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# Study of the genetic variability of peach in susceptibility to brown rot during fruit development in relation with changes in physical and biochemical characteristics of the fruit

Leandro de Oliveira Lino

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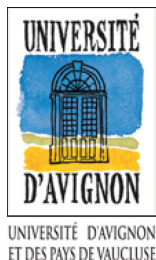
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ACADEMIE D'AIX-MARSEILLE  
UNIVERSITE D'AVIGNON ET DES PAYS DE VAUCLUSE

# THESE

Présentée pour obtenir le grade de **Docteur en Sciences**  
De l'Université d'Avignon et des Pays de Vaucluse

Spécialité : **Sciences Agronomiques**

Etude de la variabilité génétique de la sensibilité à la  
pourriture brune au cours du développement du fruit  
chez la pêche en lien avec l'évolution des  
caractéristiques biochimiques du fruit

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# INTRODUCTION GENERALE

## 1 Contexte de la thèse

### 1.1 Production mondiale et importance de la pêche pour la France et pour le Brésil

Les fruits à noyau sont importants dans l'économie agricole de nombreux pays, la production mondiale de pêches et nectarines était de 21,6 kilotonnes en 2013. La production de pêche rentre parmi les dix plus grandes productions mondiales de fruits (Statista 2013). La France est le 10<sup>ème</sup> pays producteur dans le monde et la pêche est le 5<sup>ème</sup> fruit le plus consommé dans le pays (Szódi and Rozsnay 2008). Le Brésil est parmi les trois premiers producteurs de fruits du monde (Figure 1) mais seulement le 12<sup>ème</sup> producteur mondial de pêches avec une production moyenne de 217ktonnes en 2013, très proche de celle de la France (233 Ktonnes) (FAOSTAT).

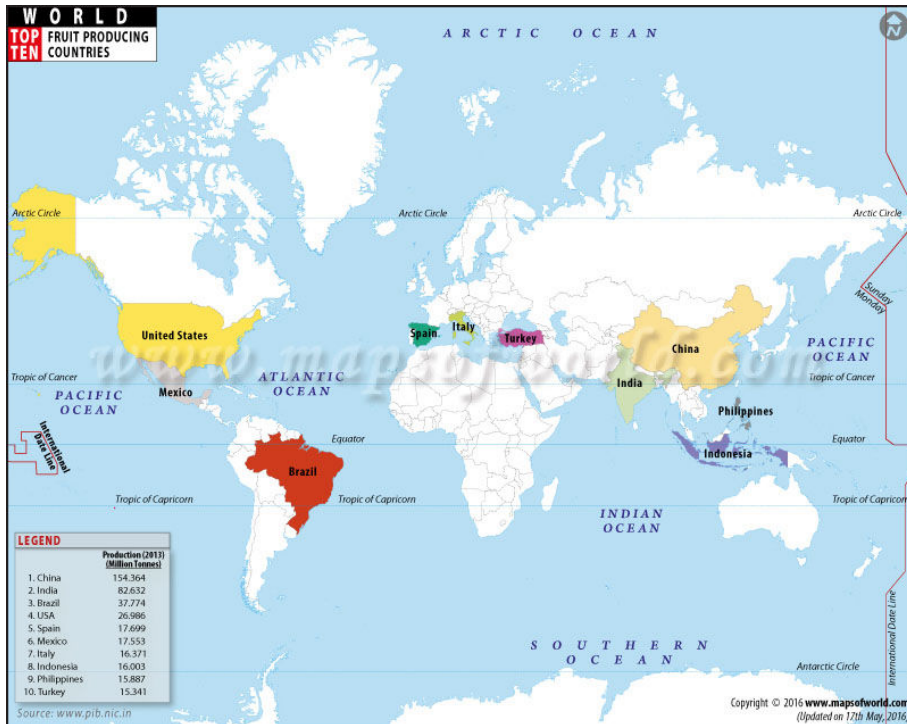
Les principales régions de production brésiliennes de pêches se trouvent au sud du pays et sont caractérisées par de fortes précipitations et une humidité relative élevée qui favorisent l'apparition de ravageurs et de maladies comme les monilioses (Fachinello, Tibola et al. 2003).

### 1.2 Caractéristiques du Pêcher

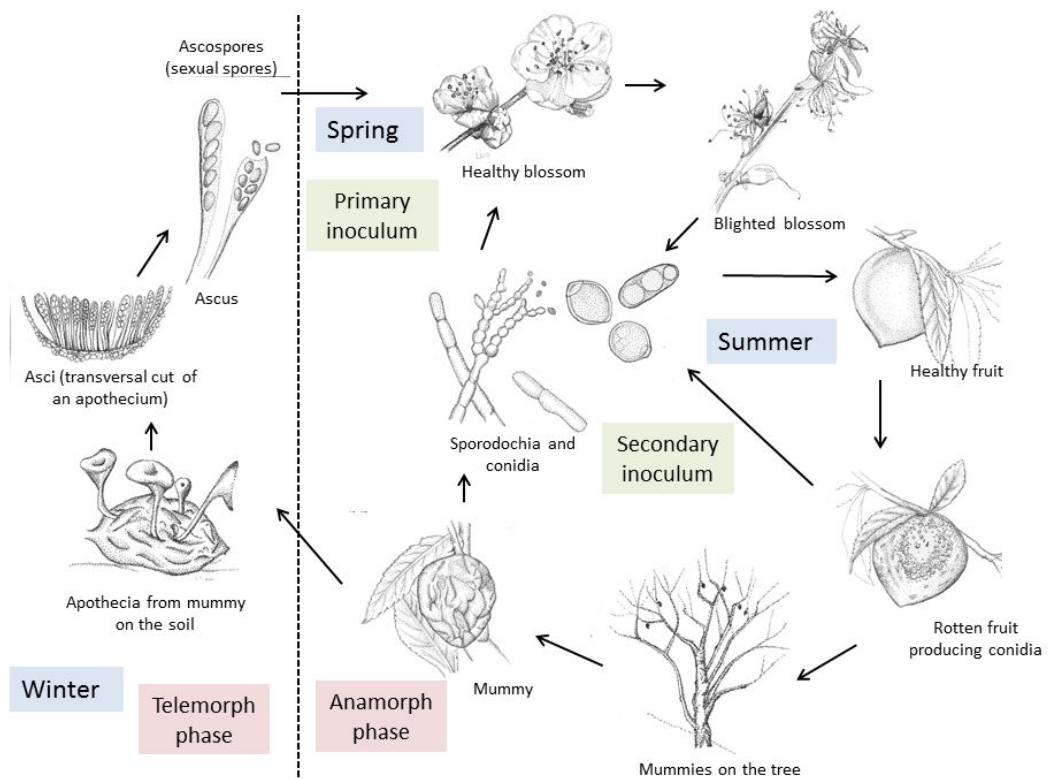
Le pêcher, *Prunus persica* L. Batsch, appartient à la famille des rosacées et au genre *Prunus* qui compte plusieurs autres espèces fruitières à fort intérêt agronomique tels que les pruniers (*P. salicina* et *P. domestica*), les cerisiers (*P. avium* et *P. cerasus*) et l'abricotier (*P. armeniaca*) (Aranzana, Abbassi et al. 2010). Le pêcher est l'espèce de ce genre la plus importante économiquement.

