commercial or physiological ripening is called "Fruit development period" (FDP).

Typically fruit weight increase follows a double-sigmoid curve, in which four main stages can be generally distinguished (Tonutti *et al.*, 1991, figure 1): SI, first exponential growth stage, characterized by a high rate of cell division and increasing fruit weight; SII, also called "pit hardening", corresponding to endocarp lignification and accompanied by a decrease in growth rate; SIII, second exponential growth and cell enlargement; SIV, fruit softening (disruption of cell walls). Between SIII and SIV ethylene accumulation increases, concomitantly with the fruit respiration rate at the SIV stage, also called "climacteric rise". However, ethylene evolution and respiration rate are highly variable among cultivars (Brady, 1993; Ventura *et al.*, 1998; Brovelli *et al.*, 1999).
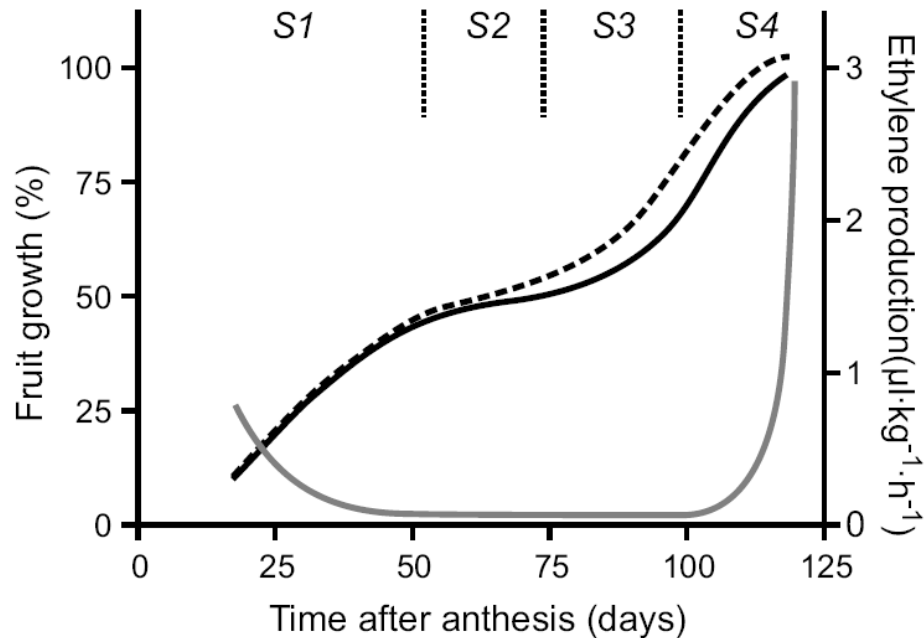
Figure 1. Evolution of fruit growth and ethylene in fruit ripening. The chart shows the changes for in 'Fantasia' (black full line) and 'Jalousia' (black broken line) cultivars, ethylene synthesis (grey full line) and the four main ripening stages (S1-S4, top of the chart). Extracted from Begheldo (2008).

FDP can range from 55 to 270 days after full bloom (DAB; Bassi and Rizzo, 1995; Caruso and Sottile, 1999), strongly suggesting the quantitative nature of this trait. In "slow ripening" cultivars, maturation processes stop in SIII (cell expansion after pit hardening) and resume only after the application of ripening-inducing treatments. This trait is monogenic recessive (*Sr/sr*). Moreover, breeding experiments suggest that FDP is not independent from other fruit quality traits and often describes a trimodal distribution, suggesting the presence of major genes regulating the FDP trait (Yamaguchi *et al.* 1984; Bassi *et al.* 1988)

1.2.4.3. Maturity and quality

Maturity greatly influences peach flavour, market life and quality potential (Crisosto *et al.*, 1995). The timing of harvest is especially a critical point for fresh peach production. Full mature fruits have a shorter post-harvest life, because of the rapid softening of climateric peach (mainly melting flesh cultivars) and the resulting susceptibility to injury caused by mechanical handling and invasion by rot fungi. Conversely, peaches harvested too early can have a low consumer acceptance due

to undesirable irregular softening and organoleptic features, as higher acidity and lower SSC compared to fully ripe fruit (Crisosto and Valero, 2008).

Maturity indexes used to define harvest timing are based mainly on background colour change, fruit firmness and size (Crisosto and Valero, 2008; Eccher Zerbini *et al.*, 1991). These methods have been historically used with good results in commercial peach orchards. However, such visual assessments do not provide a fully reliable identification of the fruit ripening stage (Ziosi *et al.*, 2008). Furthermore, variability in fruit external colour, texture and flavour obstacle the definition of a general maturity criterion applicable for all cultivars.

Recently, new non-invasive methods have been implemented to evaluate fruit ripening stage. One such instrument (DA-meter, patented by the University of Bologna) is based on the measurements of chlorophyll content, a parameter known to decrease independently of the change of red/yellow colour, weight or texture (Chalmers and van den Ende, 1975): this instrument consists in a portable spectrophotometer device to measure the I_{AD} index (index of absorbance difference or ΔA index, estimated from the difference between the two chlorophyll A peaks detected in the exocarp, Ziosi *et al.*, 2008) as an indicator of fruit maturity state. This instrument has been validated comparing results with measurements of ethylene emission, fruit quality traits, and transcript levels of ripening-related genes both at harvest and during the following shelf-life in various peach and nectarine cultivars (Ziosi *et al.*, 2008; Noferini *et al.*, 2009).

1.3. Adverse factors in peach cultivation

A range of biotic and abiotic stress factors can affect the quality and yield of peach. In general, chemical additives or energy demanding strategies are extensively used to circumvent these problems, with negative consequences for the environment, and a general loss of efficiency and the lack of sustainability.

1.3.1. Fungal diseases

Fungi cause a large number of diseases in stone fruit, compromising virtually all plant organs at different developmental stages (Adaskaveg *et al.*, 2008). In peach, fungal pathogens can infect fruit pre- and post-harvest resulting in significant damage. A list of peach fungal diseases is summarized in table 1. All these fungi are

Disease	Pathogen	Reproductive infection agent	Symptoms of economical importance	Time of Infection	Management
Brown rot / Blossom blight	*Monilinia fructicola, Monilinia laxa, Monilinia fructigena*	conidia, ascospores	Fruit decay	Pre- and Post-harvest	Temperature management, sanitation, fungicides
Powdery mildew	*Podosphaera pannosa (= Sphaerotheca pannosa)*	conidia, ascospores	Defoliation, fruit off-grades	Pre-harvest	Cultivar selection, fungicides
Leaf curl	*Taphrina defpormans*	bud conidia	Defoliation, fruit off-grades	Pre-harvest	Fungicides
Scab	*Fusicladosporium carpophilum*	conidia, ascospores	Fruit off-grades	Pre-harvest	Fungicides
Shot hole	*Wilsonomyces carpophilus*	conidia	Defoliation, fruit off-grades	Pre-harvest	Fungicides
Grey mould	*Botrytis cinerea*	conidia	Fruit decay	Post-harvest	Temperature management, sanitation, fungicides
Sour rot	*Geotrichum candidum*	arthroconidia	Fruit decay	Post-harvest	Temperature management, sanitation, fungicides

Table 1. More important peach fruit diseases caused by fungi. Modified from Adaskaveg et al. (2008).

heterotrophic and require water to grow. In this group we find members of the phyla *Zygomycota*, *Basidiomycota* and *Ascomycota* (the most frequent), differing for modes of production of sexual spores (zygospores, basidiospores and ascospores, respectively). All produce asexual spores (sporangiospores in the *Zygomycota*, conidia in the *Ascomycota* and sometimes in the *Basidiomycota*). Their survival structures, formed by mycelium tissue, are called sclerotia, or pseudosclerotia when formed also by host tissue. They are called anamorph, when are in the asexual phase, and telemorph when they are in the sexual phase (Adaskaveg *et al.*, 2008).

1.4.- Brown Rot in peach: a major problem in pre- and post-harvest.

Brown rot (BR) is one of the most serious fungal diseases in peach fruit, causing significant losses pre-harvest by infection of flowers (also called "blossom blight") and fruit, as well as extensive rot in post-harvest fruit (Bostock *et al.*, 1999). The disease affects stone fruit in warm and humid climates worldwide: heavy losses have been reported in North America on peaches, cherries and plums, and yearly losses of 1 million AUD occurred on peaches and apricots in Australia (EPPO, 2007). BR can results in fruit losses over 60% after 5 days of infection at room temperature (Tosi *et al.*, 1996). In a study conducted in organic apple (a *Pomaceae* fruit) orchards, yield losses were in the order of 41.6% at the pre-harvest stage, and 80% in the post harvest stage, where an aggravating factor was the presence of lesions in fruit (Holb, 2004).

BR is caused by species of the genus *Monilinia* (order *Helotiales*, class *Leotiomycetes*, phylum *Ascomycota*; Holst-Jensen *et al.*, 1997). *Monilinia fructicola* Winter (Honey), *Monilinia laxa* (Aderh. & Ruhl.) Honey and *Monilinia fructigena* Honey in Whetzel are necrotrophic parasites of many *Rosaceae* and *Ericaceae* fruit trees of commercial importance. In stone fruit, *Monilinia* spp. can over-winter in previously infected twigs, and peduncles or in mummified fruits. In early spring, brown fruiting bodies (apothecia, most frequently found in *M. fructicola* species) are developed from mummies that have over-wintered in the soil, producing ascospores (also meiospores or sexual spores) which, together with over-wintered conidia (also mitospores or asexual spores) can infect buds, young leaves, shoots, twigs and blossoms, to constitute primary inoculum sources for the disease. Conidia of primary inoculum can be spread by wind and by insects, such as *Drosophila* spp. (Michailides & Spotts, 1990). On immature fruit, *Monilinia* spp. can form infective structures called

appresoria to produce latent or "quiescent" infections - visible and non-visible infections that are established when environmental conditions or host physiology are conducive for penetration, but not for active growth of the pathogen (Adaskaveg *et al.*, 2000). When the conditions are favourable for the fungus, these quiescent infections get activated, penetrating directly in the cuticle and starting extensive host colonisation (Lee *et al.*, 2006). Brown rot disease is developed by somatic *hyphae* that differentiate to produce the stroma, a melanized hyphal aggregate. After extensive infection, *Monilinia* hyphae secrete cell wall-degrading enzymes macerating fruit tissues. Later, parasitized fruit desiccate, being mummified and producing a large quantity of conidia that constitute secondary inoculum for other fruit in the orchard (Batra, 1991). An illustration of the life cycle of *Monilinia* spp. is shown in figure 2.
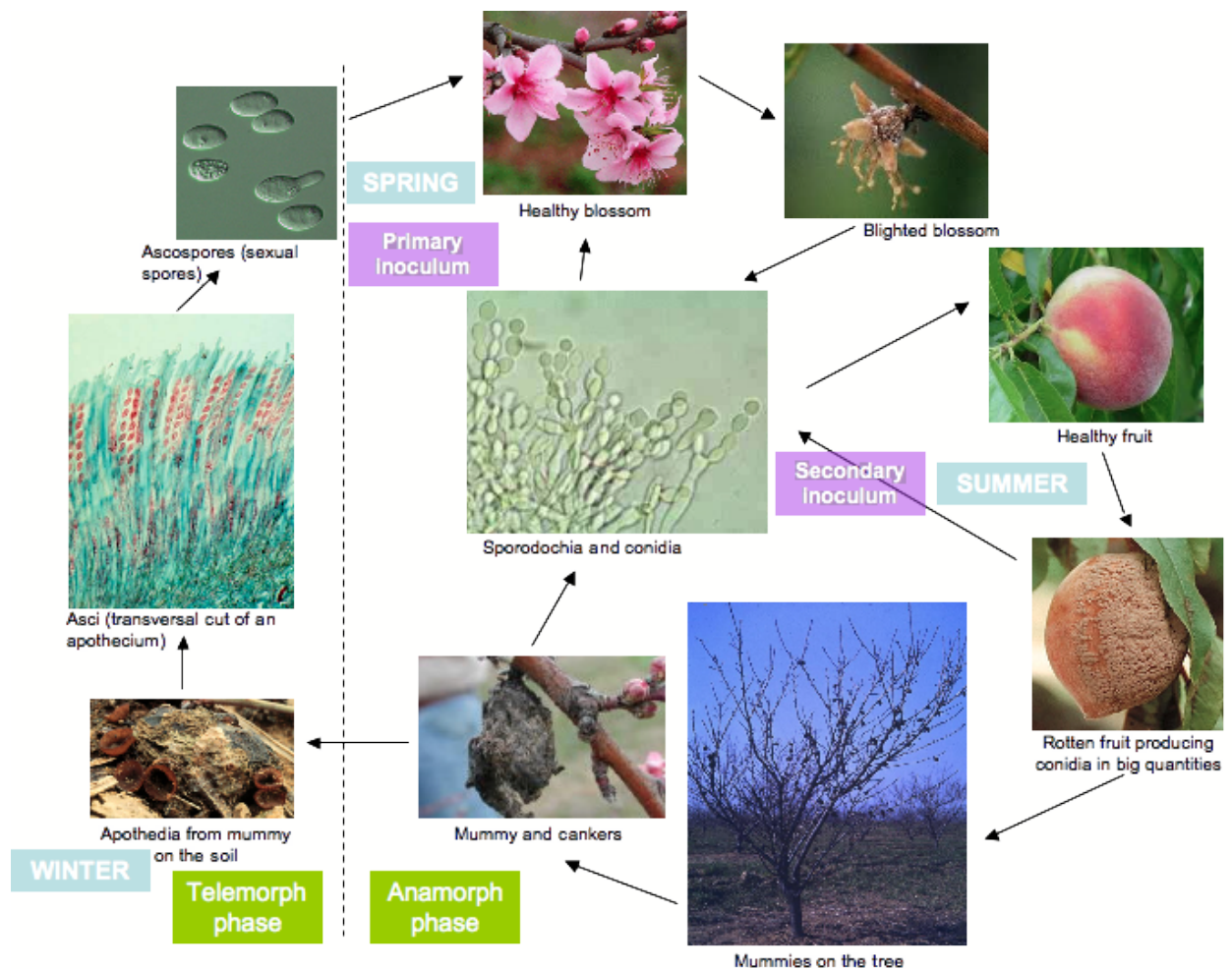

Figure 2. *Monilinia* spp. life cycle.

1.4.1. Environmental factors affecting BR impact

Several environmental factors can influence the infectivity of the fungus and the susceptibility of the plant, resulting in a range of symptoms differing from year to year.

Humidity affects both the infectivity of the fungus and fruit susceptibility. For example, the occurrence of rainfalls near the harvest period produces dramatic fruit decay (Adaskaveg *et al.*, 2008). Wilcox (1989) and Tamm *et al.* (1995) reported that temperature and wetness duration in the pre-harvest period affect infection incidence of *M. fructicola* and *M. laxa* in sour and sweet cherry blossoms, respectively. The pre- and post-harvest impact of BR depends also on the presence of wounds and micro-fractures in the cuticle and the exocarp since these tissues constitute the first physicochemical barrier against pathogens (Hong *et al.*, 1998; Borve *et al.*, 2000). In *Prunus avium*, micro-fracture or cuticular crack susceptibility depends on genotype and on seasonal rainfalls (Measham *et al.*, 2009).

Soil nutrient concentrations, *e.g.* the level of nitrogen fertilization, are positively correlated with the impact of BR infections in Californian nectarine orchards (Daane *et al.*, 1995); high concentrations of nitrogen and potassium were also associated with higher BR and scab infection rates in plum trees in tropical environments (May-De-Mio *et al.*, 2008),

Conidia are widely recognised as the most important type of BR inoculum. The quantity of secondary inoculum in the field is a key factor to generate fruit latent infections, with a direct impact on the pre- and post-harvest BR severity (Gell *et al.*, 2008). Factors that have been correlated with the production of conidia in field include low temperatures after the start of the infections: Tian and Bertolini (1999) demonstrated that low temperatures (0 to 5°C) induce the formation of a higher number of conidia per fruit; these conidia showed higher size, germination rate, and infectivity in wounded nectarines. The quantity of thinned fruit remaining in the soil that is further developed to mummies results in higher conidial loads in the orchard with a subsequent higher impact of the disease (Hong *et al.*, 1997).

1.4.2. Current and alternative strategies for BR disease control

Brown rot management is heavily dependent on the use of synthetic fungicides such as benzimidazole, thiophanate-methyl and benomyl, to protect blossoms and ripening fruit. Fungicides with local systemic activity can protect flowers and fruit,

(Adaskaveg *et al.*, 2008). However, multiple applications may be needed in the same season, when climatic conditions are expected to be favourable for the fungus; in fact, BR has been reported as the primary reason for fungicide use in stone fruits at the pre-harvest stage (Ritchie, 2000). The direct use of any pesticide on already picked stone fruits (post-harvest) has been banned in Italy (Tian and Bertolini, 1999; Pratella, 1996). The extensive use of such chemical agents can pose environmental risks and sustainability problems. In recent years, new strains of *Monilinia* spp. resistant to the most widely used fungicides have appeared in stone fruit-cultivating regions (Miessner and Stammler, 2010; Amiri *et al.*, 2009; Amiri *et al.*, 2010; Luo and Schnabel, 2008; Luo *et al.*, 2008a; Luo *et al.*, 2008b; Holb and Schnabel, 2007; Yoshimura *et al.*, 2004; among others), indicating these control methods may soon become ineffective.

Alternative environmentally friendly control methods are being designed based on demands of consumers and environmental protection agencies to reduce the use of synthetic pesticides in the environment and the amount of chemical residues on fruit. A combination of methods can result in efficient integrated strategies of disease control, avoiding or minimising the use of chemical pesticides (Spotts *et al.*, 2002).

Maintaining hygienic conditions in the orchard soil by the simple removal of thinned fruits and mummies from the past seasons, to reduce the quantity of inoculum, is a fundamental practice to diminish the incidence of BR.

The use of natural compounds that inhibit the growth of *Monilinia* spp. has been recently studied. Berberine, extracted from the medicinal herb *Coptis chinensis* (Hou *et al.*, 2010) exhibits a fungicide action specific for *Monilinia* spp.: it is effective at low concentrations (0,4 mg/ml) when applied over harvested fruit, without producing neither physical nor sensorial alterations. Plant volatile compounds, such as *trans*-2-hexenal, also have fungicide activity (Neri *et al.*, 2007). Pre-harvest application of *Phellodendron chinese* bark extract has been effective against BR development in flat peaches (Feng *et al.*, 2008).

A complementary approach that has been intensely studied is based on bio-control using *Monilinia* antagonists, such as yeasts or bacteria, alone or in combination with chemical additives. For example, *Cryptococcus laurentii* was highly efficient when used in combination with jasmonic acid for the control of *Monilinia fructicola* and *Penicillium expansum* in harvested peaches (Yao and Tian, 2005). *Epicoccum nigrum*, a component of the resident mycoflora of twigs and flowers of

peach (Melgarejo *et al.*, 1985), also gave positive results in post harvest trials, reducing the incidence of BR. However, this required four applications per season in the field or combination with chemical fungicides (Larena *et al.*, 2005) or physicochemical treatments (Mari *et al.*, 2007).

1.4.3. BR resistance sources in *Prunus* spp.

In parallel to innovation in agronomic practices, progress in sustainable control of necrotrophic fungal pathogens, such as BR in peach, can be achieved by breeding of resistant cultivars. This can be based on natural sources of genetic resistance. Molecular plant pathology studies have offered insight into interactions between fruit and necrotrophic fungi (*e.g.* the well characterized tomato-botrytis model, Cantù *et al.*, 2008-b), providing a basis for the identification of biological processes and endogenous factors associated with plant resistance in *Prunus*. This knowledge coupled with modern genetic and breeding approaches will result in the identification and localization of genomic regions associated with necrotrophic-resistance traits, and tools for their introgression into commercial cultivars.

1.4.3.1. General elements for plant resistance to necrotrophic fungi

A plant-pathogen interaction can be compared to "an open chemical warfare, whose weapons correspond to physicochemical barriers and proteins developed in what must have been a multimillion year evolutionary game of ping-pong" (Ferreira *et al.* 2007). This "chemical warfare" has been extensively described in two reviews of Dangl and Jones (2001 and 2006), in which the plant immune system is described as being two-branched. The first branch consists of slow-evolving transmembrane receptors that respond to pathogen associated molecular patterns (PAMPS). The second branch largely acts inside the cell, using the polymorphic and fast-evolving NB-LRR protein products encoded by most R genes (Jones and Dangl, 2006), which activate diverse plant responses resulting in hypersensitive plant cell death (or hypersensitive response, HR) that blocks the spreading of the pathogen and further infection (Dangl and Jones, 2001). For biotrophic pathogens (those that require a living host to complete their life cycle), this defence mechanism is very effective, but it does not affect, and even promotes the infection of necrotrophic organisms (those that kill the host and feed on the dead contents, such as *Monilinia* spp.).