Il existe des centaines de variétés de pêcher que l'on peut classer selon le type de fruit. On distingue : i) les fruits à noyau libre : pêche (duvet) et nectarine (sans duvet); ii) les fruits à noyau adhérent : pavia (duvet, utilisée pour l'industrie des fruits au sirop); iii) les brugnons (sans duvet) et les pêches plates. Tous ces fruits peuvent avoir la chair blanche, jaune ou sanguine.

Le fruit du pêcher est une drupe résultant du développement et de la différenciation des parois de l'ovaire (péricarpe) en 3 zones: l'épicarpe, pubescent chez les pêches et glabre chez les nectarines, le mésocarpe (chair), charnu et l'endocarpe qui se lignifie en un noyau dur (Zucconi 1986). La croissance du fruit en diamètre est souvent décrite (Gage and Stutte 1991) comme suivant une double sigmoïde subdivisée en trois phases (Connors, 1919). La phase initiale, exponentielle, correspond à une période de fortes divisions cellulaires; la seconde, caractérisée par une croissance ralentie du mésocarpe, commence avec la lignification du noyau (Chalmers, Canterford et al. 1975). Durant cette phase, la croissance en diamètre est faible mais la masse continue à augmenter; la troisième, exponentielle, est une période de forte expansion des cellules du mésocarpe.



**Figure 1 :** Distribution mondiale des dix principaux pays producteurs de fruits.



**Figure2 :** Résumé des principaux stades du cycle de vie de *M. laxa* au verger.

Le pêcher (*Prunus persica*) est désormais considéré comme l'espèce Rosaceae la mieux caractérisée d'un point de vue génétique. Le pêcher est diploïde, ne possède que 8 chromosomes ( $2n=16$ ) et a un génome de petite taille estimé à 265 Mpb très peu remanié au cours de l'évolution. De plus il a une période juvénile relativement courte (2 à 3 ans) comparée aux autres arbres à fruits (6 à 10 ans). La séquence du génome du pêcher (Verde, Abbott et al. 2013), de très bonne qualité est disponible depuis 2010. L'accès à la séquence du génome et aux données produites à partir de la séquence, sont en accès libre sur le site 'The Genome database for Rosaceae' (Jung, Ficklin et al. 2014). Par les nombreux avantages qu'il présente, il peut prétendre au statut d'espèce modèle pour les Prunus voire même les Rosaceae et plus généralement les espèces pérennes fruitières.

Comme tout végétal, le pêcher est attractif pour différents organismes. L'arbre est attaqué par de nombreux insectes ravageurs tels les pucerons, souvent vecteurs de maladies par les piqûres qu'ils causent. Le pêcher est aussi sensible à de multiples microorganismes comme des bactéries de la famille des Pseudomonas, des virus dont le plus connu est la Sharka, ainsi que divers champignons. Pour pallier une base génétique restreinte, les améliorateurs utilisent la diversité d'espèces apparentées, sauvages ou commerciales. Mais les programmes de sélection du secteur privé se sont longtemps concentrés sur les aspects agronomiques tels que la date de maturité ou le rendement.

L'importance économique du pêcher, sa bonne caractérisation au niveau génétique et les enjeux majeurs autour des problématiques liées à la réduction des intrants, et aux changements climatiques font du pêcher un objet de recherche très pertinent.

### **1.3 La Pourriture Brune**

Provoqué par le champignon polytrophe appartenant au Genre *Monilinia*, la pourriture brune est aussi connue sous le nom de moniliose. Ces champignons peuvent provoquer le dessèchement des rameaux, fleurs et la pourriture des fruits (Lesik 2013). Les monilioses sont les principales maladies fongiques aériennes dommageables pour les arbres fruitiers à noyau et notamment le pêcher.

Chez le pêcher et dans toutes les espèces cultivées du genre Prunus, cette maladie est un grave problème pour les producteurs à la fois avant et après la récolte. La période relativement longue d'incidence, de la floraison à post-récolte, l'apparition de résistances aux fongicides et la faible disponibilité de résistance de l'hôte, entraîne de graves pertes (Fan, Guo et al. 2010). De cette façon, des lourdes pertes ont été signalées en Amérique du Nord sur les pêches, cerises et prunes, et des pertes annuelles sont estimées à 1 million AUD pour la pêche et l'abricot en Australie (Hrustić, Mihajlović et al. 2012).

Les monilioses peuvent entraîner des pertes de fruits de plus de 60% après 5 jours d'infection à température ambiante (Tosi, Spada et al. 1996). Dans les vergers bio des fruits à noyau, la pourriture

est un facteur majeur limitant du rendement difficile à contrôler (Larena, Torres et al. 2005). Dans une étude menée dans les vergers de pommiers organiques, les pertes de rendement étaient de l'ordre de 41,6% au stade pré-récolte, et 80% dans la phase post-récolte, durant laquelle un facteur aggravant était la présence de lésions à la surface des fruits (Holb 2004).

Plusieurs espèces de *Monilinia* ont été déjà identifiées. En France, jusqu'à récemment, *M. laxa* était l'espèce la plus présente et celle causant le plus de dégâts. Cependant *M. fructicola*, apparue en France en 2001, semble se répandre rapidement sur le territoire français. Elle a d'ailleurs été supprimée en 2015 de la liste des organismes de quarantaine pour la France. Ainsi, de nos jours la distribution des différentes espèces n'est pas restreinte à un seul continent, par exemple *M. laxa* a aussi été identifiée pour la première fois au Brésil en 2008 (Souza, Fazza et al. 2008), pays où il y a une prévalence de *M. fructicola*. Plus de détails à propos des espèces, leur distribution et identification sont donnés au chapitre 1.

Les monilioses réalisent leur cycle de vie au verger, alternant une phase de reproduction sexuée et asexuée durant une année (Figure 2). Elles peuvent passer l'hiver au verger sous forme de momies. Les fruits sont résistants au moment de la lignification du noyau et sont sensibles le reste du développement.

Pour limiter l'impact des monilioses au verger, les mesures prophylactiques habituelles (élimination des foyers infectieux, ainsi que des fruits pourris) sont insuffisantes. Le principal moyen de contrôler la pourriture brune est l'utilisation de fongicides en pré-récolte (Danner, Sasso et al. 2008).