Phytopathogenic fungi combine physical structures and enzymatic actions to infect the plant tissue (Mendgen *et al.*, 1996): after spore adhesion by passive hydrophobic interactions with the cuticle, germ tubes grow on the plant surface and form appresoria that may have melanized cell walls and develop high turgor pressure for penetration. These events are accompanied by penetration-tip formation (by the accumulation of cytoskeleton components at the end of the infecting hyphae) and secretion of lytic enzymes such as cutinases (Lee *et al.*, 2010); when hyphae have entered the plant tissue, internal cell disruption is promoted by the secretion of fruit softening-related cell wall enzymes, such as polygalacturonases, pectin methylesterases, pectin and pectato-lyases, as well as laccases (that degrade lignin) (Bar-Nun and Mayer, 1990; Viterbo *et al*, 1994). Cell wall pectin-derived oligosaccharides (PDOs), generated by microbial or plant cell wall degrading proteins (CWDPs), apparently act as signaling molecules triggering defence responses (Côté and Hahn, 1994). This is followed by the induction of several additional mechanisms, such as: 1) building of passive barriers to the pathogen by the production and crosslinking to the cell wall of phenolic lignin monomers of the PAL synthetic pathway (Asselbergh *et al.* 2007), or the deposition of callose on the cell-wall (Flors *et al.*, 2007); 2) synthesis of phytoalexins (Ben-Yehoshua *et al.*, 1992; Lanza *et al.*, 1994); 3) biogenesis of several pathogen-related proteins such as chitinase (Jung *et al.*, 2005) and polygalacturonase inhibiting proteins (PGIPs; De Lorenzo *et al.*, 2001). However, PDO-dependent signalling mechanisms remain still unclear (Cantù *et al.*, 2008-a).

1.4.3.2. Peach fruit defences against BR: state of the art.

In fruit, constitutive natural barriers as the cuticle, exocarp or phenolic compounds are the first defence mechanisms against necrotrophs. The host presents to the pathogen diverse signals that result in formation of infective structures. In this section, processes that have been suggested to be involved in the BR resistance in *Prunus* species are reviewed, with supporting studies from model fruit-necrotrophs interactions.

a)   Susceptibility across ripening stage.

Susceptibility to BR in *Prunus* has been largely demonstrated to be dependent on the ripening stage of the fruit. *M. fructicola* conidial inoculations over the skin of

cvs. 'Loring' and 'Springcrest' indicated susceptibility is at the SI stage, decreases dramatically at the SII stage, with a subsequent increase from early SIII reaching maximum susceptibility at full ripening, (Biggs and Northover, 1988). Similar results were obtained from other studies with *M. laxa* (Mari *et al.* 2003; Fourie and Holz, 2003).

This phenomenon may be related to the significant changes in volatiles and organic acids (Chapman *et al.*, 1991), phenolics compounds (Senter and Callahan, 1990) and cell wall composition (Brummell *et al.*, 2004) that occur in the development of *Prunus* fruit, providing different levels of protection against BR at different ripening stages.

An important element in explaining the shift in BR susceptibility is linked to dynamics of cell wall metabolism in fruit development. During fruit ripening, especially during softening of climacteric fruits, CWDPs from the host act modifying cell wall structure (Brumell *et al.*, 2004) facilitating the action of similar pathogen-derived degrading proteins. The close relationship between necrotrophic pathogen susceptibility and softening-related cell wall disassembly has been studied in the *Botrytis*/tomato interaction (Cantù *et al.*, 2008-b): compared to wild-type, tomato mutants defective for fruit softening enzymes (*e.g.* polygalacturonase and expansin) displayed higher fruit firmness and higher pathogen tolerance at full maturation. Over-expression of cell wall modifying enzymes such as pectin methylesterases can increase *Botrytis*-resistance in wild strawberry (*Fragaria vesca*) (Osorio *et al.*, 2008), since these proteins (such as PME1 gene product) partially depolymerises oligo-galacturonides, facilitating the formation of Ca binds, which have a positive effect in the mechanical resistance of the cell wall (Micheli, 2001).

Cell-wall metabolism and softening are also associated with occurrence of skin micro-cracks or microscopic fissures in the cuticle and exocarp, which has been correlated with the impact of postharvest decay caused by *Monilinia laxa* and *Botrytis cinerea* in sweet cherry (*Prunus avium* L.) (Borve *et al.*, 2000; Sekse, 1998). Seasonal conditions influence fruit skin cracking; for example a positive correlation between skin cracking and the incidence of rainfalls in the harvesting season has been reported in sweet cherry, with a strong genotype effect in the susceptibility to this phenomenon (Measham *et al.*, 2009). Gibert *et al.* (2009) studied the effect of seasonal humidity, conidial concentration and total area of fruit cracking in nectarines, to develop a model to predict the incidence of BR at the harvest period.

The change in phenolics composition (mainly caffeic acid and its quinnate ester, chlorogenic acid) during stone fruit development has a major effect on the formation of appresoria and further quiescent BR infections (Emery *et al.*, 2000). These phenolic compounds are synthesised from the phenylpropanoid pathway (Naoumkina *et al.*, 2010). Although conidia of *M. fructicola* germinated over nectarine skin with similar frequency at different ripening stages, appresoria formation frequency was maximum at the pit hardening stage, decreased in the subsequent stages reaching a minimum level in the softening stage, where the germinated conidia showed a saprobiotic lifestyle and entered directly through microscopic fissures in the skin surface (Lee and Bostock, 2006). Expression of *M. fructicola* cutinase and polygalacturonase genes was inhibited by the exocarp redox potential (Lee and Bostock, 2007) deriving from high levels of phenolics compounds. Formation of appresoria after conidial germination (instead of a direct hyphal penetration at the pit hardening stage) was proposed to determine the development of quiescent infections, *i.e.* the fungus exists in a latent form on the fruit surface until fruit nutrients become readily available, as in full maturation (Lee and Bostock, 2006 and 2007).

Available information strongly supports direct interactions between ripening process and fruit resistance to fungal pathogens. However, given the complexity and multiplicity of events that accompany fruit ripening, it is difficult to individuate key candidate genes for improvement of pathogen resistance in stone fruit. Despite these difficulties, exploiting the existing wide variability in ripening dynamics it should be possible to identify genotypes with more effective constitutive barriers.

b) Pathogenesis-related (PR) proteins.

The concept of PR proteins was introduced to designate any protein encoded by the host plant but induced only in pathological or related situations (Antoniw *et al.*, 1980). So far, around 17 classes of PR proteins presenting diverse properties and microbial targets have been described (reviewed in: Van Loon and Van Strien, 1999; Edreva, 2005; Sels *et al.*, 2008), but only in few cases PR proteins have been implicated in fungal attack to fruit tissues.

Expression of PR protein coding genes and was shown to change following infection with necrotrophic pathogens such as *Monilinia* spp., *Botrytis cinerea* or *Colletotrichum spp*. in diverse fruit species, suggesting they act as inducible factors that promote plant defence responses (Goñi *et al.*, 2010; Derckel *et al.*, 1998;

Vellicce *et al.*, 2006; Chan *et al.* 2007; El-kereamy *et al.*, 2009). In climacteric cherimoya (*Annona cherimola*), chitinase and 1,3-β-glucanase expression (belonging to PR-Q and PR-2 classes, respectively) were associated to inhibition of *Botrytis cinerea* development (Goñi *et al.*, 2010). After *Botrytis* infection, expression of chitinase and glucanase genes was upregulated in grape berries (Derckel *et al.*, 1998). PR-Q genes were also overespressed in strawberry producing enhanced resistance to *Botrytis* (Vellicce *et al.*, 2006). In peach, treatment with salicylic acid (chemical signal for induction of pathogen defence processes) and the bio-control agent *Pichia membranefaciens* resulted in differential expression of 6 (out of 25) PR proteins and enhanced resistance to the post-harvest fungus *Penicillium expansum* (Chan *et al.* 2007). Differential expression of the PR-10 gene was also observed after *Monilinia fructicola* infection in susceptible and tolerant cultivars of European plum (El-kereamy *et al.*, 2009). Resistance to BR coincided with constitutive expression of PR-10 and phospholipase D-alpha (PLD-α, a is a major signalling component associated to various stress responses, including defence, and known to induce other PR genes), in contrast to susceptibility where PR-10 and PLD-α were induced after BR infection (El-kereamy *et al.*, 2009). Thus, gene expression differences in susceptible and tolerant genotypes may be linked to expression of other regulators, with an observed correlation of stomatal closure mediated by ABA and an increase in the concentration of hydrogen peroxide, known to be involved in several defence responses (Bhattacharjee, 2005).

In summary, current evidence suggests PR proteins represent potentially useful targets for use in genetic improvement of new tolerant cultivars, acting independently (in some cases) of the maturity state of the fruit.

c) Fruit tissue-specific resistance.

The role of exocarp and endocarp with respect to their pathogen-resistance mechanisms is relatively unknown. In an attempt to determine BR resistance levels of diverse cultivars of peach, plum and apricot, Pascal *et al.* (1994) used two tests in stone fruit cultivars: uninjured and artificially-injured inoculation. Although they found genotypic differences with both inoculation approaches, within each species rankings of the genotypes according to these two tests were not correlated. This was proposed to be due to the different barriers that the pathogen challenged: epidermal and flesh fruit resistance in uninjured fruits and artificially injured fruits, respectively.

The existence of two levels of fruit resistance at fruit maturity is an important aspect to consider when screening for BR susceptibility in different cultivars of stone fruits, since mechanisms underlying the two types of resistance are likely to differ.

Considering the multiplicity of genetic and environmental factors affecting fruit responses, BR susceptibility is a quantitative trait and different approaches have been tested to determine the most robust measurement of the trait.

1.4.3.3. Variability for BR susceptibility in stone fruits

Although complete resistance to BR has never been reported in cultivated peach, high variability in susceptibility to this disease has been found in peach germplasm. A Brazilian low-chill peach cultivar, 'Bolinha', has been reported to present one of the lowest levels of BR fruit susceptibility, when compared with other cultivars (Feliciano *et al.*, 1987). A more compact arrangement of epidermal cells, thicker cuticle, fewer trichomes, higher phenolics content (chlorogenic and caffeic acids, catechin and epicatechin) may account for the differential response to BR at harvest maturity between Bolinha and more susceptible cultivars (Gradziel *et al.*, 2003; Bostock *et al.*, 1999). Considering its poor fruit quality and high susceptibility to browning (due to the high content of phenolics), 'Bolinha' has been mainly useful as a donor of brown rot resistance (linked to low chill requirement to avoid the infections occurring at rainy harvest periods) in breeding of new canning peach cultivars (Raseira and Nakatsu, 2000).

Additional surveys of BR tolerance aimed at identifying suitable parents for breeding programs. These studies were based on different infection protocols and criteria to score and rank cultivar susceptibility; approaches also have differed with respect to the choice of germplasm and environments. Key published studies of this kind include:

• Gradziel *et al.* (1997 and 2003) used 'Bolinha' and almond accessions with high epidermal resistance as parents to generate new accessions of processing peach, using *M. fructicola* as drop inoculum in intact regions of the fruit exocarp, and considering the lesion diameter as a measure of susceptibility. Results showed that BR-susceptibility trait was heritable (the trait has a genetic component that explains a high proportion of the observed variability and thus can be transferred to next generations, see section 1.5.

• In the work by Pascal *et al.* (1994), a drop with conidial suspension of *M. laxa* was deposited directly in the mesocarp (artificial injured) or the exocarp (uninjured): taking into account the average diameter of the lesion in injured fruits and the overall percentage of infected fruit, susceptible genotypes in peach, plum and apricot were clearly distinguishable, *e.g.* 'Springred' was recognized as the most tolerant among all tested peach cultivars.

• Walter *et al.* (2004), evaluated different BR (*M. laxa*) impact methods in apricot cultivars and selections, based on average lesion surface in wounded and intact fruit, spore production in the lesions, storage lesions generated from natural field inoculum, and cuticle thickness. Different levels of correlation were revealed between screening methods and measure of the lesion area after 3 days of inoculation was identified as the most robust method capable of distinguishing apricot genotypes;

• BR susceptibility of nearly 70 accessions, including cultivars and early selections of clingstones and freestones, white and yellow fleshed peaches and nectarines, was examined in different years in Bologna and Imola orchards (Northern Italy), inoculating a conidial suspension of *M. laxa* on the whole fruit surface (thus including the effect of the skin cracking on BR susceptibility) and considering the average lesion surface and percentage of diseased fruit per genotype (Cantoni *et al.*, 1995; Bassi *et al.*, 1998; Bassi and Rizzo, 2003). In spite of high environmental variability (seasonal rainfalls and location), genetic differences in BR susceptibility were evident, and cultivars could be ranked according to their susceptibility. Using contrasting cultivars showing BR sensibility based on these tests (*e.g.*, 'Glohaven', 'Contender', 'Honey Gold', 'Kappa2', 'Elegant Lady'), they have generated controlled crosses whose progenies present different ranges of tolerance to the pathogen (Bassi and Rizzo, 2003). The percentage of infected fruits susceptibility score had a low, but significant correlation with some quality traits such as solid soluble content and fruit weight (Bassi and Hall, unpublished results). Among these crosses, progenies from 'Contender' (moderately tolerant with a 53% infected fruit) x 'Elegant Lady' (highly susceptible, with 100% infected fruit) showed a wide range of susceptibility (Bassi *et al.*, 1998). Some seedlings from 'Contender' x 'Elegant Lady' F1 progeny were more tolerant than 'Contender', indicating that this population is a valuable starting material to study the genetic bases of BR susceptibility in fresh market peach cultivars, and to identify genetic loci that may help to improve BR resistance in peach.

1.5. Molecular tools for breeding: quantitative genetics and QTL analysis for disease resistance.

Peach is a diploid species with n = 8 (Jelenkovic and Harrington, 1972) and has a comparatively small genome: 5.9 x 10^8 bp or 0.61 pg/diploid nucleus (Baird *et al.*, 1994). This is only about twice the value for *Arabidopsis thaliana* (Arumuganathan and Earle 1991).

Peach has a relatively short juvenile phase (2-3 years) compared to most other fruit tree species (Abbott *et al.*, 2002). However, such period is still long for the development of recombinant inbred lines (fully or almost-fully homozygous plant lines generated by recurrent auto-fecundation). Peach cultivars have been generated by crossing different genotypes to combine desirable traits and are characterised by a certain degree of heterozigosity. Therefore, segregation of alleles and traits is evident in F1 populations. Unlike the majority of *Prunus* species, peach can undergo both self- and cross-pollination allowing the generation of F2 populations by selfing F1 plants.

The first peach-breeding programs were directed to improve colour, firmness and attractiveness, to satisfy productive and commercial requirements such as ease of handling or shipping (Monet and Bassi, 2008). After these initial breeding efforts, modern peach breeders have focused on tree productivity, fruit quality and reduction of production costs by improving disease resistance and agronomic traits. Although the long generation time of peach (3-4 years) hampers genetic dissection of such traits, breeders and researchers have developed materials and collected data that can now be deployed to understand the inheritance of many traits of commercial and agricultural interest. In a number of cases, these have been genetically characterised as being "mendelian" (also "single" or "qualitative"), *i.e.* attributable to the action of two alleles of one locus. Some examples of mendelian genes of peach are reported in table 2. However, most fruit quality (*e.g.* flavour, weight, red over-colour), agronomic (*e.g.* tree shape and size) or pest resistance (*e.g.* powdery mildew, brown rot) traits are quantitative, *i.e.* controlled by more than one gene, strongly influenced by environmental conditions and distributed in the progeny in a continuous (not discrete) manner. The genomic regions containing genes that are associated with a determined quantitative character are called "Quantitative Trait Loci" (QTL), and its identification through QTL analysis opens to the breeders the opportunity to select

Phenotype and symbol	Genotype	Linkage group	Note	Reference
Fruit traits				
Slow ripening (Sr)	sr/sr	-	-	Ramming (1991)
Saucer or flat shape (S)	S/-	6	S/S is lethal	Lesley (1940)
Aborting fruit (Af)	af/af	6	-	Dirlewanger et al. (2006)
Blood red fruit (Bf)	Bf/-	-	Pigment appears in inmature fruit and main leaf vein; often smaller trees	Werner et al. (1998)
Rough skin (Rs)	rs/rs	-	Matte skin surface; glabrous flower buds	Okie and Prince (1982); Okie (1988b)
Nectarine (Glabrous skin, G)	g/g	5	Fuzzless	Blake (1932); Blake and Connors (1936)
Full red skin (Fr)	fr/fr	-	Expressed just in fruit	Beckman and Sherman (2003)
Highlighter (H)	h/h	-	Red colour suppression on fruit skin	Beckman et al. (2005)
White flesh (Y)	Y/-	1	Also affects calyx cup and leaf colour	Connors (1920)
Flesh texture and pit adherence (F)		4	-	
Melting freestone	F/-		-	Bailey and French (1932, 1949); Monet (1989); Peace et al. (2005)
Melting clingstone	f/f		-	Peace et al. (2005)
	f/f1		-	
	f/n		-	
Non-melting clingstone	f1/f1		-	Peace et al. (2005)
	f1/n		-	
	n/n		-	
Stony hard flesh (Hd)	hd/hd		-	Yoshida (1976); Scorza and Sherman (1996)
	hdhd/F		Stony hard, melting	Haji et al. (2005)
	hdhd/f1f1		Stony hard, non-melting	Haji et al. (2005)
Low-acid flesh (D)	D/-	7	D for "douce" (sweet in French)	Monet (1979)
Resistance traits				
Resistance to root-knot nematode *Meloidogyne javanica* (*Mj*)	Mj1/–	2	Mj1 and Mj2 are independent	Lownsberry and Thomson (1959); Sharpe et al. (1970); Lu et al. (2000)
	Mj2/–			
Resistance to root-knot nematode *Meloidogyne incognita* (*Mi*)	Mi/–	2	-	Weinberger et al. (1943); Lu et al. (2000)
Resistance to both species	Mij/	2	-	-
Green aphid resistance (*Rm*)	Rm1/–	-	-	Monet and Massonié (1994)
Leaf foliar glands (*E*)		7	Incomplete dominance	Connors (1921)
Reniform	E/E			
Globose	E/e			
Egladular	e/e		High susceptibility to Powdery mildew	

Table 2. Mendelian traits related with fruit and resistance traits discovered in peach. Modified from Monet and Bassi (2008)

from the generated hybrids just the ones that present a determined allelic configuration in a QTL-associated genetic marker. This allelic configuration is also related with a minor or major magnitude of the quantitative trait.

1.5.1. Genetic markers

Genetic markers are readily assayed phenotypes that have a direct 1:1 correspondence with DNA sequence variation at a specific location in the genome or locus. The assay for a genetic marker is not affected by environmental factors. Genetic markers are DNA sequence polymorphisms that show Mendelian

inheritance. For genome mapping, the ideal genetic marker is codominant, multiallelic, and hypervariable (*i.e.*, segregates in almost every family). However, some dominant markers are also very useful and powerful in particular situations (Wu *et al.*, 2007).

There are three types of markers:

• Morphological or classical markers, *i.e.* phenotypic traits that can be assigned clearly to discrete categories, determined by allelic variants of Mendelian or major genes.

• Biochemical markers, corresponding to allelic variations of enzymes, or isoenzymes, which are detected by electrophoresis, and distinguished by their different pattern of electroforetic migration.

• DNA or molecular markers, corresponding to nucleotide sequence variants present in the genome.

The two first categories of markers have been used in genetic mapping and breeding for decades (Collard *et al.*, 2005). However, being dependent on environmental conditions, tissue/organ and developmental stage (in the case of isozymes), these markers are prone to mistakes in the genetic assignation of the alleles ("genotyping"). An additional limitation is their relative scarcity, resulting in limited coverage and density of markers across the genome (Winter and Kahl, 1995). Advances in molecular techniques allowing detection of DNA sequence variation led to the development of a vast range of molecular markers that have largely replaced morphological and biochemical markers.

Hereon we just refer to molecular markers. Their detection is mostly based on electrophoresis or hybridization techniques. When a marker shows more than one variant across the analysed pool of samples (*e.g.*, cultivars) it is called polymorphic. Polymorphic markers may or may not allow discrimination of homozygote and heterozygote allelic configurations, *i.e.*, they can be co-dominant or dominant. Additional details on commonly used markers are given below.