#### **1.4 Traitement chimique – les conséquences pour la santé humaine et environnement**

Les maladies fongiques dévastatrices pour les cultures horticoles peuvent provoquer tellement de pertes de récolte qu'elles obligent les producteurs à mettre en œuvre une lutte chimique préventive généralisée (McConnell, Wightwick et al. 2003), (Wightwick, Walters et al. 2010). Dans les vergers de pêches commerciales, la gestion des maladies avec des fongicides peut être divisée en trois périodes de contrôle : période de dormance en sortie d'hiver, floraison au printemps et maturation des fruits avant la récolte. Au total, deux à trois applications, en utilisant généralement des fongicides systémiques ou spécifiques au site, sont effectuées au cours de chacune de ces périodes (Hamilton, Heckman et al. 2013), (Lalancette, Gager et al. 2015).

Cependant, l'utilisation régulière de fongicides présente un risque important pour l'environnement. Les résidus persistent dans le sol, migrent et finissent par rejoindre les cours d'eau (Gevao, Semple et al. 2000; Komárek, Čadková et al. 2010), entraînant des effets néfastes pour la santé des écosystèmes terrestres et aquatiques. Par exemple, des préoccupations ont été soulevées



sur l'utilisation à long terme des fongicides à base de cuivre, qui peut entraîner une accumulation de cuivre dans le sol (Komárek, Čadková et al. 2010). Cela peut avoir des effets néfastes sur les organismes du sol (par exemple les vers de terre, micro-organismes) et pose un risque potentiel pour la fertilité à long terme du sol (Wightwick, Walters et al. 2010). La lutte chimique peut également avoir des conséquences néfastes pour la santé humaine. Son utilisation excessive peut entraîner la présence de résidus sur les fruits et altérer la santé des consommateurs.

Le risque majeur est encouru par les travailleurs du secteur agricole (Remor, Totti et al. 2009) et par les habitants résidants près des vergers traités. Ainsi il semblerait que les vigneron risquent de développer de graves problèmes respiratoires chroniques du fait de l'inhalation fréquente de fongicides contenant du cuivre (Zuskin, Mustajbegovic et al. 1997), (Komárek, Čadková et al. 2010). Enfin, il existe un risque toxicologique pour la population lorsque les pesticides contaminent les sources de captation d'eau proche des vergers. Les risques des pesticides pour la santé humaine ont été l'objet de nombreuses études et revues bibliographiques (Blair, Dosemeci et al. 1993), (Kamel and Hoppin 2004), (Santić, Puvacić et al. 2005).

Par ailleurs, l'émergence de pathogènes résistants à certains principes actifs a déjà été observée chez les monilioses. La réglementation européenne en matière d'utilisation de produits phytosanitaires se durcit et il en est ainsi également de politiques nationales sur d'autres continents. Afin d'assurer la durabilité des systèmes de production horticole, notamment du pêcher, un équilibre doit être trouvé entre la maîtrise des risques de maladies fongiques et la protection de la santé humaine et des écosystèmes terrestres et aquatiques.

Des travaux ont montré que les pratiques culturales influent sur les performances sanitaires du verger. Ainsi certaines pratiques culturales peuvent permettre de réduire l'impact des monilioses. Par exemple il est désormais bien connu dans la littérature qu'un fort éclaircissage des arbres et une irrigation abondante sont des conditions très favorables au développement de l'infection (Gibert, Chadœuf et al. 2007), (Mercier, Bussi et al. 2009). Récemment une autre étude menée par Bussi, Plenet et al. (2015) a montré que la forme des arbres et la taille sont susceptibles de diminuer l'incidence des monilioses en verger de pêcher.

Ces opérations culturales, principalement l'éclaircissage et l'irrigation, affectent la croissance du fruit (Berman and DeJong 1996). Ces deux opérations sont également à l'origine de désordres physiologiques qui apparaissent sur l'épiderme du fruit, dont les fissures cuticulaires. Les opérations modifiant l'architecture de l'arbre ont pour conséquence de modifier le microclimat des fruits et donc de moduler le développement de l'infection. Cependant, le seul levier agronomique ne paraît pas suffisant pour atteindre les objectifs des politiques publiques en termes de réduction des intrants. L'association du levier génétique avec le levier agronomique apparaît comme le moyen de répondre aux enjeux de demain. Aussi devient-il urgent de développer des variétés résistantes, ou

tout au moins peu sensibles, à ces champignons. Dans cette étude, nous focaliserons sur le levier génétique.

## **2 Les mécanismes de résistance**

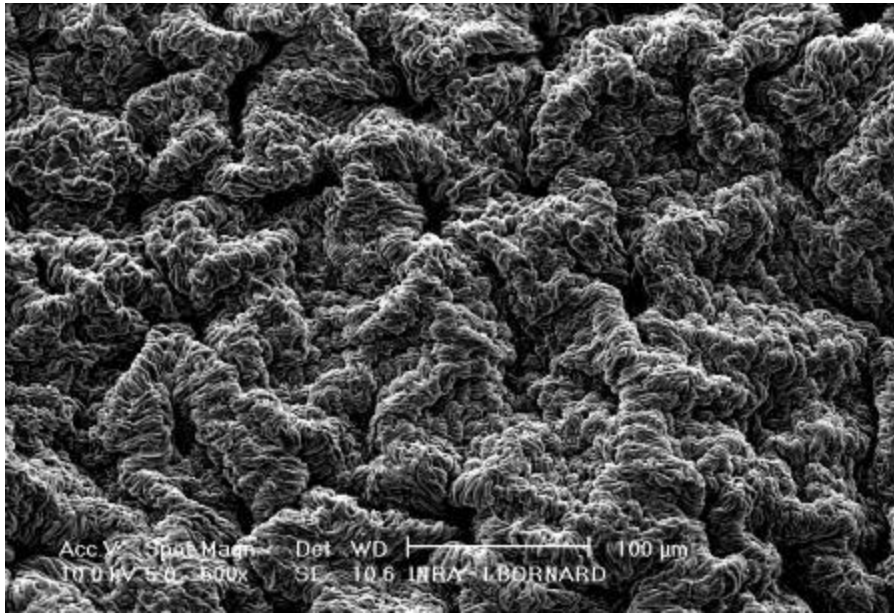
Il semblerait que la résistance du fruit aux monilioses soit essentiellement liée à la peau du fruit. Une preuve en est, le travail de (Pascal, Levigneron et al. 1994), qui montre qu'il n'y a aucune variabilité de progression de l'infection entre fruits de différentes espèces de *Prunus*, lorsqu'on infecte des fruits blessés. La pourriture brune est une maladie déjà étudiée depuis plus de 150 ans en Europe et 100 ans en Amérique. Cependant les facteurs majeurs de la résistance des fruits, et les stratégies adoptées par le champignon pour pénétrer dans le fruit, reste encore mal connus.

On peut classer deux types de défenses, la défense passive qui correspond à un processus constitutif, de base, indépendant de l'attaque du pathogène, et la défense active ou induite lors d'un phénomène d'infection, ou d'une blessure. Chacune de ces défenses peut être divisées en deux grands groupes : la défense chimique responsable des altérations dans la croissance du pathogène et la défense structurelle, comme le renforcement des barrières contre le pathogène, par exemple. Dans ce travail de thèse, nous essayerons de mieux comprendre les différentes caractéristiques physiques et chimiques qui peuvent participer à la défense constitutive du fruit contre la pourriture brune.

### **2.1 Caractéristiques physique potentiellement liées à la résistance constitutive**

Par barrière physique il faut comprendre tous les caractères qui rendent difficile la pénétration et le développement du champignon. On peut considérer l'épaisseur de la cuticule ainsi que de l'épiderme, le nombre de trichomes, de stomates ou de microfissures comme des caractères permettant de décrire la barrière physique formée par la peau du fruit. La cuticule des plantes est une couche de cires censée constituer une barrière physique efficace contre la majorité des agents environnementaux et des pathogènes qui colonisent la surface de la plante. C'est une forme de défense structurelle. Cependant, cette structure est ponctuée de stomates.

Ces orifices de petite taille présents dans l'épiderme des organes aériens du végétal, permettent les échanges gazeux entre la plante et l'air ambiant (Rogh 1977). Cependant les stomates sont aussi considérés comme porte d'entrée pour les pathogènes (Hall 1971). Chez les abricots l'invasion fongique à travers des ouvertures des stomates a été observé par (Wad and Cruickshank 1992), dans les conditions de laboratoire. Chez les pêches mûres, la surface de stomates pourrait être insignifiante par rapport à celle de microfissures et ne pas être un déterminant important de la sensibilité au pathogène, mais chez les jeunes fruits, la forte densité de stomates pourrait être l'un des facteurs pouvant expliquer leur sensibilité à la moniliose, (Oliveira Lino, Pacheco et al. 2016).



**Photo1** : photomicrographie en microscopie électronique à balayage d'une surface des fruits immature de 60 jours après la floraison.

Par ailleurs, il existe parfois un décalage entre la croissance de la cuticule au cours du développement du fruit et l'expansion de l'épiderme, ce qui cause l'apparition de microfissures

La caractérisation de cette barrière physique n'est pas facile. En effet, il est laborieux d'évaluer la densité de stomates de fruits et de caractériser le réseau de microfissures en surface d'un fruit. De plus, les techniques disponibles de nos jours ne permettent pas de mesurer l'épaisseur de la cuticule et de l'épiderme avec précision due à l'irrégularité de la surface (Gibert, Chadœuf et al. 2007) (photo 1).

Aussi, peut-on utiliser une méthodologie indirecte pour caractériser l'intégrité de la surface d'un fruit qui consiste à calculer la conductance de la surface du fruit à la vapeur d'eau. La conductance représente l'aptitude de la membrane cuticulaire à transmettre l'eau depuis les cellules de la pulpe vers l'atmosphère via les pertes transpiratoires. La conductance totale est la somme des conductances stomatique, cuticulaire et due aux microfissures cuticulaires (Gibert, Lescourret et al. 2005). Un modèle de la conductance de la surface du fruit a été proposé par Gibert, Lescourret et al. (2005). Ce modèle calcule la conductance totale de fruits en intégrant chacune de ces composantes: stomates, cuticules et fissures.

## **2.2 Caractéristiques biochimiques potentiellement liées à la résistance constitutive**

Le phénomène de résistance aux monilioses potentiellement lié aux composés présents dans la surface du fruit n'est pas encore bien connu. La surface est constituée des différentes barrières comme les cires, la cuticule, l'épiderme. Chaque tissu a une constitution biochimique différente des autres. Un certain nombre de composés a été déjà identifié dans la composition de la peau des pêches, par exemples les phénols, terpénoïdes et les alcanes, sont les plus souvent mentionnés dans la littérature, (Villarino, Sandín-España et al. 2011), (Haminiuk, Maciel et al. 2012), (Belge, Llovera et al. 2014).

Plusieurs études ont approfondi la piste des acides chlorogéniques et caféiques. Tous deux sont des acides phénoliques majeurs présents dans l'épiderme des pêches (Bostock, Wilcox et al. 1999). Leurs concentrations sont particulièrement élevées dans les génotypes à résistance élevée à la pourriture brune causée par *Monilinia fructicola* : au fur et à mesure de la maturation des fruits, leur concentration diminue et au contraire, la sensibilité au champignon augmente (Villarino, Sandín-España et al. 2011). Plus précisément, ces études ont porté sur les actions des principaux composés phénoliques, à savoir, la catéchine, la procyanidine B3, l'acide chlorogénique, l'acide néochlorogénique et l'acide caféique (Bostock, Wilcox et al. 1999) (Tomás-Barberán, Gil et al. 2001),

Par ailleurs, les acides oléanolique et ursolique sont des exemples de terpénoïdes qui semblent être des fongicides biologiques. Ces acides sont souvent et largement dominants parmi les

terpénoïdes présent dans les cires (Peschel, Franke et al. 2007), mais leurs activités comme biofongicides n'ont jamais été décrites pour le couple pêche et *Monilinia*.

Des résultats intéressants ont été trouvés par (Belding, Sutton et al. 2000) dans une enquête sur les composés de cire épicuticulaires dans les pommes contre la fuligineuse *Peltaster fructicola* et *Leptodontidium elatius*, champignons épiphytes. Dans les conditions expérimentales, les champignons ne poussent pas sur l'une des composantes épicuticulaires testées, sauf si du jus de pomme a été inclus dans l'essai. Ces résultats confirment l'hypothèse selon laquelle certains composés de l'épiderme ont un effet potentiel sur la résistance des fruits.

D'autre part certains composants présents dans la cire peuvent agir en tant que signaux de plantes au champignon, par exemple, la capacité de *Botrytis cinerea* à sécréter la lipase (EC3.1.1.3) sur la cuticule de raisin (Comménil, Belingheri et al. 1999) est un bon exemple que la façon dont certains composés peuvent devenir les promoteurs de l'infection. Des observations récentes commencent à révéler interrelations complexes entre lipides cuticulaires et l'immunité. Cela suggère que la cuticule n'est pas simplement une barrière physique, mais une défense de l'hôte dynamique avec les circuits de signalisation et des molécules effectrices, (Reina-Pinto and Yephremov 2009).

Une maladie ne se développe que s'il y a concomitance entre la présence du pathogène, de l'hôte susceptible et de conditions environnementales favorables. Il est possible de dresser une évolution générale de la sensibilité des fruits non blessés en fonction de leur stade de développement. La sensibilité évolue au cours de la maturation du fruit et en fonction du génotype.