• **AFLP markers.** Originally described by Vos *et al.*, (1995), this technique uses restriction enzymes to fragment genomic DNA, followed by enzymatic ligation of adaptors to the sticky ends of the generated fragments. The adaptors serve as target DNA for two rounds of selective amplification using primers that are complementary to the adaptors and include few nucleotides (2 or

3) at the 3' serving as selective bases to reduce the complexity of PCR products. The generated amplicons are separated in an electrophoretic matrix (denaturing polyacrylamide gels or capillary electrophoresis) and visualised by autoradiography or fluorescence methods, based on the use of radioactively or fluorescently marked primers, respectively. One run of AFLP can generate multiple bands (on polyacrylamide gels) or peaks (on capillary electrophoresis), each one representing a single locus, and visualized as a dominant marker (the presence of the band is genotyped as homozygous for one allele or heterozygous allele configurations, and the absence as the recessive allele). Although AFLP markers are theoretically expected to be distributed across the whole genome, cases of partial genome coverage have been reported. For example, when used as the sole markers in construction of peach linkage maps (see section 1.5.4) genome coverage was lower than theoretically expected (Lu *et al.*, 1998) and chromosomal distribution was not uniform, likely due to low genetic variability of the analysed sample pool. Other limitations of this method are the medium reproducibility, requirement of high amounts of genomic DNA and the relatively low throughput deriving from the complexity of the protocol (Collard *et al.*, 2005, Mueller and LaReesa, 1999). In any case, this technology has been largely used in combination with other types of markers in *Prunus*, obtaining good levels of genome coverage (Dirlewanger *et al.*, 1998; Lu *et al.*, 1998; Dirlewanger *et al.*, 2007).

• **SSR markers.** Also called "micro-satellites" (Litt and Lutty, 1989), these markers correspond to tandem repetitions of di-, tri-, tetra-, penta-, hexa- and hepta-nucleotides. The number of repetitions is variable between alleles, which can be distinguished based on size of PCR products, yielding co-dominant markers (figure 3). After their discovery in humans (Hamada *et al.*, 1982), SSR were identified in eukaryotes and prokaryotes where they appear to be ubiquitous and frequent across the genome, especially in non-coding transcribed regions (Morgante *et al.*, 2002). While detection is relatively straightforward (through a simple PCR amplification, electrophoresis band resolution and autoradiography or fluorescence detection), this technique requires the previous development of the primers flanking the SSR based on sequencing of SSR-enriched genomic or cDNA libraries or genomic/transcriptomic sequence information, where this is available; to date, nearly 600 SSR primer sequences have been reported for the
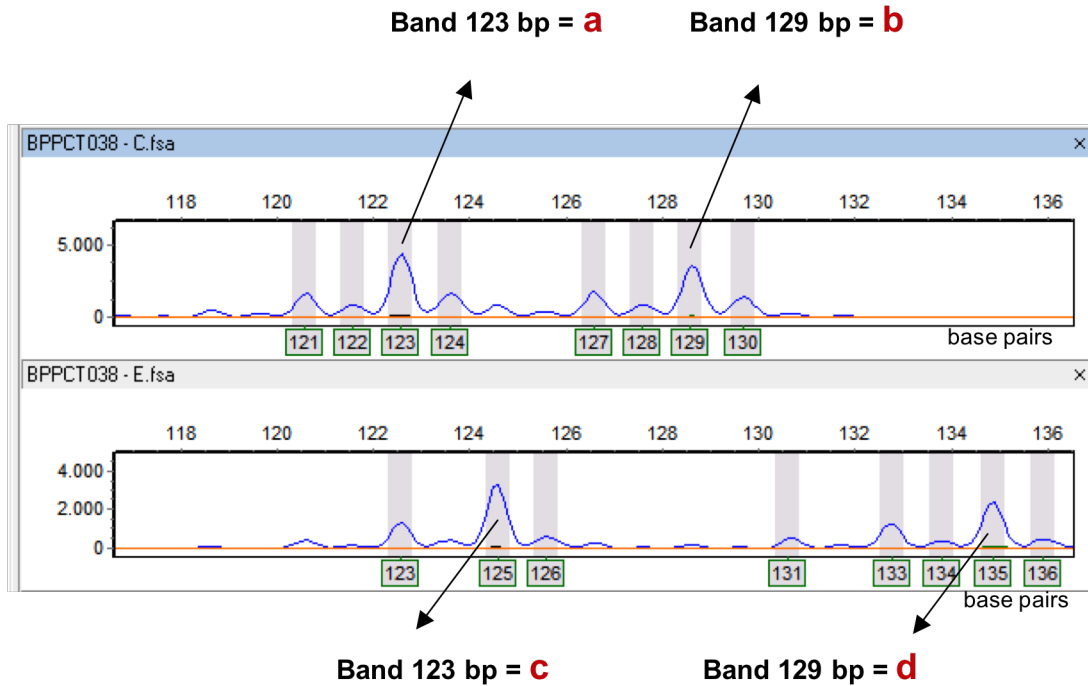
Figure 3. Electropherograms featuring typical profile of a SSR marker (BPPCT038), on 'Contender' (top) and 'Elegant Lady' (bottom) peach Cultivars. In the case of this marker, the configuration of the parents is "ab x cd".

Prunus genus (available at the Genome Database for *Rosaceae*, www.*Rosaceae*.org; Cipriani *et al.*, 1999; Sosinski *et al.*, 2000; Testolin *et al.*, 2000; Dirlewanger *et al.*, 2002; Aranzana *et al.*, 2002; Yamamoto *et al.*, 2002; Lopes *et al.*, 2002; Wang *et al.*, 2002-b; Georgi *et al.*, 2002; Decroocq *et al.*, 2003; Hagen *et al.*, 2004; Mnejja *et al.*, 2004; Vaughan *et al.*, 2004; Testolin *et al.*, 2004; Messina *et al.*, 2004; Xu *et al.*, 2004; Mnejja *et al.*, 2005; Verde *et al.*, 2005). They have been extensively used in genetics and evolution studies of various species (reviewed in Agarwal *et al.*, 2008 and Pleines *et al.*, 2009). Although cases of detection of more than one locus (Dirlewanger *et al.*, 2002) have been reported, in most cases one PCR analysis yields just one marker, implying that the cost of SSR markers is high compared to fingerprinting techniques that generate information for multiple loci in one assay (*e.g.* AFLP). However, novel high-throughput amplification approaches have been designed and applied to various plant species, generating cost-effective solutions: for instance, Hayden *et al.* (2008a and 2008b) have designed the "Multiplex-ready PCR" technique, which includes a two step PCR reaction in a single tube containing up to six unlabelled SSR-primer sets and one labelled-single colour

generic primer set, resulting in the amplification of up to six markers per colour (most used capillary electrophoresis devices, such as the ABI Prism 3730 DNA Analyzer allow the use of four different fluorophores), using just one labelled primer instead of one labelled primer per marker in the case of classical SSR analysis. This reduces significantly the cost of the assay when multiple samples have to be analysed (figure 7). These markers are extremely robust and reproducible when compared with markers such as AFLP, which are known to be tricky, presenting banding pattern changes according to the material used (Agarwal *et al.*, 2008; Pleines *et al.*, 2009), and a low reproducibility, a key condition for linkage mapping (Kumar *et al.*, 2009).
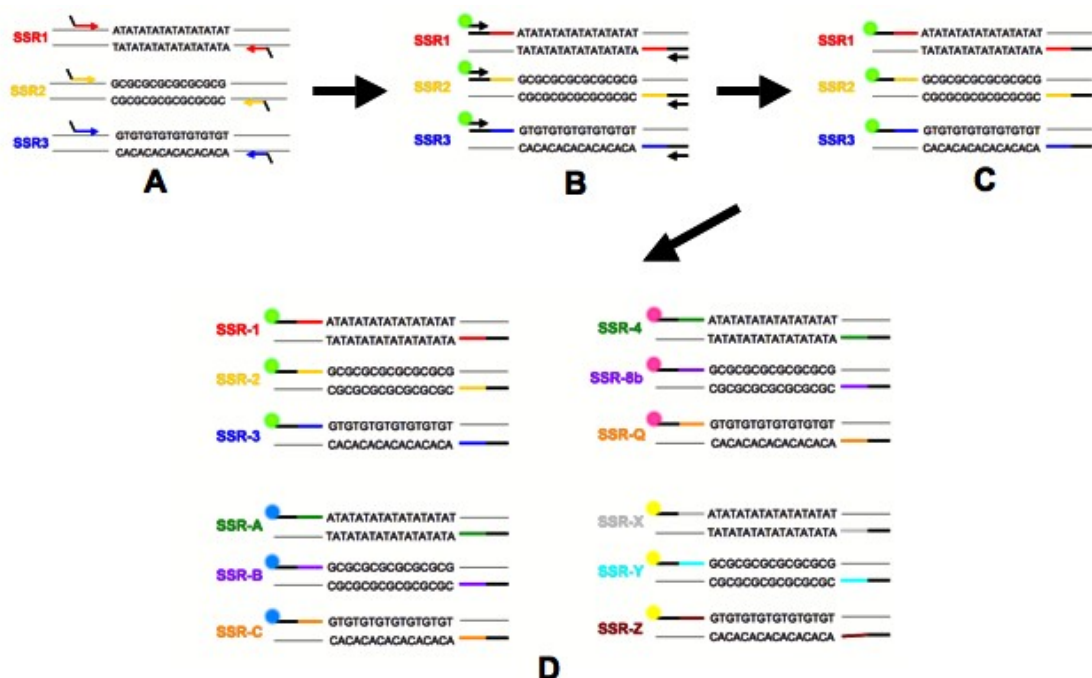
Figure 4. High-throughput, cost-effective and fluorescence based strategy for genotyping SSR markers. The protocol is composed of three to analyze 12 loci per electroforetic run. A.- In the first stage of multiplex PCR (triplex in this example), SSR loci are amplified with specific primers tagged with a short, generic "tag" sequence in their 5' end. B.- In the second stage, SSR loci amplicons are re-amplified with short and fluorescently labelled primers complementary to the "tag" sequences. C.- Each multiplex PCR reaction results in a population of three amplified SSRs (in this example) labelled with the same fluorophor. D.- Up to 4 PCR reactions (in this example, four different triplex reactions) labelled with different fluorophores are mixed before ethanol precipitation and capillary electroforetic run. Diverse SSR loci were represented with diverse colours and names (*e.g.*, SSRq, SSR-A, SSR-Q, etc.). Modified from Hayden *et al.* (2008).

• **SNP markers.** Single nucleotide sequence variations (substitutions, insertions and deletions) provide abundant co-dominant markers. They present high variability and abundance across plant genomes (Varshney *et al.*, 2009). Even if they have been developed more recently than the other named types of markers they have been quickly adopted and applied for a wide range of objectives, *e.g.*, linkage mapping (Chagne *et al.*, 2008), association genetics (Chu *et al.*, 2009), genome evolution (Garvin *et al.*, 2009), etc. SNP discovery is performed through whole genome/transcriptome re-sequencing of different cultivars (Varshney *et al.*, 2009), either from sequencing specifically targeted locus (*e.g.*, candidate genes or ESTs; Costa *et al.*, 2010). SNP discovery available technical approaches are sequencing on capillary electrophoresis, but new available technologies usually called "next generation sequencing" (NGS, reviewed in Lister *et al.*, 2009; Bräutigam and Gowik, 2010; Metzker, 2010) up to 30 Gbp per run (achieved by Applied Biosystems SOLiD platform; Metzker, 2010). Diverse chemical methods of sequence generation have been recently reviewed in Metzker (2010). SNPs may fall within gene coding and non-coding sequences, as well as in intergenic regions. If it falls in a coding region, the SNP can generate a synonymous mutation or a non-synonymous mutation that results in an aminoacid change (missense mutation) or in a stop codon (nonsense mutation). Therefore, besides serving as genetic markers, SNPs may be associated with phenotypic variation thus providing functional markers. NGS technologies open the possibility to detect markers in the whole genome or transcriptome with a high density with a price affordable if entire populations have to be analysed. For SNP genotyping, diverse and new technologies also available (reviewed in Kim and Misra, 2008), allowing the simultaneous analysis of hundreds of markers for thousands of individuals.

1.5.2. Linkage Mapping.

Linkage is the tendency for genes (or markers) to be inherited together because of their physical proximity to each other, phenomenon that lays the foundation for construction of genetic maps and the subsequent molecular dissection of quantitative traits using the map (Wu *et al.*, 2007). A linkage map represents a "road map" of the chromosomes derived from two different parents (Patterson *et al.*, 1996), indicating the position and relative genetic distances between markers and trait loci along

chromosomes. Dense genetic maps based on molecular markers provide a starting point for QTL mapping, since provide the localization of the found QTL, and in some cases the interval of the chromosome in which marker/phenotype associations are statistically sound. Patterson *et al.* (1991) determined for the first time in tomato the chromosomal locations of 29 fruit quality QTLs by using an RFLP-based linkage map and correlating it to the phenotypes of F2 and F3 progenies.

After the discovery of linkage by Bateson and Punett (1902), Thomas Hunt Morgan and his student Alfred Sturtevant proposed in 1913 that the greater the distance between two linked genes, the greater the chance that non-sister chromatids would undergo crossing-over (meiotic recombination) in the region between the genes. Based on the number of recombinants, it is possible to obtain a measure of genetic distance between the genes. The frequency of recombination can be estimated analysing the progeny of a sexual cross between two genetically different parents.

Collard *et al.* (2005) have generalised the construction of a linkage maps in three basic steps: production of a mapping population, identification and genotyping of polymorphic markers and linkage analysis of markers.

1.5.2.1. Mapping population

The first step in construction of a linkage map is the choice of parents that differ in one or many traits of interest. For preliminary mapping projects the size of the population can typically range between 50 and 300 individuals. These are tractable numbers, for example, when performing QTL analyses, in which all the individuals must be phenotypically evaluated in replicated experiments.

Different types of bi-parental mapping populations are currently used in plants (shown in figure 5, Collard *et al.*, 2005). In *Prunus* species several populations have been developed for linkage studies and QTL analyses purposes, for example: F2 progenies of peach rootstocks in Blenda *et al.* (2007) and peach in Dirlewanger *et al.* (2007) and Yamamoto *et al.* (2005); and F1 progenies of apricot in Piralovà *et al.* (2010) and Lambert *et al.* (2007), and in almond in Sánchez-Pérez *et al.* (2007). Also, inter-specific crosses with *P. davidiana* has been used to introgress pathogen resistance and variability in quality traits in peach by the generation of F1, F2 and advanced BC2 populations (Quilot *et al.*, 2004; Foulogne *et al.*, 2003).
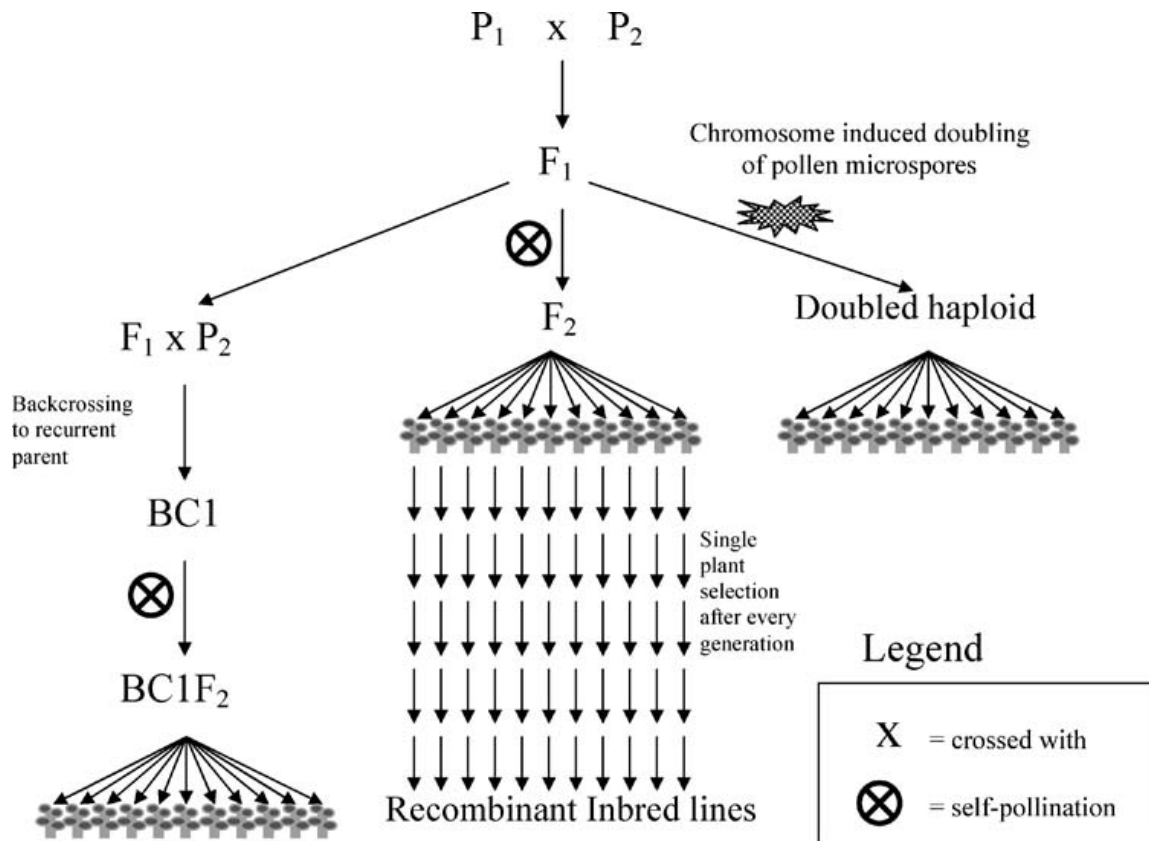
Figure 5. Most recurrent population structures in self-pollinating species, from Collard *et al.* (2005)

1.5.2.2. Polymorphic markers

In order to maximize the number of heterozygous markers segregating in the population, genetically diverse parents need to be selected based for example on different pedigree origins. This is particularly critical in the case of F1 populations, where genotypic evaluation using molecular markers will generate two maps (one for each parent) and a high proportion of heterozygous markers is required to achieve adequate high genome coverage and provide statistical support for QTL analysis. Markers that are heterozygous in both parents can be used to anchor the two parental maps, which may be merged when such markers are in sufficient numbers.

Markers for linkage map construction can also be selected on the basis of already known chromosomal positions. In *Prunus* species, hundreds of markers have been positioned in genetic maps, including the Texas x Earlygold (TxE) *Prunus* reference map (Joobeur *et al.*, 1998) generated from an inter-specific cross between

peach and almond. This map contains the position of 536 markers that are transferable to *Prunus* species (and in some cases to other taxons, Aranzana *et al.*, 2003-a; Dirlewanger *et al.*, 2004-a; Dominguez *et al.*, 2003). Information about these markers is available from www.*Rosaceae*.org. These markers can be used as anchors for map construction in other progenies, allowing adoption of a common linkage group terminology and verification of marker order within each linkage group (Abbott *et al.*, 2008).
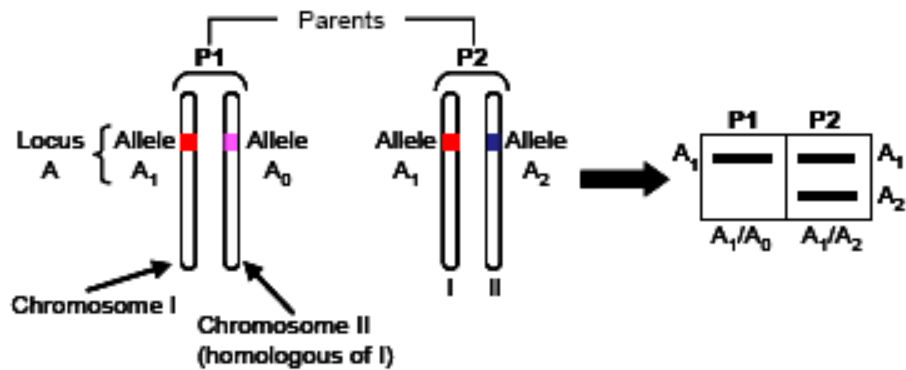
After selecting markers based on heterozigosity in the parents and/or position on reference maps, they have to be screened on the whole population or "genotyped": segregation data will finally be used to calculate recombination frequencies and build the map.

Peach crosses between outbred parents often generate *full-sib* families (Maliepaard *et al.*, 1997), displaying different types of segregation. Depending on the marker genotype patterns in the parents, a specific segregation pattern in the progeny will be expected as illustrated in table 3 and figure 6. However, segregation distortion can occur if a marker is linked to a sub-lethal gene or due to bias in the selection of individuals for genotyping, resulting in discrepancies between observed and theoretical segregation patterns (Sayed *et al.*, 2002; Xu *et al.*, 1997). For this reason, potential segregation distortion needs to be verified using a *Chi-square* test.