Ainsi pour mieux comprendre ce mécanisme complexe il faut investiguer les facteurs liés à chaque stade de développement et vérifier si la susceptibilité est liée à l'absence d'une barrière physique, pas bien développée (fruit immature) ou endommagée, par des microfissures par exemple (fruit à maturité), ou si elle est liée à l'absence de composés inhibiteurs (phénols, triterpénoïdes, cires, etc au stade des fruits immatures) ou à une dilution de ces composés provoquée par l'effet de grossissement des fruits (maturité).

### **2.3 Les mécanismes de résistance induite**

Dans certains cas, les fruits peuvent répondre par la mort des cellules autour du point d'infection, la formation de phellogène à la marge de lésions des rameaux, par exemple sur les arbres fruitiers à noyaux et les pomoïdées (Zwigart 1970) et la subérisation des cellules vivantes dans les fruits au tour du point d'infection sont des exemples de mécanismes de défense induite.

Les mécanismes de résistance induite sont très peu connus chez la pêche. Généralement ils comprennent, les inhibiteurs de protéinases, par exemple cutinases, en réponse aux attaques du champignon. Contrairement aux composés qui peuvent intervenir dans la résistance constitutive, les protéines nécessitent une énergie importante pour leur synthèse. .

Dans une investigation sur le rôle possible des gènes Ppdfn1 dans la défense des fleurs et des fruits du pêcher contre *M. laxa*, Nanni, Zanetti et al. (2013) montrent que le Ppdfn1 présente une activité antifongique à travers des interactions spécifiques avec les lipides de la membrane des champignons. D'autres exemples de mécanismes de résistance induite sont la production de dérivés réactifs de l'oxygène et la mort cellulaire programmée (pour plus d'information, voir Chapitre 1).

### 3 La recherche de sources de résistance

Actuellement il n'existe pas de variétés vraiment résistantes à la pourriture brune, disponibles pour les producteurs. Dans la littérature 'Bolinha' est le génotype brésilien le plus connu comme résistant aux monilioses. Ce cultivar, de type pavie, a été utilisé dans les programmes d'amélioration des fruits par l'Embrapa Pelotas (Brésil) et comme sujet d'étude dans autres institutions. Bolinha, est mentionné comme possédant un fort niveau de résistance (Feliciano, Feliciano et al. 1987) qui pourrait être lié d'une part à une cuticule épaisse et d'autre part à la présence de composés inhibiteurs de la germination ou de la pénétration mycélienne.

Afin de générer de nouveaux cultivars nécessitant moins de fongicides, l'identification des gènes ou loci associés à la résistance aux monilioses permettrait de progresser dans l'intégration des allèles favorables dans les programmes d'amélioration. La recherche de QTL (*Quantitative Trait Locus*) est un outil permettant d'identifier les loci impliqués dans un caractère quantitatif. Plusieurs groupes de recherches dans le monde s'appliquent à ce défi mais seules deux publications font état de détection de QTL de résistance aux monilioses.

A l'Université de Californie-Davis, les études génétiques de la résistance à la pourriture brune ont porté sur l'introgession de résistance à la pourriture brune de l'amandier. (Martínez-García, Parfitt et al. 2013) ont évalué une population issue d'un hybride pêcher x amandier sur 3 années en inoculant des fruits blessés ou non, avec une goutte de 10 $\mu$ L contenant une suspension de conidies de *M. fructicola* à la concentration de 2,5 x 10<sup>4</sup> spores par mL. Aucune résistance de la chair n'a été détectée sur les fruits blessés infectés. La détection de QTL portant sur le test sur fruits non blessés a quant à elle permis de détecter des QTLs sur les groupes de liaison 1 et 4 (Martínez-García, Parfitt et al. 2013).

Les travaux menés au sein du programme de sélection de l'Université de Milan (Italie) ont porté sur une descendance pêcher avec une évaluation sur 2 années de la résistance de fruits blessés ou non, avec une goutte de 10 $\mu$ L contenant une suspension de conidies de *M. fructicola* à la concentration de 5 x 10<sup>6</sup> spores par ml (Pacheco, Bassi et al. 2014). Cette étude a permis de détecter des QTL différents pour la résistance de la chair (fruits blessés) et de l'épiderme (fruits non blessés),

notamment sur les groupes de liaison 2 et 4 pour la première et sur les groupes de liaison 3 et 6 pour la seconde.

Le programme d'amélioration du pêcher à l'INRA-Avignon focalise une majeure partie de ses travaux sur la résistance aux ravageurs, en utilisant notamment des pêchers sauvages tels que *Prunus davidiana* comme sources de résistance. *P. davidiana* a montré son intérêt comme source de résistance à l'oïdium, au puceron vert et à la sharka (Rubio, Pascal et al. 2010) ; (Foulongne, Pascal et al. 2003) ; (Sauge, Lambert et al. 1998). Plus récemment, l'équipe Génétique Intégrative et Innovation chez les *Prunus* (G2IP) travaillant sur les *Prunus* au sein de l'unité GAFL a développé des travaux sur les monilioses, en interaction avec l'unité Plantes et Système de cultures Horticoles (PSH) (Quilot-Turion, Ould-Sidi et al. 2012). L'objectif est d'identifier des sources de résistance et de progresser dans la caractérisation de la variabilité génétique de la résistance. Ces informations ont aussi vocation à être intégrées dans le modèle 'Fruit Virtuel' développé par PSH, de façon à concevoir, par le biais de simulations, des systèmes de production innovants moins sensibles aux monilioses, définis comme des ensembles idéotypes-pratiques et obtenus par optimisation conjointe des composantes génétiques et techniques du modèle 'Fruit Virtuel'.

## **4 Présentation de la thèse**

### **4.1 Hypothèses**

Différentes caractéristiques physiques et biochimiques des fruits peuvent jouer le rôle de facteurs de résistance aux monilioses. Ces facteurs peuvent se succéder au cours de la croissance des fruits. Ces caractéristiques peuvent être sous contrôle génétique. Dans une perspective de création variétale, il apparaît judicieux de combiner différents facteurs de résistance provenant éventuellement de différents géniteurs.

### **4.2 Objectifs de la thèse**

Le but de cette étude est i) d'étudier les facteurs de résistance du fruit à *M. laxa* à différents stades de croissance des fruits et ii) leur contrôle génétique, par l'étude d'une descendance interspécifique de cartographie.

Pour l'étape (i), on étudiera la probabilité d'infection à *M. laxa* au cours du développement du fruit en lien avec les caractéristiques structurales et biochimiques des fruits de quelques cultivars, et on vérifiera l'importance des stomates et de la conductance cuticulaire dans la résistance à la pénétration des champignons *M. laxa* sur les fruits immatures de la descendance interspécifique.

Pour l'étape (ii), on examinera la résistance à *M. laxa* des génotypes de la descendance interspécifique à maturité, par des tests au laboratoire et en verger. On décrira la variabilité

génétique de la composition et des teneurs des composés d'épiderme et de surfaces au sein de la descendance interspécifique. On étudiera les relations entre les teneurs en composés d'épiderme et de surface et la probabilité d'infection à maturité au sein de la descendance interspécifique. Enfin on recherchera la présence de QTLs de résistance aux monilioses à différents stades et leur co-localisation avec des QTLs des caractéristiques physiques et biochimiques des fruits dans la descendance interspécifique cartographiée à l'aide de marqueurs SNP et Microsatellites (SSRs).

### 4.3 Le matériel végétal

La population utilisée dans cette thèse a été créée à l'INRA d'Avignon dans le cadre d'un programme d'amélioration de la résistance à plusieurs ravageurs du pêcher (sharka, oïdium, puceron vert et cloque). Cette population est issue d'un croisement interspécifique entre un pêcher sauvage, *P. davidiana* (clone P1908), source de résistance face aux ravageurs mais possédant une très faible valeur agronomique et une variété commerciale de nectarine à chair jaune Summergrand® (*P. persica*). Suite à ce croisement, un hybride SD40 présentant un bon niveau de résistance à l'oïdium a été sélectionné pour effectuer un rétrocroisement avec la variété Summergrand donnant lieu à la famille BC1.

Un mélange des pollens de cette famille a servi à féconder la variété commerciale Zéphyr® (*P. persica*), une nectarine à chair blanche. C'est la population appelée BC2 (pour Back Cross 2) issue de ce dernier croisement qui a fait l'objet de cette étude. Cette population s'apparente à un double back cross si l'on considère que les allèles proviennent soit de *P. persica*, soit de *P. davidiana*. Cependant ce n'est pas un double back cross au sens strict car les deux parents *P. persica* utilisés sont différents, bien que très proches comparés à *P. davidiana*. Les génotypes que l'on peut rencontrer à un locus donné sont décrits dans le Tableau 1.3. Dans la population BC2, tous les génotypes portent un des deux allèles de la variété Zéphyr alors que l'allèle de *P. davidiana* est présent chez environ ¼ des individus.

### 4.4 Plan de la thèse

Cette thèse est organisée en cinq chapitres principaux.

**Le chapitre 1** est une synthèse bibliographique sur la pourriture brune chez les *Prunus*, publiée dans le journal *Journal of Agricultural and Food Chemistry* (Oliveira Lino, Pacheco et al. 2016). Une brève description de la taxonomie, morphologie et distribution géographique des *Monilinia* spp est proposée, avec des focus particuliers sur les caractéristiques du fruit, les étapes de développement de l'infection. Les principaux facteurs biochimiques et moléculaires de résistance de l'hôte à la pourriture brune sont discutés et les différents programmes d'amélioration des plantes pour la sélection de génotypes résistants aux monilioses sont expliqués. L'importance économique du genre



*Prunus*, la réglementation des fongicides et les pertes provoquées par la pourriture brune sont également abordés dans cette synthèse.

Le **chapitre 2** présente les résultats expérimentaux de l'étude de la probabilité d'infection au cours de chaque stade de développement du fruit, en lien avec les caractéristiques structurales et biochimiques du fruit, pour quelques cultivars. Outre le suivi de la masse des fruits et leur conductance cuticulaire, les cires ont été analysées pour la première fois chez des fruits et les composés de surface ont été recueillis, identifiés et dosés. Quelques composés ont été identifiés pour la première fois à la surface des fruits de pêche.

Cette étude nous a permis de mettre en évidence des facteurs qui pourraient être à l'origine de la résistance ou de la susceptibilité à chaque stade de développement du fruit.

Les résultats obtenus confirment que pendant le stade I, les fruits immatures sont très susceptibles à la pourriture brune. En revanche, pendant le durcissement du noyau les fruits sont résistants et redeviennent susceptibles à la fin de croissance à l'approche de la maturité physiologique. Aussi ces deux stades de susceptibilité font l'objet des deux chapitres suivants.

Au **chapitre 3** nous avons exploré les différentes caractéristiques physiques (structurales) du fruit immature en relation avec la sensibilité à *M.laxa*. A ce stade précoce et susceptible, les cires ne sont pas déposées et la surface du fruit ne contient que très peu de composés biochimiques. La conductance cuticulaire et le nombre de stomates par génotype (uniquement pour les nectarines) ont été mesurés. Les infections contrôlées de ces jeunes fruits ont révélé un symptôme inattendu: une tâche claire est apparue à la surface de certains fruits à l'endroit du dépôt de la goutte de suspension de spores, tâche qui n'a jamais progressé au-delà de 5 millimètres de diamètre. La conductance cuticulaire est apparue significativement liée à la probabilité d'infection, en revanche le nombre de stomates n'a pas montré de lien significatif avec la probabilité d'infection. Des QTL (*Quantitative Trait Loci*) contrôlant la résistance des fruits à la pourriture brune au stade I, la conductance cuticulaire et le nombre de stomates ont été identifiés.

Deux études ont été réalisées à la maturité physiologique des fruits de la descendance interspécifique et font partie des chapitres IV et V.

Le **chapitre 4** présente les résultats de trois années de tests d'infection réalisés en verger et au laboratoire. Cette population a affiché une forte variabilité de résistance à *M. laxa*. Malgré une faible stabilité entre les années, un haut niveau de résistance a été identifié pour certains génotypes et deux QTLs déjà décrits dans la littérature ont été détectés.

Le **chapitre 5** est une étude réalisée en 2015 pour explorer la variabilité des composés chimiques présents dans l'épiderme et à la surface des fruits à maturité au sein de la descendance interspécifique. Des composés phénoliques, terpènes et dérivés de terpènes ont été quantifiés par

HPLC. La relation entre la résistance à la pourriture brune et la présence et / ou les teneurs de certains composés de l'épiderme et le contrôle génétique de ces composés ont été étudiés.

Enfin, la **conclusion générale** des travaux présentés débouche sur des perspectives ouvertes.

## 5 Publications et communications scientifiques

### 5.1 Publications

Oliveira Lino, L., Pacheco I., Mercier, V., Faoro, F., Bornard, I., Bassi, D., Quilot-Turion, B. 2016. Brown rot strikes Prunus fruit: an ancient fight almost always lost. *Journal of Agricultural and Food Chemistry*, 64: 4029–4047.

Oliveira Lino, L., Génard, M., Signoret, V., Quilot-Turion, B. 2016. Physical host factors for brown rot resistance in peach fruit. *Acta Horticulturae* 1137. ISHS 2016, 105-122. DOI 10.17660/ActaHortic.2016.1137.15.