Cross (parental genotypes)	Expected genotypes in the progeny	Expected segregation	Mapping usefulness
ab x cd	ac, ad, bc, bd	1:1:1:1	anchor marker (segregating and thus mapable on both parents)
ef x eg	ee, eg, ef, fg	1:1:1:1	anchor marker (segregating and thus mapable on both parents)
hk x hk	hh, hk, kk	1:2:1	marker phase must be known to be mapable
lm x ll	ll, lm	1:1	segregating for first parent only
nn x np	nn, np	1:1	segregating for second parent only

Table 3. Segregation of codominant markers in outbred F1 crosses.
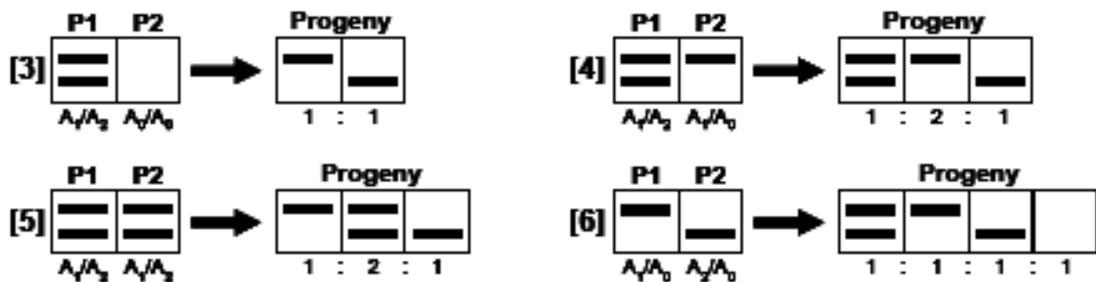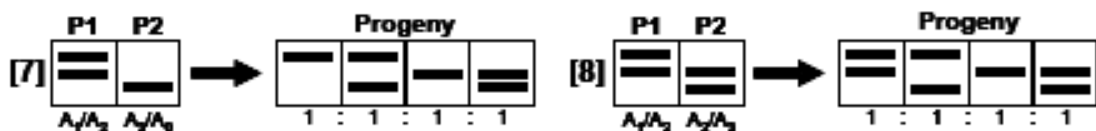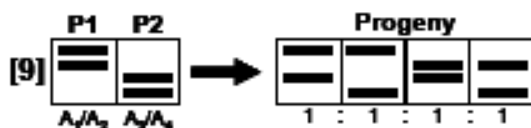
Figure 6. Genotypic configuration observed in heterozygote crosses, modified from Lespinasse (1999). Considering 2 heterozygote parents P1 and P2 at the locus A, 9 informative genotypic configurations for mapping can be distinguished. The configuration [1] is observed with the dominant markers as the configuration [2]. This last configuration cannot be used with Mapmaker software in pseudo-testcross strategy because it is impossible to determine the parental origin of the alleles in the progeny. The configurations [6], [7], [8] and [9] are fully informative.

1.5.2.3. Linkage analysis

In small-scale experiments (two or three point analysis), recombination frequencies between two or three markers can be easily calculated, but linkage map construction involves analysis of a high number of markers (in order of 10s-1000s) requiring the use of specialized software. Linkage significance is verified using odd ratios (*i.e.*, the ratio of linkage likelihood and non-linkage likelihood). This ratio is expressed as the logarithm of the mentioned ratio and is called LOD (for logarithm of odds; Collard *et al.*, 2005). Usually, to affirm that a marker subset map on the same chromosome, the minimum considered LOD score for the subset is 3 (*i.e.*, likelihood of that the markers are linked is 1000 times higher than the likelihood of they are not linked).

In outbreeding species such as peach, linkage analysis and calculation of recombination frequencies are more complicated than in crosses between homozygous parents: markers may exhibit different numbers of segregating alleles and dominance, and one or both parents may be heterozygous; furthermore, the marker phases in the parents are often not known (arrangement of the alleles on homologous chromosomes within an individual, which in theory must be known in linkage theory; Wu *et al*, 2007). Statistical estimators of maximum-likelihood for recombination frequencies are different for each marker pair type of the table 3. Details of how these can be calculated are presented in Maliepaard *et al.* (1997), including formulas to calculate maximum-likelihood estimators for recombination frequency in all the possible marker pair types, and marker ordering algorithms.

After calculating recombination frequencies, these must be converted into genetic distances (unit: centimorgans, cM) and for this purpose mapping functions are available. These functions have different assumptions. For example the Haldane map function (Haldane, 1919) assumes that crossovers occur at random and independently of each other, so they are equally probable at any point between the loci. This assumption does not consider the interference phenomenon (the occurrence of a crossover in a region affects the probability of a second crossover in the vicinity) and thus is not very accurate for long marker distances. The Kosambi function considers this interference phenomenon in genetic distance estimation (Wu *et al.*, 2007).

The most common specialized software packages currently used to generate linkage maps are the freely distributed MAPMAKER/EXP 3.0 (Lander *et al.*, 1987), and the commercial Joinmap 4 (Van Ooijen, 2006).

1.5.2.3.a. Some examples of linkage maps in *Prunus*

Peach genetists have generated linkage maps and used them in QTL analysis of a number of traits. For instance, the TxE reference map (Joobeur *et al.*, 1998; Aranzana *et al.*, 2003-a; Dominguez *et al.*, 2003) has been constructed using an F2 population of 111 seedlings (from the MB1-73 almond x peach hybrid; Joobeur *et al.*, 1998) and includes 536 markers; the wide genetic divergence between the parents means that this cross is highly polymorphic and the corresponding map provides excellent coverage of the 8 chromosomes of the *Prunus* genus, with a total distance of 519 cM and average density of 0.92 cM per marker (figure 7). Several intra or inter-specific *Prunus* maps have been anchored to the TxE reference map (Abbott *et al.*, 2008). Using only 6 TxE F2 individuals, Howad *et al.* (2005) have developed the "bin mapping" strategy, a useful tool to quickly and efficiently assign sequences of interest to sub-chromosomal regions in *Prunus*.

Prunus maps based on F1 populations include the cross between 'R1000' and 'Desmayo Largueta' almond cultivars for the genetic study and QTL analysis of quality traits, such as kernel taste, in-shell weight, shell hardness, kernel weight and double kernel (Sanchez-Perez, 2007-a, -b and 2010). They used 167 seedlings genotyped to construct the parent linkage maps with 56 codominant markers that covered the eight linkage groups of *Prunus* for both parents.

Dirlewanger *et al.* (2004-b) used an F1 population of 101 seedlings originated from the three-way cross between Myrobalan plum (*P. cerasifera* clone P.2175) and the almond (*Prunus dulcis* Mill.)-peach (*Prunus persica* L. Batsch) hybrid clone GN22 ['Garfi' (G) almond × 'Nemared' (N) peach], with the aim of combining the root-knot nematode resistance from Myrobalan and peach and other rootstock traits from almond, peach and plum. The maps included two morphological markers and 166 co-dominant markers covering the eight *Prunus* linkage groups.

Other publicly available *Prunus* maps include F2 and BC2 populations derived from crosses between peach and *P. davidiana* (Foulogne *et al.* 2003; Quilot *et al.*, 2004) to study quality and resistance traits.

The Genome Database for *Rosaceae* (GDR www.*Rosaceae*.org, 2010) hosts 7 maps for *Prunus* inter-specific crosses, 4 for almond, 3 from cherry, 3 from apricot, and 9 for peach, based on different numbers of markers, individuals and population types.
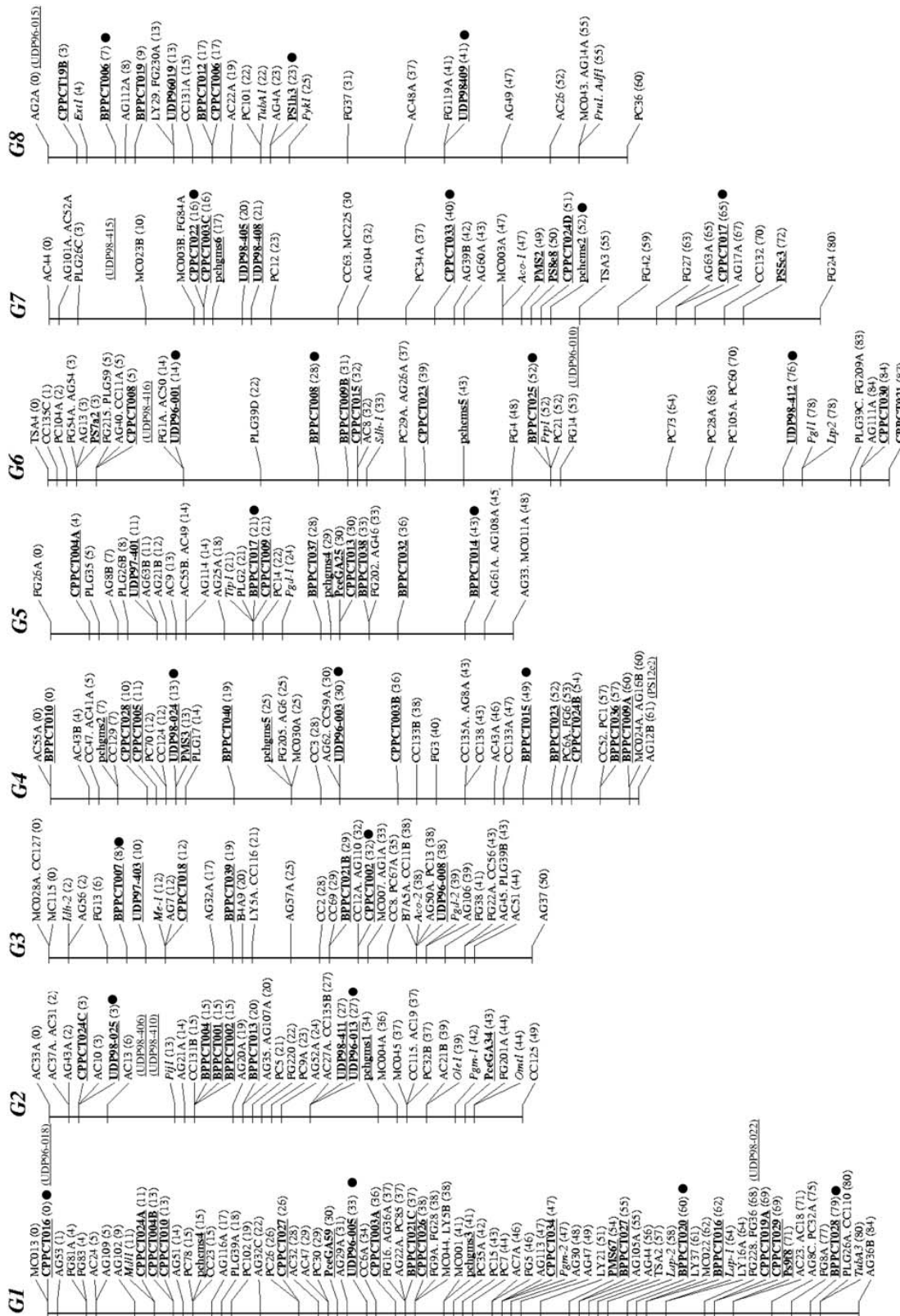
Figure 7. TxE *Prunus* reference map extracted from Aranzana el al. (2003-a). Linkage groups are indicated on the top of the figure (G1-G8). Marker names are indicated at the right of the corresponding linkage groups and the numbers in parenthesis indicate their genetic position (in cM). Underlined markers correspond to SSRs. The SSRs belonging to the proposed reference set (Aranzana et al., 2003-a) are marked with a black point at the right side of the marker name.

1.5.3. QTL analysis

QTLs are genetic loci where functionally different alleles segregate and cause significant effects on a quantitative trait (Salvi and Tuberosa, 2005). The findings of QTL studies completed to date indicate an L-shaped distribution of QTL effects, *i.e.* most QTLs have a small effect and only a few show a strong effect (Brem and Kruglyak, 2005). QTL analyses associate phenotypic differences with alleles of a set of markers of known chromosomal location, and reveal the approximate location of a gene affecting the quantitative character.

The population used in QTL analysis can be a germplasm pool or a biparental cross, associated to a linkage map. In the first case, the QTL analysis is called "association mapping" or "linkage disequilibrium mapping", thoroughly described in Gupta *et al.* (2005), and the second is the linkage-based QTL analysis, that will be briefly described below.

Marker genotypes are used to classify individuals in marker-specific groups, based on the alleles that each individual has, then these groups are analysed to assess if they possess significant differences with respect to the measured traits. If a determined marker serves to partition the population into significantly different phenotypic categories, we can say that the marker is linked to a QTL controlling the trait (Tanksley, 1993; Young, 1996).

There are three methods for linkage-based QTL analysis, each one with multiple variants depending on the distribution of the trait in the population, or the number of QTLs to detect.

Single-marker analysis (also 'single-point analysis') is the simplest method for detecting QTLs associated with single markers. The statistical methods used for single-marker analysis include t-tests, analysis of variance (ANOVA) and linear regression. Linear regression is most commonly used because the coefficient of determination (R2) from the marker explains the phenotypic variation arising from the QTL linked to the marker (Collard *et al.*, 2005). These analyses assume that the trait is normally distributed in the sampled population. Non-parametric tests such as the Kruskal-Wallis test, allow the association of allelic categories to not normally distributed traits, as frequently happens with disease resistance scores (*e.g.* PPV resistance QTL analyses in apricot: Pilarovà *et al.*, 2010; Marandel *et al*, 2009-a). In such cases, a non-parametric version of the one-way ANOVA is performed. Software such as MapQTL 6.0 include an implementation of this test for QTL analysis on

populations that are generated from two heterogeneously heterozygous and homozygous diploid parents with linkage phases unknown (Van Ooijen, 2009), as is often the case in peach, Since this approach is based on single markers, it does not require a linkage map. However, linkage maps can be useful, as suggested by Van Ooijen (2009), because a segregating QTL must reveal a significance gradient in the linkage group towards the marker with closest linkage to the QTL, and this can be visualised using the linkage map.

Interval mapping methods are considered more powerful than single marker analysis, since they use the interval between two linked markers, and then considering the possibility of recombination between markers and QTL (Lander and Botstein, 1989). When this statistical model is integrated with linear regression to determine the R2 of an interval and use of additional linked markers, it gains power and precision in the interval determined as linked to the QTL (Collard *et al.*, 2005). In MapQTL 6.0 (Van Ooijen, 2009), maximum likelihood and regression-based algorithms are included to calculate trait-marker associations also considering intervals of various linked markers; in addition, a multiple QTL model is included in which the interactions between QTLs detected by interval mapping are calculated when markers strongly linked to QTLs are designed as "covariates".

1.5.3.1. Some examples of QTL analysis in *Prunus*.

Several QTL analyses have been reported in *Prunus* species for a wide range of traits of agronomical and commercial importance. Fruit quality traits have been extensively dissected by QTL analysis in *Prunus*. Etienne *et al.* (2002), Quilot *et al.* (2004 and 2005), Dirlewanger *et al.* (1998 and 2007) have associated traits such as sugar content, fruit weight, acidity, blooming and maturity date to specific genomic regions, using different intra-specific peach crosses. Major genes such as flat shape, nectarine, and sub-acidity were mapped and their positions were anchored to the TxE reference map (Dirlewanger *et al.*, 2007). Eduardo *et al.* (in press) have detected a strong pleiotropic effect of maturity date on fruit quality traits as fruit weight, fruit acidity, soluble solid content, and skin over-colour, using F1 and F2 populations derived from intra-specific crosses of peach. Recently, fruit size has been analysed with the QTL approach in sweet cherry by using a population derived from the cross between an 8 g-weighted commercial cultivar and a 2 g-weighted wild forest clone: parameters such as fruit size, mesocarp cell number, cell length, and pit

size were analysed, detecting a QTL cluster for the first two traits on chromosome 2 and for pit size on chromosome 6 (Zhang *et al.*, 2009).

Ogundiwin *et al.* (2008) and Cantin *et al.* (2010) have used the bin mapping approach and QTL approach, respectively, to identify 3 regions on peach chromosome 4 associated with enzymatic browning and chilling injury susceptibility. This was the basis for identification of a gene encoding the leucoanthocanidin dioxygenase (PpLDOX) enzyme was identified as the gene potentially responsible for a QTL associated to browning susceptibility, as is putatively involved in the enzymatic oxidation of phenolic compounds.

Fruit quality traits have been extensively dissected by QTL analysis in *Prunus*. Etienne *et al.* (2002), Quilot *et al.* (2004 and 2005), Dirlewanger *et al.* (1998 and 2007) have associated traits such as sugar content, fruit weight, acidity, blooming and maturity date to specific genomic regions, using different intra-specific peach crosses. Major genes such as flat shape, nectarine, and sub-acidity were mapped and their positions were anchored to the TxE reference map (Dirlewanger *et al.*, 2007). Eduardo *et al.* (in press) have detected a strong pleiotropic effect of maturity date on fruit quality traits as fruit weight, fruit acidity, soluble solid content, and skin over-colour, using F1 and F2 populations derived from intra-specific crosses of peach. Recently, fruit size has been analysed with the QTL approach in sweet cherry by using a population derived from the cross between an 8 g-weighted commercial cultivar and a 2 g-weighted wild forest clone: parameters such as fruit size, mesocarp cell number, cell length, and pit size were analysed, detecting a QTL cluster for the first two traits on chromosome 2 and for pit size on chromosome 6 (Zhang *et al.*, 2009).

Regarding disease resistance QTL mapping, Piralova *et al.* (2010) have recently reported the association of the resistance to PPV with the upper region of chromosome 1 in apricot cultivar 'Harlayne' using both non-parametric and parametric QTL analysis based on an F1 population. Using F1 and F2 populations from an inter-specific cross between peach cv. 'Rubira' and P. davidiana clone P1908, the PPV-resistance in P. davidiana has been associated to a total of nine involved regions; six of these were consistent in the QTL analyses on both F1 and F2 populations (Rubio *et al.*, 2010), although high and significant environmental effects were also apparent. Marandel *et al.* (2009-a and -b) have co-localised a PPV-resistance QTL in linkage group 1 of cv. 'Harlayne' (explaining between 5 and 39% of

the phenotypic variation) with an expressed gene coding for the eukaryotic translation initiator factor, eIF4E. Six powdery mildew-associated QTLs have been identified by the use of interspecific crosses (F1, F2 and BC2) between commercial cultivars of peach and *P. davidiana* (Foulogne *et al.*, 2003); these QTLs have been also validated in diverse environments.

These examples support the feasibility of genetically dissecting quantitative characters in *Prunus* species through QTL analysis. Identification of the genomic regions associated to traits of interest provide valuable information for marked assisted selection programs, as well as a basis for the isolation of the underlying genes and a better understanding of the genetic, cellular and physiological mechanisms controlling phenotypic expression.

1.6. Aim of the project

The main objective of this PhD project has been to find molecular markers associated with the tolerance to brown rot caused by *Monilinia* spp. in peach. To achieve this, a linkage-based QTL mapping approach has been taken, using the F1 segregating population (110 seedlings) from the 'Contender x Elegant Lady' intra-specific peach cross, available in our research group.

The first specific objective has been to construct a linkage map of both parents. The chosen molecular markers to be included in the map were SSRs. After screening nearly 300 SSRs on the parents and four progeny seedlings, we selected high quality markers that were heterozygous in at least one parent. The resulting map (CxEL) was compared to the TxE *Prunus* reference map, to assess marker order and level of genome coverage.

The second specific objective was to phenotypically evaluate the population over two seasons. A phenotyping protocol was developed to clearly determine the impact of the artificial infection of an 'Elegant Lady' fruit-isolated strain. Three treatments were considered in order to evaluate: 1) the tolerance to post-harvest rot generated by natural quiescent field infections, by measuring the proportion of infected fruits per seedling not subjected to artificial infection (control); 2) the resistance effect of the exocarp tissues, by the inoculation over an intact region of the fruit skin, and subsequent measurement of the proportion of infected fruits per genotype and mean fruit infected surface; and 3) the resistance effect of the mesocarp tissues by the inoculation in an artificial wound of the fruit, and the

subsequent measurement of the proportion of infected fruits per genotype and mean fruit infected surface. Infection measurements were registered for the whole population for two different harvest seasons (and thus being affected by different environmental conditions) and statistical analyses allowed to reveal genotypic and environmental differences and correlations between the diverse infection measurements. Depending on the distribution of phenotypic data, an estimation of the genetic variability was calculated to roughly estimate the heritability of the trait in the analysed population.

The third specific objective was to carry out QTL analysis using the generated linkage map and population phenotypic data. Markers found to be strongly linked to QTLs will be valuable tools for marked assisted selection of new seedlings with improved BR-tolerance.

At the same time this work will provide basic knowledge about the locations of some of the genetic factors involved in the tolerance to *Monilinia* spp., serving as a basis of a more detailed genetic dissection of this important trait.

2. Materials and Methods

2.1. Plant material

In this study, trees of the cultivars 'Contender', 'Elegant Lady' and 168 seedlings of an F1 population derived from the cross between the mentioned cultivars (hereon "CxEL") were studied in the years 2009 and 2010. The trees are present at the experimental orchards "Francesco Dotti" of the University of Milan (Arcagna, Montanaso Lombardo, Lodi, Italy). The seedlings were grafted on the GF305 seedling rootstock and planted with a spacing of 1 m within and 4 m between rows, trained as slender spindle (one stem with short upper lateral scaffolds, and larger bottom lateral scaffolds). Two replicate trees per each genotype were planted together. Dormant pruning was performed yearly and standard cultural practices applied. The fruits were thinned before pit hardening to a load of only 40-80 fruits per tree according to tree vigour, in order to allow a full expression of fruit size not limited by competition. Treatments against leaf curl were performed three times in autumn (before, during and after leaf fall); insecticide (white oil) and antifungal treatments (copper against leaf curl, bupirimate against powdery mildew and ziram for blossom blight) were applied at bloom. No treatment against BR was applied during and after petal fall, in order to add external resistance factors on fruits.

'Contender' is one of the most reliably cropping peaches from North Carolina (introduced by D.G. Werner in 1987), producing a freestone, yellow fruit of about 200 g with a melting, high-quality flesh type, round shape, resistant to browning, and field tolerant to brown rot and bacterial spot (Okie, 1998). Its flower type is non-showy and their fruits matured by the first week of August in the year 2009 and the second week of August in 2010.

'Elegant Lady' is a popular Californian cultivar (introduced by G. Merrill in 1979), but somewhat buds are tender so production is erratic; produces an attractive freestone, yellow peach fruit of about 220 g with a melting, high-quality flesh type, round shape, being very susceptible to brown rot, leaf curl and bacterial spot (Okie, 1998). Has a high cold-requirement of 750 CU. The flower type is non-showy and their fruits matured by the second week of August on 2009 and 2010. Pedigree of both cultivars is shown in the figure 8.
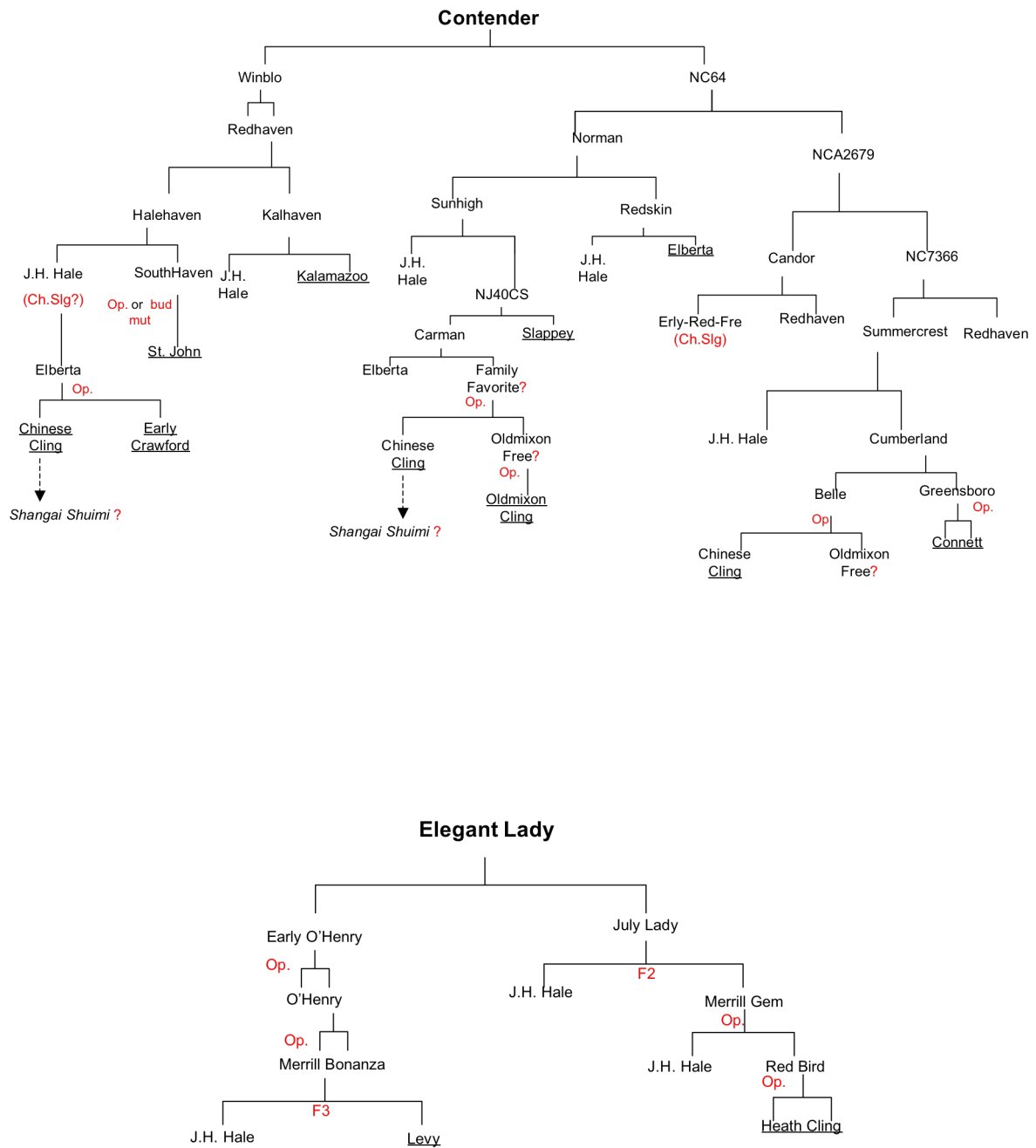
Figure 8. Pedigrees of the parent cultivars used in this study: 'Contender' (seed parent, top) and 'Elegant Lady' (pollen parent, bottom), extracted from Okie (1998). Underlined cultivar names represent those accessions for which no pedigree information was available. Red symbols represent the type of cross that originated the cultivar: "Ch.Slg" for chance seedling, "Op." for open-pollination, "bud mut" for bud mutation, "?" to design not confirmed origins, "F2" for F2 seedling and "F3" for F3 seedling.

The CxEL population segregates for BR susceptibility, maturity date, flower type, soluble solid content (SSC) and fruit weight (Bassi *et al.*, 1998; Bassi and Rizzo, 2003).

2.2. Isolation and molecular identification of *Monilinia* spp. field strain.

Two *Monilinia* spp. (called Arc1 and Arc2,) isolates were obtained from a rotten plum fruit (cv. 'TC Sun') present in the same farm, for artificial inoculation. The surface of the fruit was thoroughly disinfected by 3 cycles of washing in a solution containing 0,5% NaOCl and 0,05% Tween20 for 2 minutes and rinsing in sterile distilled water for 1 minute. A piece of 1 cm^2 of the infected fruit was put on the centre of a 9 cm diameter-plates containing 4 ppm of tetracycline (PDA-tet) and incubated at 22ºC, 74% of relative humidity and 12 hour-photoperiod for 4 days in order to develop mycelial growth. Mycelial plugs of approximately 4 mm^2 were transferred to plates containing PDA-tet for three times to discard possible fungal contaminants and to make stocks to be stored at 4ºC and periodically renewed.

Deoxyribonucleic acid (DNA) extraction of the isolate Arc1, Arc2, *Monilinia fructigena* CBS492.50 and *Monilinia laxa* CBS10150.3 was made using the PowerPlant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) and lyophilized mycelium obtained from 20 ml of liquid culture of each strain in potato dextrose broth containing 4 ppm of tetracycline (PDB-tet). Ten nanograms of each isolate DNA were used to identify *Monilinia* strains Arc1 and Arc2, according with the PCR protocol of Hughes *et al.* (2000).
Mycelial plugs were used also for infect peeled and disinfected apples (cv. 'Golden Delicious') in order to generate abundant conidia to be used in further artificial inoculations.

2.3. Phenotyping

Between 40 and 60 intact and healthy fruits per seedling (according to fruit availability) were harvested at commercial maturity based the use of I$_{AD}$ index of difference in the fruit-chlorophyll absorbance (Ziosi *et al.*, 2008), taken with the DA-meter instrument (Synteleia S.R.L., Italy). In some precocious CxEL seedlings, an I$_{AD}$ value minor than 0,4 indicated they had reached physiological maturity based on fruit colour change and manual evaluation of firmness. In this way, I$_{AD}$ was monitored each two days in five fruits per seedling, and maturity date (and hence the harvest)

was determined to be the day in which fruits of a determined seedling presented a mean I_{AD} value equal or under 0,4. Firmness and colour were sensory checked for each harvested seedling to confirm the validity of the I_{AD} parameter. I_{AD} was measured with the DA-meter non- invasive and portable instrument (Sinteleia Sr., Italy).

The infection procedure was based on the protocols reported in Pascal *et al.* (1994), Gradziel *et al.*, (2003) and Walter *et al.* (2004), and is described as follows.

Harvested fruits were disinfected in order to reduce the probability of *Monilinia* spp. or other post-harvest fungal infection that interfered with our measurements. Disinfection consisted in a two minute-immersion in an aqueous solution containing 0,5% NaOCl and 0,05% Tween20, followed by a 70% ethanol spray. After 12 hours of air-drying, fruits were artificially inoculated with a conidial suspension drop (10 µl) containing $2x10^4$ - $5x10^4$ spores on a field-isolate of *Monilinia fructigena* isolate A1 (see section 2.2) on the centre of the sun-exposed fruit cheek, where the fruit crack probability is lower than in pedicel cavities and stylar regions (Gibert *et al.*, 2005). The spores were counted on a Malassez counting-chamber and the concentration was adjusted with aqueous 0,05% Tween20. Three inoculation ways were performed, to assess genetic factors associated to resistance of skin and flesh fruit tissues:

- Inoculations on the skin, to test the BR susceptibility due to the barriers present the skin;
- Inoculations in the flesh, through wounding the skin with a pipette tip, in order to test the BR susceptibility due to the barriers present in the flesh;
- a mock inoculation of 10 µl of water over the fruit skin (hereon "Control"), to determine the impact of natural quiescent infections from the field in the whole fruit.

Each seedling-treatment combination contained 10 - 22 replicate fruits that were incubated in a room equipped with an air conditioning device for 5 days at 25ºC, inside a plastic-sealed fruit box (to maintain high humidity).

Data of seedling identification, treatment, year, maturity date, fruit and infection diameter (d_F and d_{Inf}, respectively), presence or absence of lesions from artificial (skin and flesh) and natural (Control) infections were recorded in Microsoft Excel 97 worksheets. Fruit and infection diameter were measured with a manual calliper. For the artificial inoculation treatments (skin and flesh) only the lesions

originated from the point of inoculation were considered, and fruits presenting lesions in other points of the fruit were disregarded. IVF was considered only for the artificial inoculum treatments (SkinInf and FleshInf), and not for the control treatment, since multiple infections per fruit often occurred, making difficult to measure lesion diameter.

These data were used to score the BR susceptibility by considering two methods:

1) calculating the frequency of BR infected fruits per seedling-treatment combination (hereon "%IF");

2) calculating the average percentage fruit volume affected by the artificial inoculation in each seedling-treatment combination (hereon "IVF"). To this purpose a spherical shape of peach fruit was assumed for all the analysed seedlings. Then, to calculate the fruit and infection volumes (V_F and V_{Inf}) we used the following equations:

$$h = (d_F/2) - [(d_F/2)^2 - (d_{Inf}/2)^2] \quad \text{(Equation 4)}$$

$$V_F = 4 \pi r^3 / 3 \quad \text{(Equation 5)}$$

$$V_{Inf} = \pi h^2 [3 (d_F/2) - h] / 3 \quad \text{(Equation 6)}$$

$$IVF = V_{Inf} \times 100/ V_F \quad \text{(Equation 7)}$$

This protocol was used to test the BR susceptibility over a total of 142 different seedlings and in both parents. In 2009 and 2010, 105 and 115 seedlings of CxEL, were analysed respectively. The obtained dataset included two years of analysis, three treatments and two BR susceptibility scores, defining twelve diverse sub-datasets. Each sub-dataset included corresponding susceptibility score measured in each treatment and year.

2.4. Data analysis

All the statistical analyses were performed using the R version 2.12.1 (R Development Core Team, 2010), including frequency histograms to determine sub-datasets distributions and matrices to determine the correlation between sub-datasets. Spearman method was used to determine the significance of the correlations. Paired Wilcoxon signed-rank test (the non-parametric version of t test) was used to determine the significant difference between sub-datasets. To assess

significant difference between genotypes and determine the genetic variance component of the measured BR susceptibility phenotype, the whole dataset including infection data of each fruit replicate (presence or absence of lesion scored as 1 or 0) for the analysed seedlings was submitted to a logistic regression analysis using the "lmer" function of the lme4 R package (Bates, 2005). Two models were tested:

infart ~ MD + trat + (1 | year) + (MD | genot)   (Equation 8), and

infart ~ trat + (1 | year) + (MD | genot)   (Equation 9)

where "infart" is the presence or absence of infection (scored 1 or 0), "MD" is the maturity date, "trat" is the infection treatment, "year" is the year of analysis, and "genot" is the seedling identification. On both models the variable "year" and "genot" are treated as random factors in order to determine their associated variances, and MD was treated as a "genot"-nested variable. Model described by the equation 8, considers maturity date as a fixed factor. As binary data was the output of the logistic regression model, residual variance was assumed to be equal to 1. ANOVA between both models was used to test the difference between them and evaluate which explains better the obtained results.

2.5. Population genotyping

DNA extractions from young leaves of the parents and 110 CxEL seedlings were carried out using the PowerPlant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). SSR amplifications were performed following the multiplex-ready PCR protocol (Hayden *et al.* 2008-a and -b; figure 4) with some modifications. In this protocol, the forward and reverse SSR-specific primers used were synthesized adding at their 5' end the sequence 5'-ACGACGTTGTAAAA-3' and 5'-CATTAAGTTCCCATTA-3', respectively. The protocol also included the use of short, generic primers tagF (fluorescently labeled with FAM, VIC, NED or PET fluorescent dyes) and tagR (unlabelled) whose sequences were 5'-ACGACGTTGTAAAA-3' and 5'-CATTAAGTTCCCATTA-3', respectively. PCR reactions contained 1-20 ng of genomic DNA, 1x PCR reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-Cl pH 8,8 and 0.1% Tween-20), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 100 nM of each tag primer, 40 nM of SSR-specific primers (Sigma Life Sciences, Italy) in either singleplex or multiplex reactions, 0.01 U of EuroTaq DNA polymerase (EuroClone, Italy) and

sterile distilled water to complete 8 µl of final volume. The amplification program consisted in an initial denaturation step of 2 min at 95°C; 20 pre-amplification cycles of 30 s at 92 °C, 30 s at 60 °C, 30 s at 72 °C; 40 amplification/labeling cycles of 15 s at 92 °C, 30 s at 54 °C, 30 s at 72 °C; and a final extension of 5 min at 72 °C followed by 25 min at 25 °C. After the amplification, the PCR products were diluted with 10 µl of distilled water, then 5 µl of dilution labeled with a different dye was pooled in a 1:1:1:1 proportion (multi-pooling), ethanol-precipitated and re-suspended in 20 µl with distilled water. The capillary electrophoresis run was made loading a mix consisting of 2 µl of purified PCR multi-pool, 10 µl of formamide and 0.15 µl of GeneScan500 LIZ-250 size standard in an ABI Prism 3730 DNA Analyzer was used (Applied Biosystems), according manufacturer's indications. Allele size was manually determined using the GeneMarker demo version 1.70 (SoftGenetics) and scored in a Microsoft Excel 97 worksheet in two independent events. Using the multiplex-ready approach with "tagged" SSR primers, the expected size of the alleles corresponded to approximately 30 base pairs larger than reported allele sizes.

A total of 342 SSR primer pairs derived from different species of *Prunus* genus (table 4) was screened in the parents. SSR markers yielding distinguishable peaks and showing to be heterozygous in at least one of the parents were tested afterwards in a sample subset composed by the DNA of the two parents and four CxEL selected seedlings (two tolerant and two susceptible, based on the data of Bassi and Rizzo, 2003). Markers that confirmed good amplification quality, showed missing data rate less than 10% and gave an expected segregation in the four selected seedlings were then qualified as suitable to be genotyped in the whole CxEL population.

The markers to be mapped were also selected on the basis of their position on the available *Prunus* linkage maps using the comparative mapping information available in the Genomic Database for *Rosaceae* (GDR, http://www.*Rosaceae*.org, "search markers" and "CMap" tool).

2.6. Linkage mapping

Genetic linkage analysis and map construction was performed with JoinMap 4 (Van Ooijen 2006) using a dataset generated by genotyping 78 co-dominant SSR markers in 110 F1 individuals of the CxEL progeny.

CxEL map was calculated considering simultaneously the maternal and paternal meiosis, by using the "Create Population Node" option of JoinMap 4.0, since

a great number of anchor markers were found among the analysed studied parents. Additionally, this method can deduce the linkage phase of *hkxhk* segregation type markers. Markers showing segregation distortion were not excluded from the analysis. The pairwise recombination fraction threshold was fixed to 0.40, and the recombination fraction values were transformed into genetic distances (centimorgans) by using the Kosambi mapping function. Linkage groups were defined with a minimum LOD value of 5.0 (the probability that the markers were linked is 10^5 times higher than the probability they were not), using the independence LOD as grouping test statistic. Linkage maps were drawn using the MapChart 2.1 software (Voorrips, 2002). Linkage groups nomenclature was the same as in the TxE *Prunus* reference map (Dirlewanger *et al.* 2004-a).

2.7. QTL analysis

QTL analysis was carried out using the software MAPQTL 6.0 (Van Ooijen 2009). Phenotypic data included 2009 and 2010 datasets, both containing data of maturity date (MD), control-%if, skin-IVF, skin-%IF, flesh-IVF and flesh-%if. The CxEL map was used to locate the found associations on peach chromosomes.

In a first analysis, the non-parametric Kruskal–Wallis (KW) rank-sum test was used in order to search phenotype-marker associations without assume a normal distribution of the phenotypic data. A stringent significance level of p=0.005 was adopted as threshold for the detection of a QTL for the individual test in order to obtain an overall significance level of about p=0.05, as suggested by Van Ooijen (2009). In addition, associations of phenotype with groups of more than one marker located adjacently in the linkage map were regarded as sound, for individual markers with p<0,05.

In order to obtain a rough estimation of the genomic interval containing QTL and its contribution to the phenotypic variance, "interval mapping" approach (IM) was used. After a genome-wide test of 10,000 permutations (p<0,05), threshold LOD scores of 2,5 in all datasets were chosen to accept QTL significance.

QTLs were drawn using the MapChart 2.1 software (Voorrips, 2002).

3. Results

3.1. Phenotypic analyses

A phenotyping strategy was set up in order to generate BR-resistance phenotypic data to be used in QTL analysis. The strategy was designed by establishing infection conditions, in terms of: i) inoculum uniformity, to ensure that all seedlings were infected by a unique and known pathogen that formed part of the orchard mycoflora; ii) identification of the infected fruit tissue, to clearly associate the resistance data to the inoculated tissue; and iii) uniformity of the post-inoculum condition. In this way, we divided phenotyping activities in: isolation of the pathogen for the artificial infections, setup the infection procedure and measurement of BR-resistance in the progeny.

3.1.1. Isolation and identification of a *Monilinia* spp. field strain.

Two *Monilinia* spp. strains (labelled as Arc1 and Arc2) were isolated from rotten tissue from plum fruit and cultured in PDA plates (figure 9A). In order to check their identity, DNA samples extracted from Arc1, Arc2 *Monilinia fructigena* CBS492.50 and *Monilinia laxa* CBS10150.3 (among other fungal isolates) were PCR-amplified using three primer pairs reported in Hughes *et al.* (2000). Primer pairs Mfc-F1/Mfc-R1, Ml-Mfg-F2/Ml-Mfc-R1 and ITS1/Mfg-R2 detect *M. fructicola*, *M. laxa* or *M. fructigena* ITS-ribosomal DNA specific sequences, yielding bands of 280, 280 and 460 bp, respectively. A fourth primer, ITS1-ITS4 amplifies a common fungal ITS-ribosomal DNA sequence set, and was used as DNA quality control.

A Eukaryotic universal primer set (ITS1-ITS4) yielded bands for all extracted DNAs, thus confirming the good quality of the extracted samples (figure 9C). No amplification was produced by the *M. fructicola*-primer set in any DNA sample (figure 9b). Arc1 and Arc2 isolates yielded a 480 bp band samples only with *M. fructigena* primers (figure 9B).