### 5.2 Communications orales

Oliveira Lino, L., Génard, M., Signoret, V., Quilot-Turion, B. 2015. Host factors for brown rot resistance in peach fruit. Innohort ISHS Symposium: Innovation in Integrated & Organic Horticulture, Avignon University, France, June 8-12, 2015.

Pacheco, I., de Oliveira Lino, L., Ciacciulli, A., Lama, M., Foschi, S., Faoro, F., Signoret, V., Bassi, D., Quilot-Turion B. 2015. Assessing brown rot resistance in peach fruit. XIV Eucarpia Symposium on Fruit Breeding and Genetics. 14-18 June 2015, Bologna, Italy.

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## Chapitre 1: Synthèse bibliographique sur la pourriture brune chez les *Prunus*

La pourriture brune provoquée par les champignons du genre *Monilinia spp.*, est un des plus graves problèmes économiques pour le marché des fruits à noyau en raison des pertes dramatiques occasionnées, principalement en post-récolte. Une littérature riche décrivant l'infection par *Monilinia spp.* permet d'indiquer que l'environnement influe de manière significative le développement de l'infection dans le verger. Cependant, des progrès sont nécessaires pour limiter durablement cette maladie: l'agent pathogène est capable de développer une résistance aux pesticides et la plupart des programmes de recherche de résistance des plantes à cette maladie ont échoué. La résolution de ce problème devient importante en raison de la nécessité de réduire les traitements chimiques et de diminuer les résidus sur les fruits. Ainsi, les recherches ont récemment recommencé, en explorant un large éventail de stratégies de contrôle des maladies (par exemple génétique, chimique, physique). Réaliser une synthèse de la diversité de cette information est difficile, car les études ont évalué différentes espèces de *Monilinia* et *Prunus*, avec diverses stratégies et protocoles. Aussi, le but de cette revue est de présenter la diversité et la distribution des agents pathogènes provoquant la pourriture brune, en se concentrant sur les mécanismes biochimiques de l'infection à la fois au niveau du champignon et du fruit, et de présenter les sources de résistance connues chez les *Prunus*. Dans cette revue, nous compilons les informations actuellement disponibles afin de mieux comprendre les mécanismes liés à la résistance de cette maladie.

Ce chapitre a été co-écrit avec Igor Pacheco-Cruz, dans le cadre du projet européen FruitBreedomics. Il est publié dans le Journal of Agricultural and Food Chemistry.

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# Brown rot strikes *Prunus* fruit: an ancient fight almost always lost

## Abstract

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

## 1 Introduction

The genus *Prunus* has hundreds of species with some economically important members, including the cultivated almond, peach, plum, cherry and apricot. The five most important countries for the production of these fruits are China (10.7MTon), the United States (2.9MTon), Italy (1.9MTon), Spain (1.4MTon) and Greece (0.8MTon) (FAOSTAT, 2015).

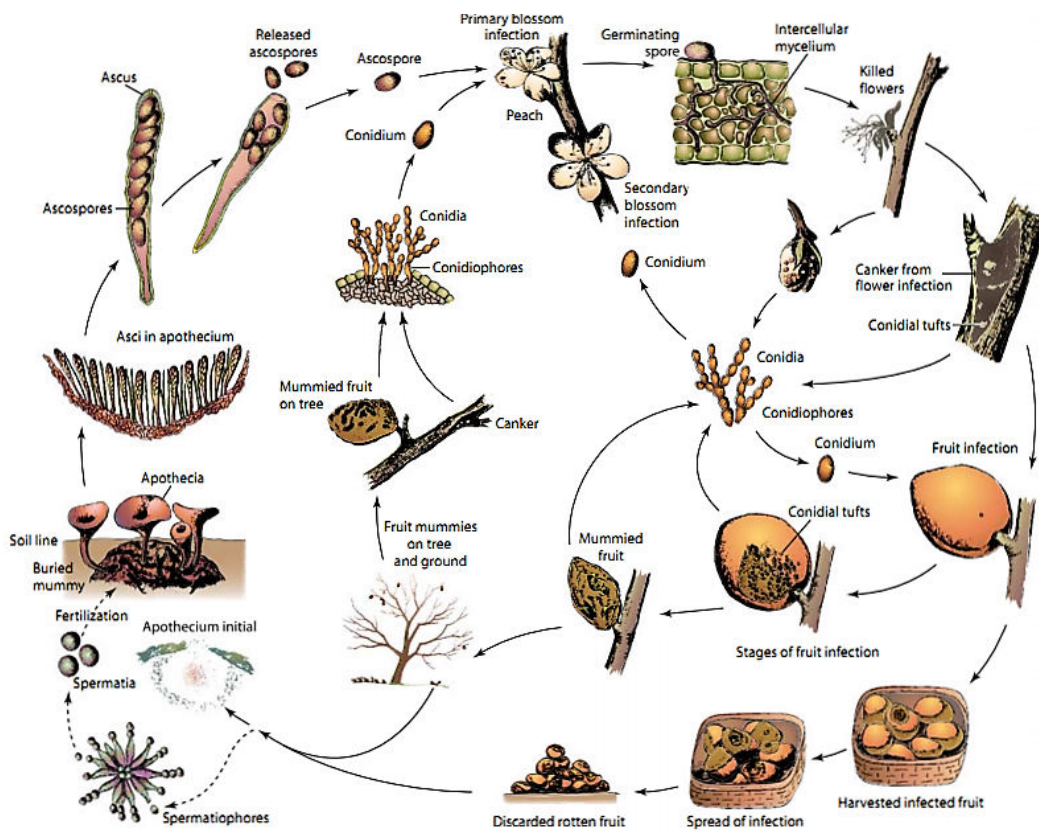
Different cropping practices are employed for the production of this variety of fruit, according to their different environmental and nutritional requirements. In addition, the broad range of pests has to be controlled to reach a high quality final product. This latter point is a crucial issue in current fruit cropping, since the demand of fresh fruit with reduced residual quantities and the regulation of fungicide use has become stricter in EU countries, after the release of the European Directive 2009/128/EC which indicates the use of Integrated Pest Management (IPM) as mandatory (EPPO, 2009) (Colla, 2012). In the United States, the government has strongly promoted IPM in order to reduce chemical pesticide input with the creation of Regional IPM Centres, resulting in progressive decreases in pesticide use and toxicity for humans (EPA, 2011) (Chou et al, 2015). Reduced pesticide applications have been advised in China (Zhu et al, 2011), Brazil and other countries (Fan et al, 2010).