3.1.2. Phenotypic data

The protocol used for BR-resistance phenotyping was established from previously reported experimental conditions (Pascal *et al.*, 1994; Gradziel *et al.*, 1998; Walter *et al.*, 2004), and were quickly set up directly in the progeny.
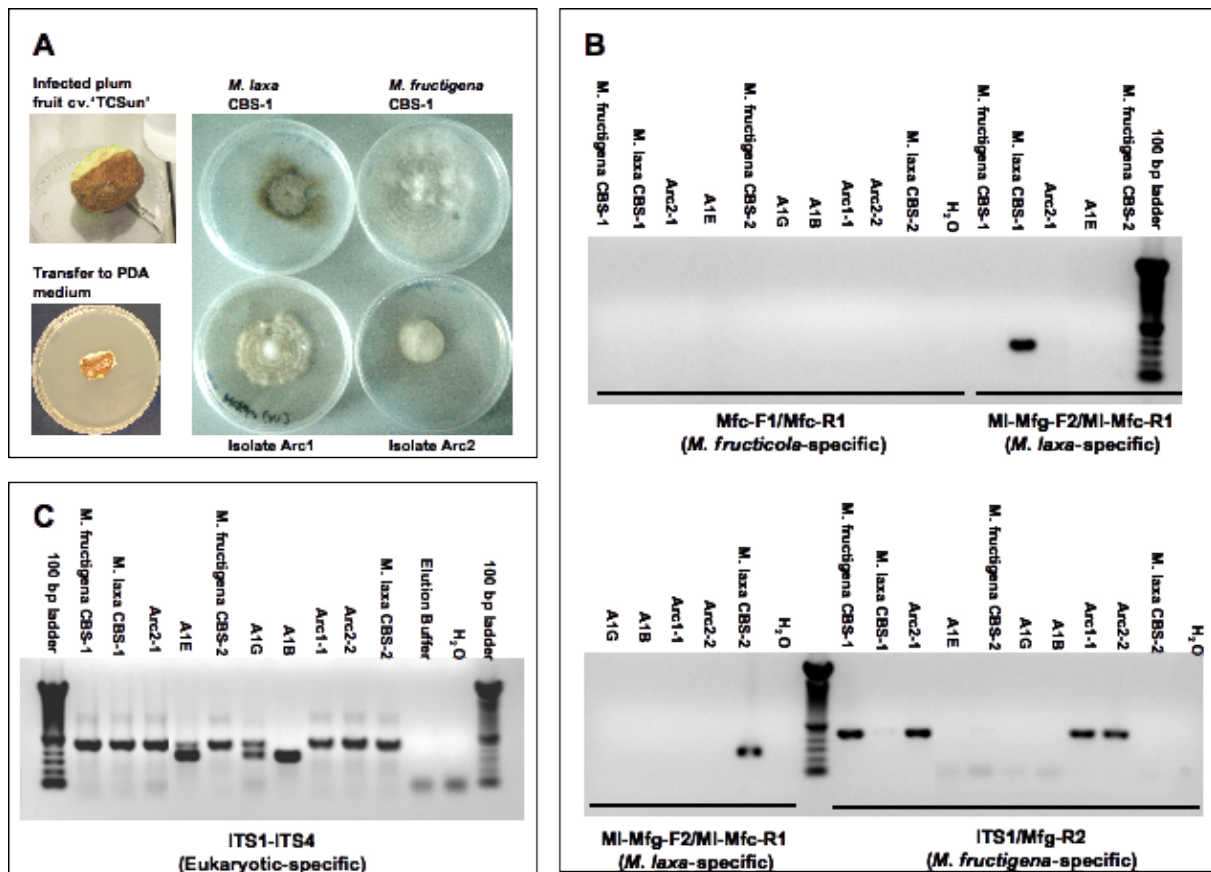
Fig. 9. Arc1 and Arc2 *Monilinia* spp. strains isolation and identification. A, isolation from infected fruits from field and growth in PDA-tet plates. B, identification of Monilinia strains Arc-1 and Arc-2 with species-specific PCR from Hughes *et al.* (2000). C, DNA quality control by Eukaryotic PCR.

Two different artificial inoculation treatments measured BR-resistance in two fruit tissues (skin and flesh) with two different infection scores (IVF and %if). Each of these "tissue x score" combinations defined a trait, in such a way that "skin-%if" and "flesh-%if" traits indicate the frequency of infected fruits per genotype in the skin and flesh inoculations, respectively; in the same way, "skin-IVF" and "flesh-IVF" indicate the seedling-average lesion volume in the skin and flesh inoculations, respectively.

A third, control treatment measured field inoculum resistance, and only the %if score was considered, defining the "ctrl-%if" trait.

All resistance (n=4) and maturity date (MD) traits (total 5 traits) were measured in two years (2009 and 2010), giving place to 10 sub-datasets.

3.1.2.1. Distribution of resistance traits

Histograms for the traits measured in the two years of study are shown in figure 10. All traits described a similar non-normal distribution, in which resistance intervals of extreme values (0-10% or 90-100% of the individuals) were often obtained.

Most of the progeny exhibited low infection from field inoculum: 40% of the tested seedlings in 2009 and 80% in 2010 fell in the class 0-20% of infected fruits.

Skin susceptibility (skin-IVF) behaved similarly in both years. Around 60% of the tested seedlings showed the highest skin-%if (90 – 100% of infected fruit) in 2009, while in 2010 around a 50% showed the lowest rate (0 to 10%), suggesting a strong environmental effect on this trait.

Flesh-IVF showed a similar frequency in all infection classes, in both years. The flesh-%if showed similar distributions across years, with a high quantity of seedlings with infection in all tested fruit, but in diverse frequencies (~ 80% of seedlings fell in the 90%-100% class in 2009, and ~50% in 2010).
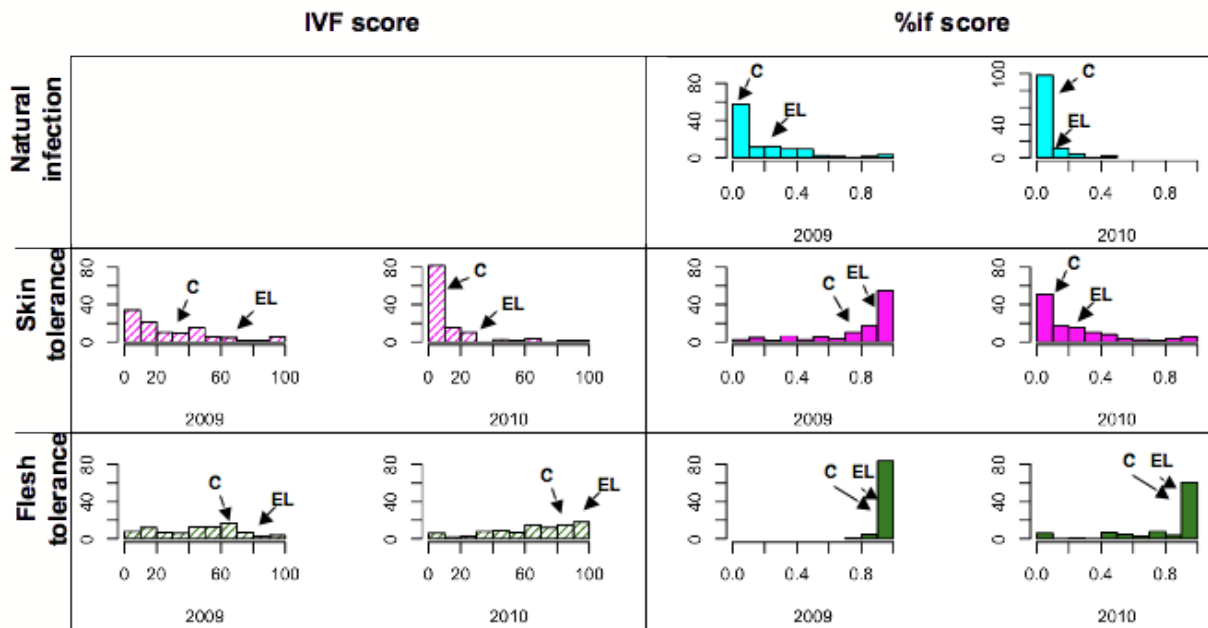
Fig. 10. Frequency histograms of CxEL F1 progeny for BR-tolerance traits. Each sub-dataset is represented by an different histogram. Trait is indicated at the left of each graph row and by the bar colour: cyan for natural infection BR-resistance, magenta for skin BR-tolerance, green for flesh tolerance, and black-diagonal lines for maturity date. BR score considered is indicated on the top of each graph-pair column and by the bar fill effect: IVF score is represented by diagonal line-filled bars and %if is represented by full bars. Years of sub-datasets are indicated at the bottom of each histogram. Frequency is expressed in the y-axis as the number of individuals falling in phenotypic interval (x-axis).

'Contender' parent always appeared more tolerant than 'Elegant Lady'. Depending on the trait and score, the parents fell in different infection classes respect to the progeny distributions (figure 10). Transgressive segregation of BR-resistance was observed in some traits: skin resistance in 2009, and flesh resistance in both years.

MD showed non-normal distributions in both years (figure 11). Transgressive segregation for this trait could be also observed, since maturity dates of the parents are close and progeny showed a range of 38-42 days, depending on the year. A two-weeks delay on 'Contender' ripening was registered in 2010.
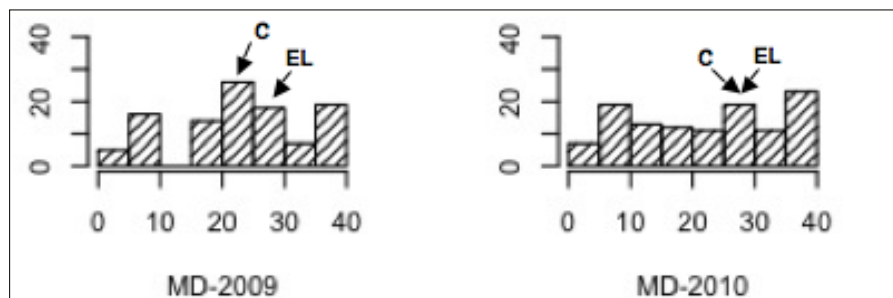
Fig. 11. Frequency histograms of CxEL F1 progeny for maturity date. Years of sub-datasets are indicated at the bottom of each histogram. Frequency is expressed in the y-axis as the number of individuals falling in phenotypic interval (x-axis).

3.1.2.2. Resistance traits correlations

Significant differences between all traits were confirmed by the paired Wilcoxon signed-rank test ($p<0,0005$). This test is the non-parametric version of the t-test, and served to search for similarities between all traits and seasonal measurements.

3.1.2.2.1. Correlations between years

Correlations were found for MD, and for skin and flesh resistance (only when measured as IVF, table 4). MD presented the highest correlation between years (R^2 = 0,92 with $p<0,0005$). Skin and flesh resistance (IFV) showed R^2 values of 0,29 and 0,39, respectively ($p<0,05$), suggesting that they could be controlled by a significant genetic component.

3.1.2.2.2. Correlations between traits

Natural infection resistance correlated only in 2009 with skin-IFV and flesh-IFV (R^2 =0.34 and 0.28, respectively, table 4). Skin and flesh resistance correlated in both years for both scores (except skin-%if vs. flesh-%if on 2009). Between skin and flesh resistance (IVF), correlations of 0.55 and 0.47 were observed in 2009 and 2010, respectively, suggesting possible common resistance mechanisms on both tissues.

Maturity date significantly correlated with all resistance traits (except field infection resistance in 2010), showing correlations ranging from 0.26 (for skin-%if in 2009, $p<0,05$) to 0.72 (for flesh-IVF in 2009, $p<0,0005$; table 4).

Trait	Correlation (2009-2010)
MD	0,92***
Control	NS
Skin-IVF	0,29*
Skin-%if	NS
Flesh-IVF	0,39**
Flesh-%if	NS

		MD	Control %if	SkinInf IVF	SkinInf %if	FleshInf IVF
Control %if	2009	0,34**	1			
	2010	NS	1			
SkinInf IVF	2009	0,46***	0,34**	1		
	2010	0,32**	NS	1		
SkinInf %if	2009	0,26*	NS	0,74***	1	
	2010	0,37**	NS	0,27*	1	
FleshInf IVF	2009	0,72***	0,28*	0,55***	0,30*	1
	2010	0,45***	NS	0,47***	0,52***	1
FleshInf %if	2009	0,28*	NS	0,23*	NS	0,36**
	2010	0,63***	NS	0,26*	0,31*	0,60***

Table 4. Correlations in CxEL phenotypes. Top table shows correlations between years. Bottom table shows correlations between traits in each year. Significance codes: NS non significative, 0.0005 '***' , 0.005 '**', 0.05 '*'.

3.1.2.3. Genetic and seasonal components of BR-resistance variance

Logistic regression is used for prediction of the probability of occurrence of an event (in our case infection occurrence) by fitting data to a function curve (logit function logistic curve), and also can estimate the variance associated to the resulting presence or absence of infection. Two simple logistic regression models were tested to estimate variance components on fruit infection (equations 8 and 9; see section 2.4). Both models indicated the treatments, genotypes and years as significant effects on infection. ANOVA assessed the difference between the two models ($p<1e-7$). Eq. 9 model resulted the most accurate, because of its lower Akaike Information Criterion (AIC; Akaike, 1974) of goodness-of-fit of 5386 compared to 5412 of Eq. 8 model.

Eq.9 model treated MD both as a fixed effect (or explanatory variables treated as non-random) and as a variable nested (or dependent) to genotype, resulting to be a significant fixed effect. The model allowed the estimation of genetic and seasonal (year) variance components of 3,32 and 2,13, respectively (table 5). As assumed variance of residual effects is 1 in logistic regression, the genetic component of the variance, or broad-sense heritability, was equivalent to a 51,5%.

These results suggest that the BR-resistance trait, although being affected by seasonal factors, may be genetically improved.

(Eq.9) infart ~ MD + trat + (1 | year) + (MD | genotipo)

Goodness-of-fit:

AIC	BIC	logLik	deviance
5386	5442	-2685	5370

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
genot	(Intercept)	**3,319268**	1,821886	
	MD	**0,006834**	0,082668	-0,859
year	(Intercept)	**2,131136**	1,459841	

Number of obs: 8497, groups: genot, 143; year, 2

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)	
(Intercept)	-4,87555	1,07	-4,56	5,20E-06	***
MD	0,06356	0,01113	5,71	1,12E-08	***
FleshInf	6,7904	0,15469	43,9	~0	***
SkinInf	3,54041	0,11359	31,17	~0	***

Table 5. Output of Eq.9 model. AIC index, variance components and significance of fixed effects are indicated with bold letters. Signiicance codes: 0 '***' , 0.001 '**', 0.01 '*', 0.05 '.'.

3.2. Marker transferability and diversity

In order to construct a linkage map for QTL analysis of BR resistance, we initially selected and tested 344 SSR markers from various *Prunus* species, identifying 317 that yielded distinguishable peaks in at least one parent of the CxEL F1 population (table 6). The percentage of heterozygous or monomorphic markers in both parents was 58,8% and 46,8%, respectively, while 38,4% of the tested markers were heterozygous in at least one parent. Peach SSRs compared to SSR derived from other *Prunus* species, produced a higher amplification rate (96% vs. 86%), similar heterozigosity (39% vs. 38%) and higher quantity of markers heterozygous in both parents (or "anchor markers", 17% vs. 8%).

Genomic DNA-derived SSRs, compared to EST-SSRs, showed the same level of amplification (92%), but a higher level of heterozigosity (44% vs. 20%) and a higher quantity of anchor markers (15% vs. 8%).

Ninety-one and 89 SSR markers were heterozygous in 'Contender' and 'Elegant Lady', respectively. Ninety SSRs were genotyped in the CxEL population, but only 78 markers fulfilling our technical requirements (see section 2.5) were used for CxEL map construction. From these, 54 are heterozygous in 'Contender', 50 in 'Elegant Lady' and 25 in both parents simultaneously (hereon named "anchors"), corresponding to nearly one-third of the genotyped markers.

3.3. Linkage analysis

The anchor markers found in the population parents were distributed in the eight *Prunus* linkage groups, based on the marker position tool available on GDR ("search marker" in www.*Rosaceae*.org). This situation allowed the construction of a consensus linkage map for the CxEL F1 population (hereon "CxEL map").

The CxEL map was anchored to the TxE *Prunus* reference map by common markers present in both (figure 12). Tentative positions of markers that were not mapped in TxE population but were mapped in other *Prunus* linkage maps were determined using the GDR comparative mapping tool (CMap, http://www.*Rosaceae*.org/node/41).

The CxEL map covered a total genetic distance of 371,7 cM, corresponding to 73% of the 519 cM covered by the TxE reference map. Thirty-two markers allowed direct alignment to the TxE map (figure 12). CMap allowed approximate localization

SSR Designation	Species	Sequence origin	Tested SSRs	Amplified SSRs	Homozygous	Monomorphic	Heteroz. in C	Heteroz. in EL	Heteroz. in C or EL	Heteroz. in C and EL	References
AMPA	P. armeniaca	EST	10	9	5	5	3	2	4	1	Hagen et al. (2004)
AMPA	P. armeniaca	Genomic	6	6	3	3	2	2	3	1	Hagen et al. (2004)
ASSR	P. armeniaca and P.	EST	8	8	6	6	0	2	2	0	Xu et al. (2004)
BPPCT	P. persica	Genomic	41	40	20	16	15	10	22	5	Dirlewanger et al. (2002)
CPDCT	P. amigdalus	Genomic	24	23	8	7	7	12	15	4	Mnejja et al. (2005)
CPPCT	P. persica	Genomic	28	27	15	15	7	9	12	5	Aranzana et al. (2002)
CPSCT	P. salicina	Genomic	22	21	14	14	4	6	7	3	Mnejja et al. (2004)
EMPA	P. avium	Genomic	10	7	5	5	1	1	2	0	Clarke and Tobutt (2003); Clarke et al. (2008)
EMPaS	P. avium	Genomic	9	8	3	3	3	1	5	0	Vaughan et al. (2004)
EPPCU	P. persica	EST	31	29	24	23	2	5	5	2	Howad et al. (2005)
EPPISF	P. persica	EST	26	23	19	18	4	5	4	3	Vendramin et al.(2007)
LDOX	P. persica	Genomic	1	1	1	1	0	0	0	0	Ogundiwin et al. (2008)
M	P. persica	EST	8	8	5	4	3	1	3	1	Yamamoto et al. (2002)
MA	P. persica	Genomic	29	29	11	9	16	11	18	11	Yamamoto et al. (2002)
PceGA	P. cerasus	Genomic	2	2	2	2	0	0	0	0	Cantini et al. (2001); Downey and Iezzoni (2000)
pchcms	P. persica	EST	3	3	2	0	1	1	1	1	Sosinski et al. (2000)
pchgms	P. persica	Genomic	16	16	8	6	8	2	8	2	Sosinski et al. (2000); Wang et al. (2002); Georgi et al. (2002); Verde et al. (2005)
PMS	P. avium	Genomic	2	2	2	2	0	0	0	0	Cantini et al. (2001)
Prp-SSR	P. persica	EST	3	2	1	1	0	1	1	0	Xu et al. (2008)
PS	P. avium	Genomic	3	1	0	0	0	1	1	0	Sosinski et al. (2000); Joobeur et al. (2000); Cantini et al.
ssrPaCITA	P. armeniaca	Genomic	22	18	10	8	3	9	8	3	Lopes et al. (2002)
UDA	P. amygdalus	Genomic	4	4	2	2	2	0	2	0	Testolin et al. (2004)
UDAp	P. amygdalus	Genomic	16	10	4	4	2	1	6	0	Messina et al. (2004)
UDP	P. persica	Genomic	26	26	14	9	9	9	12	6	Cipriani et al. (1999); Testolin et al. (2000)
Total of P.persica SSRs			212	204 (96,2)	122 (57,5)	102 (48,1)	57 (26,9)	36 (17)	70 (33)	65 (30,7)	
Total of SSRs from other species			132	113 (85,6)	63 (47,7)	59 (44,7)	24 (18,2)	11 (8,3)	32 (24,2)	26 (19,7)	
Total of Genomic SSRs			261	241 (92,3)	126 (48,3)	107 (41)	69 (26,4)	37 (14,2)	89 (34,1)	80 (30,7)	
Total of EST SSRs			83	76 (91,6)	59 (71,1)	54 (65,1)	12 (14,5)	10 (12)	13 (15,7)	11 (13,3)	
TOTAL			344	317 (92,2)	185 (58,8)	161 (46,8)	81 (23,5)	47 (13,7)	102 (29,7)	91 (26,5)	

Table 6. SSR features in the parents of the CxEL population. Numbers in parenthesis represent the percentages of the tested SSRs
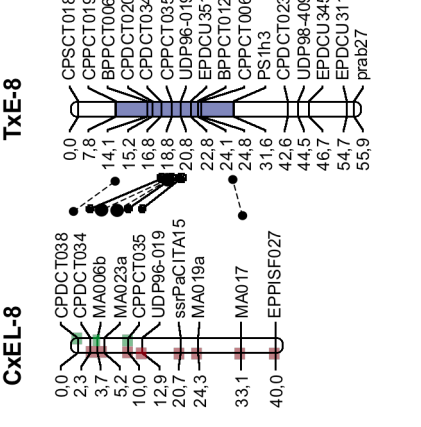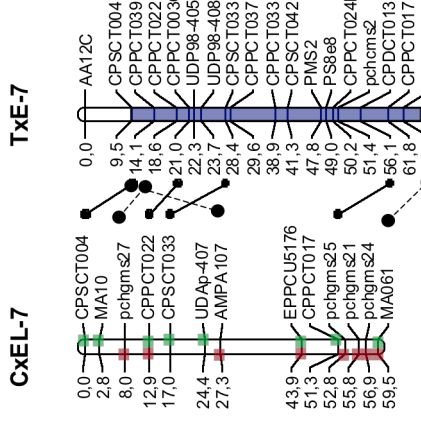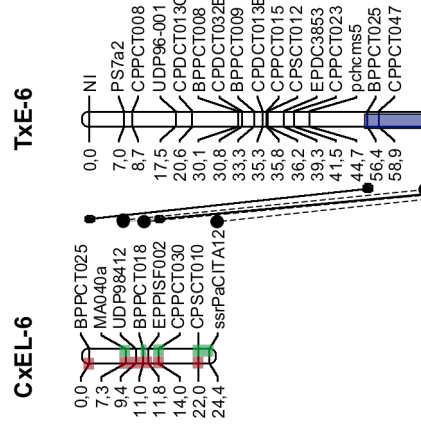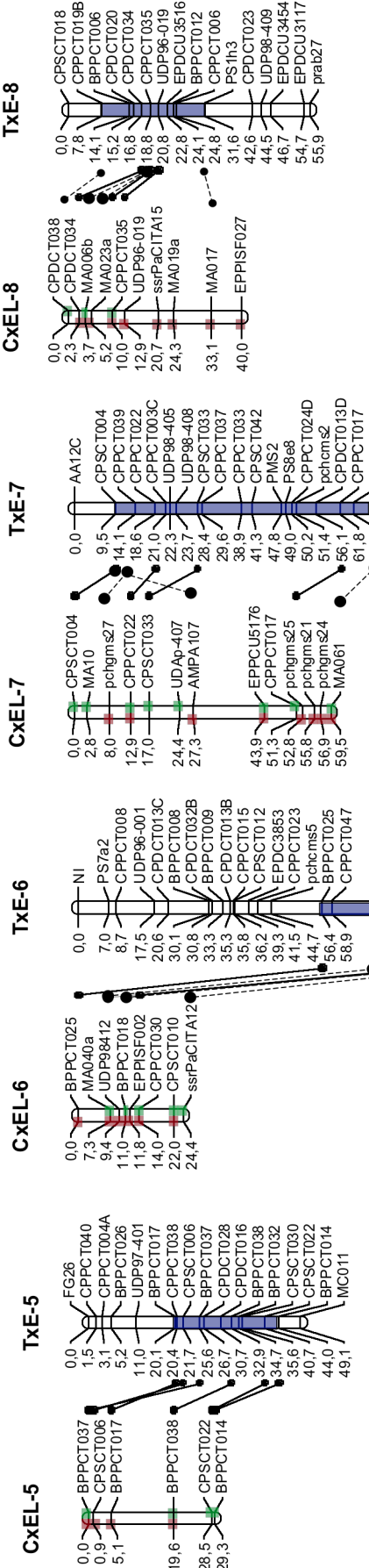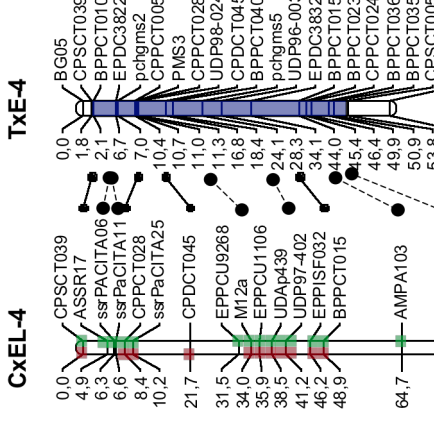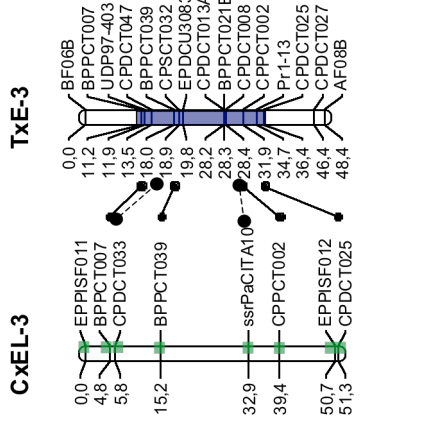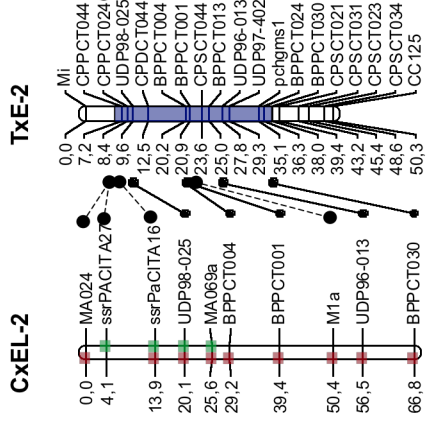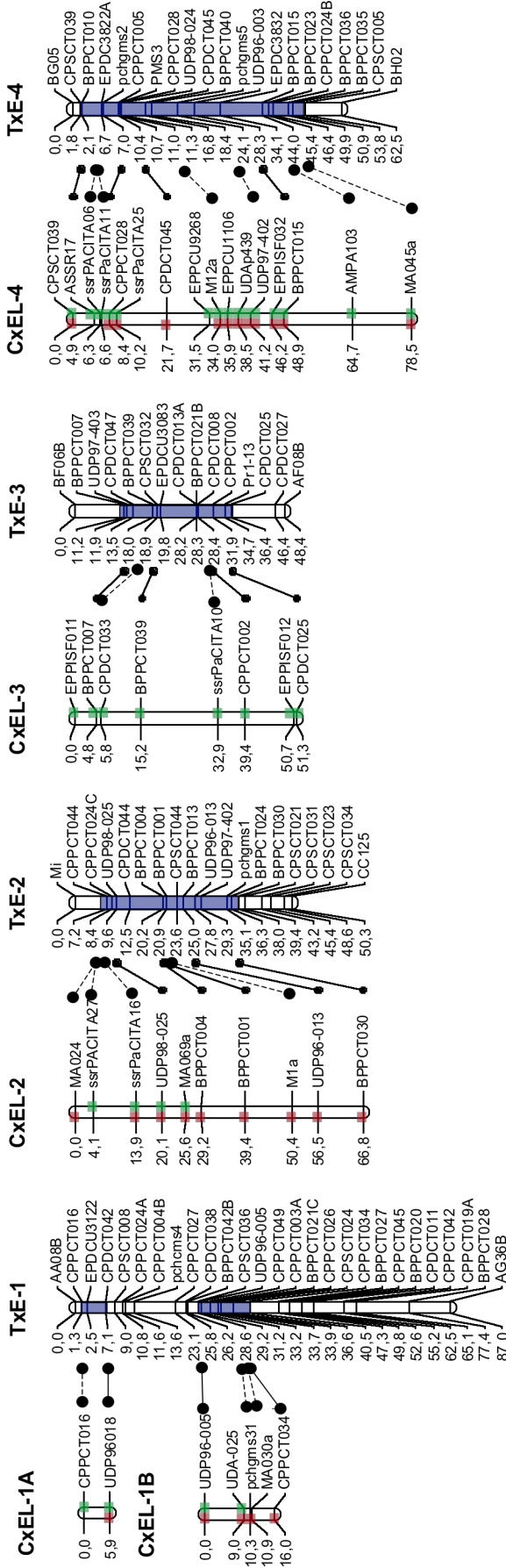
Fig. 12. Genetic linkage map of CxEL F1 progeny anchored to *Prunus* reference map (TxE map, Dirlewanger *et al.*, 2004). The name of each linkage group (LG) is followed by their respective number according with the nomenclature of *Prunus* chromosomes. Markers are listed to the right of each LG and the genetic distances are listed at the left of each one. Heterozygosity of the markers is represented inside the linkage groups by squares of red colour when the marker is heterozygous for 'Contender' parent, or green,when is heterozygous for 'Elegant Lady'. Solid lines are used to anchor common markers of B or O maps with TxE, while dotted lines are used to indicate the tentative position of the markers in the TxE map, estimated using comparative mapping tool (CMap, http://www.bioinfo.wsu.edu/gdr/cmap/index.php). Reference map regions that are covered by the CxEL map are represented by blue colour inside the TxE linkage groups. Presence of segregation distortion with p<0,005 is represented by the symbol "SD" at the left of the corresponding marker.

of 26 markers in the TxE map, while for the remaining 20 markers no precise map information was available in the GDR.

The CxEL map included nine linkage groups instead of the expected eight groups of the TxE map. Two CxEL linkage groups were anchored to separate segments of LG1 of TxE map. These groups were called CxEL-1a and CxEL1b, according to their relative position on the TxE map. Considering this, the groups of CxEL map covered all the eight groups of the TxE reference map. CxEL-3 was the unique group composed by markers that were heterozygous only for 'Elegant Lady', while no heterozygous markers were found in this group for 'Contender'.

Within linkage groups, marker order was conserved between the CxEL and reference maps, with two exceptions: BPPCT037 on group CxEL-5 and AMPA103 on group CxEL-7.

The average density of the map was of 4,7 cM/marker. The genetic distances within pairs of adjacent markers ranged between 0,3 and 17,7 cM (ssrPACITA06 and ssrPACITA11 in CxEL-4, and BPCCT039 and ssrPACITA10 on CxEL-3, respectively). Other gaps longer than 15 cM were found in CxEL-4 (15,8 cM between BPPCT015 and AMPA103) and CxEL-7 (16,6 cM between AMPA107 and EPPCU5176).

A high level of segregation distortion was found for eight markers on the group CxEL-4: M12a (with distortion probability $p<0,05$), CPDCT040 ($p<0,05$), ssrPaCITA10 ($p<0,01$), CPDCT045 ($p<0,01$), BPPCT015 ($p<0,01$), MA015a ($p<0,005$), CPSCT039 ($p<0,001$), AMPA103 ($p<0,0005$), ASSR17 ($p<0,0001$),

CPPCT028 (*p*<0,0001) and ssrPACITA06 (*p*<0,0001). Lower segregation distortion was also noted for markers on group CxEL-3. All these markers were included in the linkage analysis since Joinmap 4.0 performs grouping based on the independence LOD score, which is not affected by segregation distortion (Van Ooijen 2006).

3.4. QTL analysis

Two QTL analysis approaches were used to identify genomic regions underlying variation for BR-resistance in the CxEL F1 progeny: Kruskal-Wallis single marker analysis (KW, figure 13) and regression-based interval mapping approach (IM, figure 14). Results are compared in table 7.

3.4.1. Kruskal-Wallis rank-sum test

QTLs with *p*<0,005 were found near the centre of group CxEL-4 for all the analysed traits in the two years and considering the two BR-resistance scores (figure 13). For both years, MD was found to be strongly associated to all markers inside the EPPCU9268-BPPCT015 interval (*p*<0,0001). All BR-resistance traits showed significant associations with this same MD-linked region.

Trait	Year	LG	KW Marker	KW min p value	PT Genome-wide threshold for p<0,05	IM max. LOD	IM position max. LOD (cM)	IM nearest marker	IM nearest marker position (cM)	IM R² (%)
Ctrl-%if	2009	4	UDP97-402	<0,0005	2,7	2,97	35,9	EPPCU1109	35,9	16,3
	2010	4	-	-	2,8	-	-	-	-	-
Skin-IVF	2009	4	EPPISF032	<0,005	2,5	2,57	46,23	EPPISF032	46,23	14,2
		2	M1 (a)/UDP96-013 (a)	<0,01 (c)		2,28 (c)	56,49 (c)	UDP96-013 (c)	56,36 (c)	12,7 (c)
	2010	4	EPPCU1106	<0,0001	2,7	4,97	33,47	M12a	34	21,4
		2	M1a	<0,005		2,91	50,36	M1a	50,36	13,2
Skin-%if	2009	4	EPPISF032	<0,001	3,3	2,6 (c)	46,24 (c)	EPPISF032 (c)	46,24 (c)	14,4 (c)
		2	UDP96-013	<0,01 (c)		4,2	56,36	UDP96-013	56,49	22,2
	2010	4	EPPCU9268/ EPPCU1106/ UDP97-402 (a)	<0,005	3	6,62	32,47	M12a	34	27,5
		2	M1a/BPPCT030 (a)	<0,01 (c)		3,34	50,36	M1a	50,36	14,9
Flesh-IVF	2009	4	UDAp-439/ EPPISF032 (a)	<0,0005	3,5	6,41	45,24	EPPISF032	46,24	35,2
	2010	4	UDAp-439	<0,001	3,4	6,2	45,24	EPPISF032	46,24	30
Flesh-%if	2009	4	EPPCU1106/ EPPISF032 (a)	<0,005	3	-	-	-	-	-
	2010	4	UDP97-402	<0,001	3,6	5,45	46,24	EPPISF032	46,24	26,9
MD	2009	4	EPPCU9268- BPPCT015 (b)	<0,0001	3,4	15,1	39,5	UDAp-439	38,5	59,5
	2010	4	EPPCU9268- BPPCT015 (b)	<0,0001	3,5	20,3	39,5	UDAp-439	38,5	62,5

Table 7. Summary of the QTLs detected for each scoring dataset by Kruskal–Wallis test (KW) and Interval mapping (IM).
(a) Indicates in KW analysis that the trait is associated to the indicated markers
(b) Indicates in KW analysis that the trait is associated to an interval limited by the indicated markers
(c) The indicated QTL has a significance value under the considered threshold for the trait

One QTL for field infection resistance was found in 2009 near markers UDP97-402 and EPPISF032.

QTLs for skin resistance were detected in the MD-linked region of CxEL-4, and in the distal region of CxEL-2. For the skin-IVF trait QTLs located near markers CPDCT045 and UDAp-439 were detected in 2009, and near EPPISF032 in 2010. A QTL on marker M1a was found on CxEL-2 in 2009, and a weaker association with the trait ($p<0,01$) was found for M1a and UDP96-013 in 2010. Skin-%if QTLs were detected on marker EPPISF032 in 2009, and on EPPCU9268, EPPCU1106 and UDP97-402 in 2010 (CxEL-4); QTLs of low significance for skin-%if ($p<0,01$) were observed near markers M1a and BPPCT030 in 2009 and UDP96-013 in 2010 (CxEL-2).

QTLs for flesh BR-resistance were mapped in the MD-linked region of CxEL-4 on both years and considering the two scores. Flesh-IVF QTLs were detected in both years near markers EPPCU9268, EPPCU1106, UDAp-439, UDP97-402 and EPPISF032, while BPPCT015 was correlated to the trait only in 2009. Similarly, flesh-%if associated in 2009 with markers EPPCU1106 and EPPISF032, while in 2010 was correlated to M12a, EPPCU1106 and UDP97-402.

3.4.2. Interval mapping QTL analysis

Interval mapping QTL analysis is suitable for normally distributed phenotypic data. Although the BR-resistance data did not show normal distributions, IM QTL analysis was performed in order to estimate the explained phenotypic variance of QTLs found by KW. The results of this analysis are detailed in table 7 and figure 14.

In agreement with results of KW analysis, a major QTL for MD trait was found in the central region of CxEL-4, explaining a 62,5% and a 59,5% of phenotypic variance in 2009 and 2010, respectively.

QTLs for all resistance traits were mapped in the MD-linked region on group CxEL-4. The explained phenotypic variance of these QTLs ranged between 16,3% and 35,2%.

A QTL explaining 16,3% of natural infection BR-resistance variance (ctrl-%if) was found only with 2009 data, and located in the interval between markers UDP97-402 and AMPA103 of CxEL-4 group.
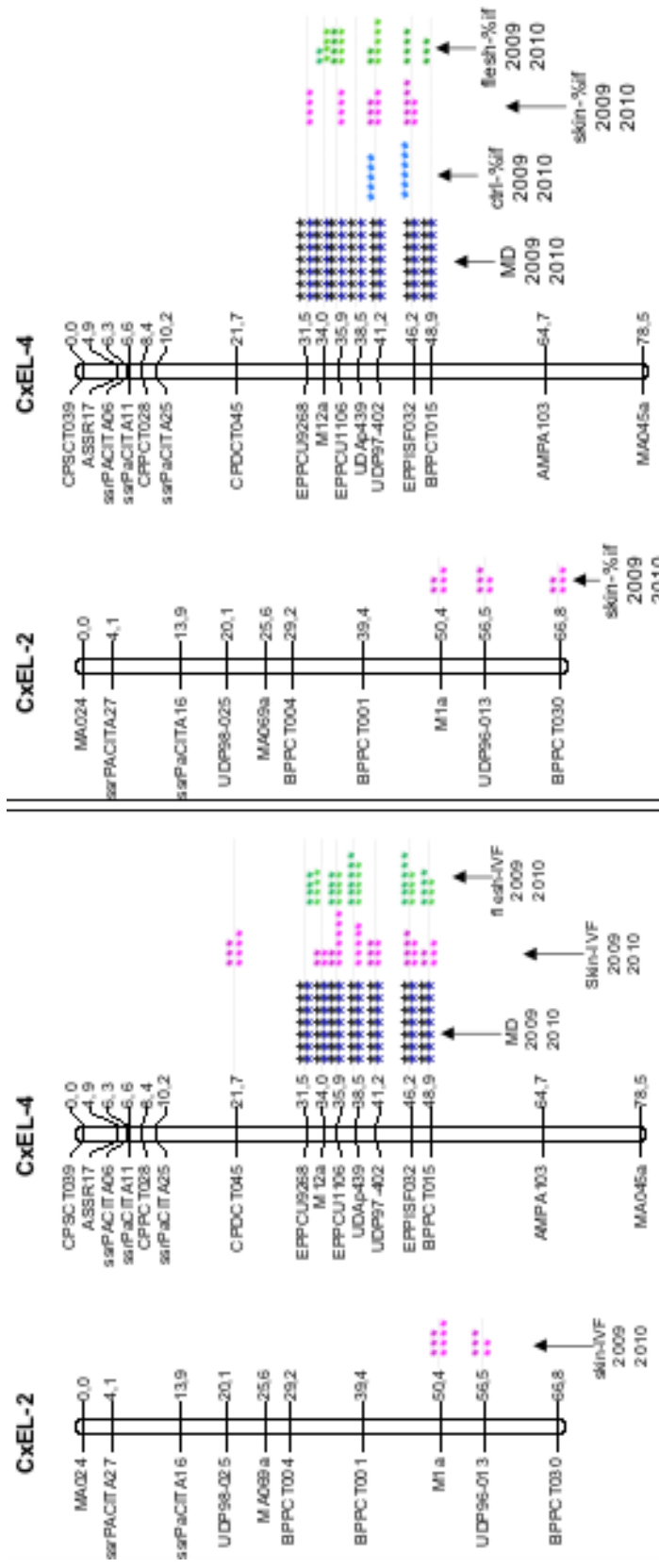
Fig. 13. Kruskal-Wallis single marker analysis of BR-tolerance measured using IVF score (left panel) and %if score (right panel). The names of the linkage groups (CxEL, on the top of each one) are followed by their respective number according with the nomenclature of *Prunus* chromosomes. Markers are listed at the left side of each linkage groups and the genetic distances at the left of each one. Significant marker-trait associations were represented as horizontal asterisks series at the right side of each corresponding marker. Year replicates are represented by asterisks series vertically grouped next to the corresponding marker. Traits are distinguished by the asterisks colour: light-blue for natural inoculum BR-tolerance, magenta for skin BR-tolerance, green for flesh BR-tolerance and black/blue for maturity date. Significance of the associations is represented by the length of the asterisks series: "**********" for p<0,0001, "*********" for p<0,0005, "********" for p<0,001, "*****" for p<0,005, "***" for p<0,001, and "*" for p<0,05.
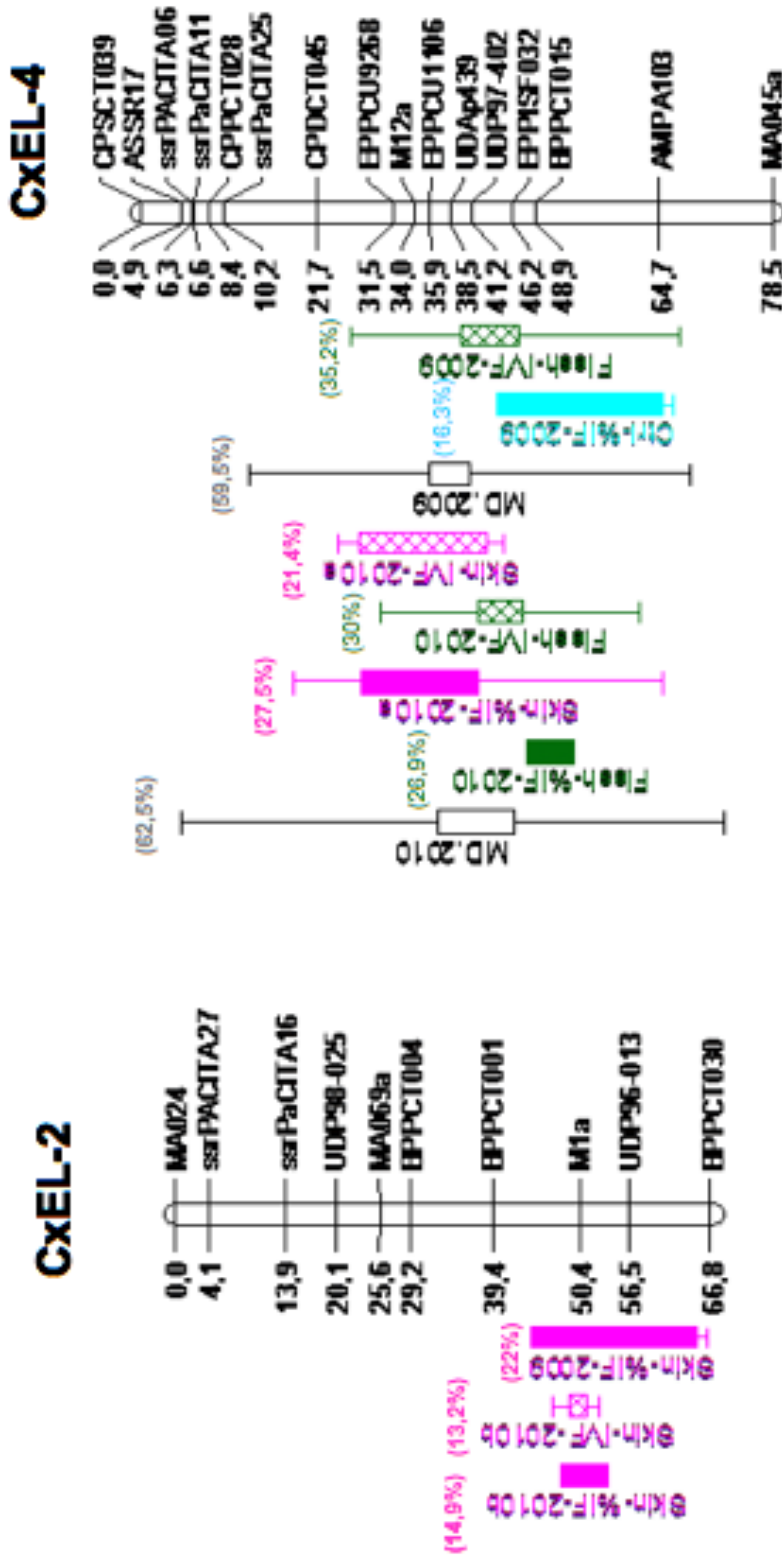
Fig. 14. Location of putative QTLs controlling BR-tolerance, determined by interval mapping. The names of the linkage groups (CxEL, on the top of each one) are followed by their respective number according with the nomenclature of *Prunus* chromosomes. Markers are listed at the right side of each LG and the genetic distances are listed at the left of each one. QTLs are drawn at the left of each corresponding linkage group and were represented in such a way that the thick line represents the "LOD minus one" interval (maximum LOD score minus one) and the thin line represents the whole significance interval of the QTL (see Section 2.7). When the LOD minus one value was minor than the significance LOD threshold, the QTLs were represented with a thick line along the significance interval. The abbreviations of the traits are the same used in the text or in the tables and are followed by the year in which they were taken and by a small letter if a single dataset determined more than one significant QTL (e.g., Skin-IVF-1010a). QTLs for diverse traits are represented with different colours: cyan for natural inoculum BR-tolerance, magenta for skin BR-tolerance, green for flesh BR-tolerance and unfilled for maturity date.

As in KW analysis, two QTL clusters were found for skin resistance, one in the MD-linked region of CxEL-4 and other in the distal region of CxEL-2. Regarding CxEL-4, QTLs in the interval between CPDCT045 and AMPA103 were found only in 2010, explaining 27,5% and 21,4% of the phenotypic variance of skin-IVF and skin-%if, respectively. With respect to the CxEL-2 linkage group, QTLs for skin-%if were detected between BPPCT001 and BPPCT030 markers, explaining 14,9% - 22% of the trait variance. One QTL for skin-IVF was found in the same region only in 2010, explaining 13,2% of the phenotypic variability.

For flesh resistance, three QTLs were detected in CxEL-4, within the MD-associated region (figure 14): two QTLs for flesh-IVF in both years, accounting for 30% - 35,2% of the total phenotypic variability, and one QTL for skin-%if, found only in 2010 between markers EPPISF032 and BPPCT015, explaining 26,9% of the phenotypic variability.

4. Discussion

4.1.   Identification and use of *M. fructigena*.

Using the *Monilinia* spp. identification protocol reported by Hughes *et al.* (2000) we could classify two field-isolates (Arc1 and Arc2) as belonging to *M. fructigena* (figure 9). Isolate Arc1 was then used in artificial inoculations on the CxEL F1 progeny. *M. laxa* has been the most used species in BR-related studies in Europe (Mari *et al.*, 2007; Neri *et al.*, 2007; Bassi and Rizzo, 2003; Thomidis and Michailides, 2010; Casals *et al.*, 2010a; Gibert *et al.*, 2009; Gell *et al.*, 2008; Larena *et al.*, 2005), probably because this species is present in most regions where stone fruit trees are cultivated. *M. fructigena* has been found outside Europe in only a few cases (De Cal and Melgarejo, 1999) and causes brown rot also in pome fruit (Byrde and Willetts, 1977). To our knowledge, the present work is the first using *M. fructigena* to study peach susceptibility in Europe.

4.2.   SSR marker genotyping and linkage map

High levels of SSR transferability have been found in rosaceous species (Mnejja *et al.*, 2010, Gasic *et al.*, 2009). The multiplex-ready strategy used for SSR genotyping allowed screening and mapping of a high number of markers (92% of the screened markers, table 6) with cost- and time-saving efficiency, as previously described in apricot and cherry (Hayden *et al.* 2008a and b). Observed heterozigosity rates for peach-derived SSRs were higher than for other *Prunus*-derived SSRs, and higher for genomic-SSRs than for cDNA-SSRs. This trend has been also reported in other genetic studies in peach (Dirlewanger *et al.*, 2007) and other *Rosaceae* (Mnejja *et al.*, 2010).

Both parents exhibited similar and low heterozigosity values (27% for 'Contender' and 26% for 'Elegant Lady'), a common situation in modern peach cultivars: Aranzana *et al.* (2003-b), using a set of 16 SSRs to genotype 212 commercial peach cultivars, determined an average heterozigosity of 35% (table 6).

A unified map could be constructed for the CxEL F1 progeny (figure 12), due to the high proportion of anchor markers found in the genotyped marker set (32% of 78 SSRs). Anchor markers were found in all CxEL linkage groups (LG), except in CxEL-3 that lacked heterozygous markers in 'Contender'; similarly, heterozygous markers in 'Elegant Lady' covered only partially CxEL-2 and CxEL-8. The coverage observed

in CxEL (74%) is inferior compared to other intra-specific peach linkage maps where a similar or higher genetic distance was covered relative to the TxE map (Yamamoto *et al.* 2005; Dirlewanger *et al.* 2007; Blenda *et al.* 2007). It should be noted that is common to observe complete absence of entire LGs in peach genetic maps (*e.g.*, G8 in Dirlewanger *et al.* 2007 and G7 in Blenda *et al.* 2007). The lack of coverage of extensive chromosomal segments is due to homozygosity/homomorphism of genomic regions, which were likely fixed during the breeding process because of the recurrent use of the same genetical background of the parents used in this work. For instance, "J.H. Hale" accession is present five and three times in 'Contender' and 'Elegant Lady' pedigrees, respectively (figure 8). The case of 'Contender' and 'Elegant Lady' is an example of the "identity by descent" phenomenon, also reported in peach by Illa *et al.* (2009), that could explain the narrow genetic variability in modern peach cultivars (Scorza *et al.*, 1985).

In the regions covered by the CxEL map, marker density was 4.7 cM/marker, with three not covered gaps (in LGs CxEL-3, CxEL-4 and CxEL-7). Considering that the gain in QTL precision is not proportional to the increase of the marker density beyond 10–15 cM (Darvasi *et al.* 1993; Kearsey and Farquhar 1998), we considered this map saturated enough for QTL detection.

Strong segregation distortion was found in LGs CxEL-3 and CxEL-4. Clustering of these markers can be interpreted as the effect of sublethal genes that may be present in the corresponding regions. Markers along the entire LG CxEL-4 displayed segregation distortion with $p<0,05$. In this case, based on parental allele distribution, we observed that all the under-represented alleles in the progeny came from 'Contender'.

4.3.   Correlations and variance components of traits

MD showed significant correlation between the two seasons analysed (92% with $p<0,0005$, table 4). This high correlation suggests that a major genetic component in variance for this trait could be found in the considered cross. MD positively correlated in both years with all BR-resistance traits: correlation between control-%if and MD (34% with $p<0.005$) is consistent with the hypothesis that earliness is proportional to field-pathogen avoidance (Topp *et al.*, 2008). Artificial inoculation traits (skin and flesh infections) are also correlated with MD, suggesting that ripening time could have an effect on BR-resistance. Using an F2 derived from the cross 'Contender' x

'Ambra', Eduardo *et al.* (in press) have found strong correlation of MD data between two years (94%), as well as correlation with fruit quality traits. Other examples correlating MD and other fruit traits have been reported for *Prunus* interspecific crosses (Quilot *et al.*, 2004) and apple (Kenis *et al.*, 2008).

Skin-IVF and Flesh-IVF correlated between the two years, with values of 29% (p<0,05) and 39% (p<0,005), respectively, suggesting a significant genetic component controlling these traits.

In both years, correlations were detected between skin-IVF and flesh-IVF values (55% and 47% in years 2009 and 2010, with p<0.0005), consistent with previous results indicating a significant correlation between infection area in wounded and not wounded apricots infected by *Monilinia* spp. (47% with p<0,01) (Walter *et al.*, 2004). In contrast, Pascal *et al.* (1994) observed that the rankings of the genotypes according to the two tests were not correlated. Walter *et al.* (2004) discussed that this difference in the results was due to technical differences inherent to inoculation procedures with Pascal *et al.* (1994). Instead, in the work of Pascal *et al.* (1994) the mentioned "low correlation" is referred only to observed discrepancies in the ranking of the genotypes after each treatment, and not to a linear regression-based determination of correlation of each genotype between two treatments. The presented results of Walter *et al.* (2004) agree with our observed correlation between skin- and flesh-resistance, suggesting the existence of common resistance mechanisms acting in both tissues.

Genetic and seasonal components of BR variability in CxEL have been estimated as 51,5% and 33,5%, respectively; using generalized linear models (logistic regression function), since the analysed traits are non-normally distributed (parametric tests such as ANOVA precisely estimate variances on normally distributed datasets). A complementary approach based on data transformation of BR-resistance traits aimed to obtain normal distributions and proceed with parametric analysis of variance did not give positive results (data not shown). The logistic regression method has been successfully used in a germplasm collection of *Saccharum* spp. to determine variance components of diverse morphological traits (Balakrishnan *et al.*, 2000).

4.4. Specific QTL clusters for flesh and skin BR-resistance.

Similar results were obtained with parametric (interval mapping QTL analysis, IM) and non-parametric (Kruskal-Wallis test, KW) analyses for both years of phenotypic analysis (figures 13 and 14).

QTL analyses showed that peach skin and flesh resistance are associated to different QTL patterns that share one MD-QTL as a common element. Two QTL clusters for skin resistance were found: one in the LG CxEL-2 and the other in CxEL-4 that collocates with the MD-QTL. The QTL cluster detected for flesh resistance also collocates with the MD-QTL. These QTL patterns were stable over two years of phenotypic evaluation in some traits, *e.g.* lesion volume in flesh infections (flesh-IVF) and percentage of infected fruits in skin infections (skin-%if; figures 13, 14 and table 7).

The high-significance QTL for maturity date found in CxEL-4 explained around a 60% of the phenotypic variability (figure 14). As MD did not distributed normally, the reliability in the explained phenotypic variability value of QTL cannot be ensured, although this value was quite similar on both years (59,5% in 2009 and 62,5% in 2010) and showed a high seasonal correlation (92%). This QTL showed maximum LOD scores near marker UDAp-439, concordant with the MD-QTL found in Eduardo *et al.* (in press) that located between markers M12a and EPPISF032, in a map based on the 'Contender' x 'Ambra' F2 intra-specific peach progeny (CxA). In our map, UDAp-439 is situated between these two markers. Moreover, this trait behaved as a mendelian gene (explaining almost 100% of the variability), allowing to be used as a codominant marker and thus to directly determine its position in CxA (Eduardo *et al.*, in press). Similarly, in advanced peach x *P. davidiana* crosses, Quilot *et al.* (2004) detected a MD-QTL explaining a 39% of the phenotypic variability, on the centre of the group 4. In apple (another *Rosaceae* fruit crop) Kenis *et al.* (2008) located a QTL explaining between an 11% and 18% of the variability of the MD trait in the LG 10 of *Malus*, a LG that has been shown to be syntenic with LG 4 of *Prunus* (Dirlewanger *et al.*, 2004-a).

4.4.1. Skin resistance QTLs

Skin-resistance can be considered as the resistance to fungal penetration to the fruit, as is the seedling-specific rate of infected fruits. The QTL detected on CxEL-2 (more frequently between M1a and UDP96-013 markers) for skin-%if and skin-IVF

can be attributed to skin factors that avoid the fungal penetration to the fruit. Skin resistance factors have been previously reported to be accounting for the high BR-tolerance in peach Brazilian cultivar 'Bolinha', *e.g.*, the compact arrangement of epidermal cells, thicker cuticle, fewer trichomes and higher phenolics content (Feliciano *et al.*, 1987; Bostock *et al.*, 1999; Gradziel *et al.*, 2003).

Few traits have been genetically associated with this region of the *Prunus* chromosome 2 in the literature. In the "*Prunus* resistance map" (Lalli *et al.*, 2005), two resistance gene analogs (RGAs) have been anchored to LG 2. One of these RGAs is situated in the lower region of TxE-2 linkage LG (marker AC19, 10cM under UDP96-013), collocating with a QTL for powdery mildew resistance (Foulogne *et al.*, 2003). However, RGAs mapped in Lalli *et al.* (2005) correspond to genes encoding for proteins containing nucleotide binding site-leucine rich repeat (NBS-LRR) domain, which have been related predominantly to the regulation of resistance responses to biotrophic pathogens (Jones and Dangl, 2006), that are different to the responses against necrotrophic pathogens (Broekaert *et al.*, 2006). Similar to our results, a PPV-resistance QTL has been detected to collocate with marker M1a in *P. davidiana* P1908 linkage LG 2 (Rubio *et al.*, 2010).

A possible relationship between fruit size and BR resistance has been suggested, since in peach bigger fruit has a thinner skin than small fruits (Bassi and Rizzo, 2003). Also, an important QTL cluster containing QTLs for fruit size and fruit cell number has been detected in the centre of LG 2 of sweet cherry (Zhang et al., 2009), and could be.

The markers in the skin-%if QTL of the LG CxEL-2 (M1a and UDP96-013) show similar genotype classes in the parents (lm x ll type, table 3). After IM QTL analysis, the group of seedlings containing the "ll" allele in M1a were indicated as being the more resistant. However, despite a QTL was detected, those markers do not allow to resolve which allele ('Contender' or 'Elegant Lady') has a greater effect in the BR-tolerance phenotype.

Search for more codominant markers in order to get more information about the segregation of the alleles involved in the expression of this QTL, coupled with studies on F2 populations to estimate the dominance and/or epistasis effect are needed to reach a higher knowledge about the genetic control of BR resistance encoded by this QTL.

Moreover, the recent release of the first version of peach genome (available at http://www.*Rosaceae*.org/peach/genome) can be a useful tool in searching candidate genes in the BR-resistance QTL regions and designing SNP markers in order to saturate these regions.

4.4.2. Resistance QTLs collocating with MD

Flesh resistance co-located with the mentioned MD-QTL of LG CxEL-4. On both years, KW analysis showed significant associations of flesh-IVF trait with the markers inside the EPPCU9268-EPPISF032 interval (p<0.005) and IM detected a QTL with maximum LOD value near EPPISF032 marker, explaining 35.3 and 30% of the phenotypic variance in 2009 and 2010, respectively (table 7, figures 13 and 14). Additionally, QTLs for skin-IVF and skin-%if have been detected in this interval but only for one year.

The observed collocations of BR-resistance with MD-QTL are supported by the significant correlations between these traits, mentioned in section 4.2 (table 5).

Two possible explanations can be given to this collocation: i) the QTLs controlling MD and flesh-resistance are tightly linked, or ii) the QTL controlling MD has a pleiotropic effect over flesh resistance. The tight-linkage hypothesis may be tested by further saturation of the MD-QTL region, with more markers and testing more individuals. Also QTL analysis in F2 populations would help to clear this hypothesis.

The MD pleiotropic effect explanation agrees with published works cited before in *Prunus* and *Malus* species (Eduardo *et al.*, in press; Quilot *et al.*, 2004; Kenis *et al.*, 2008), since fruit quality QTLs have been often found to collocate with fruit MD-QTL. The QTL analyses of CxA F2 population show that QTLs for all the studied fruit quality traits collocate with MD-QTL (Eduardo *et al.*, in press). QTL controlling diverse chilling-injury (CI) susceptibility traits have been mapped in an MD-QTL analogous position of linkage LG 4 of a 'Venus' x 'Big Top' F1 population (VxBT-4, Cantín *et al.*, 2010). For both mealiness and graininess susceptibility traits, the authors found three QTL peaks, inside a 39.5 cM interval in the centre of VxBT-4, mapping near a QTL for harvest date. One of these peaks collocated with the *endoPG* marker (Morgutti *et al.*, 2007), which did not segregate in our CxEL F1 progeny (data not shown).

Flesh-IVF trait can be considered as the resistance to fungal spread after skin penetration, because measures the rotting speed (infection volume in a given period)

of an already penetrated pathogen. Ripening is the summation of biochemical and physiological changes that occur at the final stage of fruit development (Giovannoni, 2001). BR-resistance decrease in stone fruit between pit hardening and physiological maturity (Biggs and Northover, 1988; Mari *et al.* 2003; Fourie and Holz, 2002) has been suggested to be due to a concomitant drop in the fruit phenolics content (Lee and Bostock, 2006 and 2007). According to our results and the results presented in the literature, we suggest that the relationship between maturity date and flesh BR-resistance in CxEL can be due to the difference between cultivars in which in their fruit barriers change along ripening process. Experiments measuring phenolic compounds content (*e.g.*, caffeic and chlorogenic acid) at equivalent maturity levels on CxEL seedlings that present contrasting BR-tolerance could eventually confirm this hypothesis. Another hypothesis that could explain this relationship between MD and flesh resistance could be due to differences in CxEL in their susceptibility to micro-cracks generation, phenomenon that has been reported to be proportional to the fruit growth velocity in the last stages of ripening (Gibert et al. 2005 and 2009).

5.   Conclusions and future prospects.

The current work presents a genomic dissection of the inheritance of brown rot (BR) resistance factors, based in an F1 progeny from the peach interspecific cross between 'Contender' x 'Elegant Lady' (CxEL) cultivars, differing for BR-resistance.

A linkage map was constructed, containing 78 SSR markers that partially cover the eight *Prunus* chromosomes in a total genetic distance of 371.7 cM.

A *Monilinia fructigena* strain was isolated, identified and used in the fruit inoculation. Phenotypic analysis was performed over two harvest seasons, by an artificial inoculation procedure that measured skin and flesh resistance to BR. Significant correlations were found between the data obtained in both years of and between the two traits. Maturity date was found to be highly correlated with all the resistance traits.

QTL analysis using genotypic and phenotypic data from CxEL revealed two QTL clusters, one associated skin resistance associated QTL on the LG CxEL-2, and a flesh resistance associated QTL, collocating with a strong MD-QTL on the LG CxEL-4. These results suggest that resistance to BR has at least two main components: one responsible for the fungal penetration avoidance, and an earliness-associated factor responsible for fungal spread after penetration.

The regions defined in this analysis contain markers that will serve as a starting point for QTL validation in other available populations (F2 from CxEL individuals, and other crosses segregating for BR-resistance) and the narrowing of the discovered QTL regions by the use of the recently released peach genome sequence. Markers M1a (CxEL-2) and UDAp-439 (CxEL-4) may be also considered as strong candidates for its use in marker assisted selection (MAS) for BR-resistance breeding in peach (and *Prunus*) experimental crosses.

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