Among the plethora of pathogenic agents attacking *Prunus* crops (and other Rosaceaeous), brown rot (BR) is the economically most important disease of stone fruits (Hu et al, 2011). *Monilinia* spp. are able to infect various plant organs, causing blossom blight, twig blight, and BR in immature and mature fruits, the latter being the most sensitive host phenological phase. The relatively long period of incidence, extending from bloom to post-harvest, the multiplicity of climatic and cropping factors favouring disease spread, the occurrence of diverse fungicide resistances in some BR agents and the poor availability of host resistance, result in severe, unavoidable and sometimes unpredictable losses in the fruit market (Fan et al, 2010). According to Martini and Mari (Martini & Mari, 2014), the worldwide yearly value of *Monilinia* losses is 1,7 thousand million Euro; in the United States, yearly losses are estimated to be 170 million USD for peach, cherry and plum production (RosBREED, 2016); and in Australia yearly losses are estimated at 1 million AUD for peach and apricot crops (EPPO, 2007). Under laboratory conditions, BR can result in losses of over 60% of peaches and nectarines after 5 days of infection at room temperature.

To avoid these damages, *Monilinia* spp. diseases are controlled by chemical methods. Fungicide applications are necessary to diminish BR damage in humid seasons, but lead to sustainability challenges in pome and stone fruit cropping, as there are many fungicide-resistant strains (*Monilinia fructicola*, see below). An important research field has been dedicated to the epidemiology of BR, as well as aspects related with traditional chemical control and emerging alternative control strategies (e.g. tree management) (Mercier et al, 2008), (Bussi et al, 2015), compatible with IPM and organic agriculture (biologic agents, post-harvest bio-chemical-physical agents). These topics are thoroughly reviewed and discussed in recent publications (Rungjindamai et al, 2014), (Martini & Mari, 2014). Significant efforts are being invested in order to characterize and enhance fruit resistance to BR for the generation of new varieties with reduced requirements of application of exogenous methods for BR control. These have been included as important objectives of international collaborative initiatives for new cultivar development around the world, such as Fruit Breedomics European project and ROSBREED American initiative.

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





**Figure 1.** *Monilinia* spp. life cycle. Figure used with permission of Elsevier Limited, Oxford, UK (Copyright George N. Agrios, 2015)



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

## 2 *Monilinia* spp. fungi cause brown rot

### 2.1 Taxonomy

The agents causing BR are polytrophic fungi belonging to the Phylum Ascomycota, Class Leotiomycetes, Order Helotiales, Family Sclerotiniaceae, Genus *Monilinia*. They attack members of the Rosaceae and Ericaceae families (Holst-Jensen et al, 1997a), (Honey, 1928). The generic name *Monilinia* includes those members of *Sclerotinia* that produce moniloid conidia and pseudosclerotia.

Of the 35 species of the genus *Monilinia* Honey, three are the main species that are pathogenic to pome and stone fruits: *Monilinia fructicola* (G. Winter) Honey; *Monilinia laxa* (Aderhold & Ruhland) Honey and *Monilinia fructigena* (Aderhold & Ruhland) Honey (van Leeuwen & van Kesteren, 1998). At least two species have been described to be important pathogens of Ericaceae: *Monilinia vaccinium-corymbosi* causing mummy berry of blueberry (Ehlenfeldt et al, 2010) and *Monilinia oxycocci* causing cottonball of cranberry (McManus et al, 1999). According to phylogenetic analyses based in rRNA sequences of *Monilinia* and *Sclerotinia* species, the separation of the genus in two sections is consistent: *Junctoriae*, attacking Rosaceae hosts, and *Disjunctoriae* attacking Ericaceae hosts (Holst-Jensen et al, 1997a) (Holst-Jensen et al, 1997b), (Holst-Jensen et al, 1998), moreover, partial congruence found in the branching topologies of hosts and pathogen phylogenies, lead to suggest the hypothesis of co-speciation between them (Holst-Jensen et al, 1997a). In this review, we will focus on *Monilinia* spp. and BR in stone fruits.

The disease cycle of *Monilinia* species is represented in Figure 1. Primary inoculum sources in the spring are overwintering BR fruit mummies either on the tree, which produce asexual fruiting structure (sporodochia) and spore (conidia) or on the orchard floor, which produce sexual fruiting structures (apothecia) and spores (ascospores). The spores are dispersed by wind and rain to susceptible host tissues, and germinate under favourable wetness and temperature conditions. In general blossom blight reduces the crop load in fruit crops, but it can destroy the crop at flowering in susceptible almond cultivars. The infections of blossoms typically remain attached and the infection spreads into the peduncle and down into the twig. The infection continues with the formation of a twig canker that often develops a gumdrop as a host response. Conidia form on infected tissue and serve as secondary inoculum for infection of immature and mature fruit (loos & Frey, 2000). Infections on immature fruit, after the endocarp lignification, may give place to conidia, providing additional inoculum.

### 2.2 Differentiation of *Monilinia* species

By observation with naked eye, it is possible to identify the differences between the three agents of monilioses in fruit in orchard conditions (Mercier, 2009). *M. fructigena* has colour ranging

from white to light beige, large (1.5 mm on average) conidiospores tufts, and disposition in concentric circles in the fruit. *M. fructicola* has brown-coloured, medium size (1 mm on average) conidiospores tufts and 10% black spots. *M. laxa* can be distinguished by greenish-grey conidiospores tufts less than 0,5 mm on average that cover the whole infected surface. However the differentiation in fruit between *M. laxa* and *M. fructicola* may sometimes be difficult and the use of molecular techniques is required (Figure 2).

Studies to identify the *Monilinia* species reported that, in culture medium with potato dextrose and agar (PDA) at 22°C, *M. laxa* is characterized by concentric rings of mycelium with lobbed margins, while in *M. fructigena* it is possible to observe fragmented radial colonies. Differences in colony growth rates between the three species were observed (20–25°C). The highest growth rate on PDA was found for *M. fructicola*, followed by *M. fructigena* and *M. laxa* respectively. However, *M. laxa* showed the biggest lesion growth rate on peach fruit (Hu et al, 2011). In culture medium it is possible to analyse characters as conidial size and germ tube morphology. These methods have been used since 1920 and their simplicity makes them useful still (Ioos & Frey, 2000). Differences in conidia size among the species are reported. On average the conidia size of *M. laxa* is smaller compared to *M. fructigena*, 13x9 µm and 22x12 µm, respectively. *M. fructigena* produces one or two germ tubes per conidium, and *M. laxa* and *M. fructicola* isolates consistently produce only one germ tube per conidium (Hu et al, 2011).

Several molecular biology techniques (mostly based in the Polymerase Chain Reaction, PCR) have been used to develop reliable and sensitive methods to identify and detect *Monilinia* species. Fulton and Brown (Fulton & Brown, 1997), proposed the study of the small sub unit of ribosomal DNA (rDNA) to differentiate *Monilinia* isolates from the three major species. Many PCR protocols for *Monilinia* spp. identification, based on the comparison of internal transcribed spacers, sequence between the 18S small and the 28S rDNA subunits of *Monilinia* genes, have been proposed (Ioos & Frey, 2000) (Boehm et al, 2001), (van Brouwershaven et al, 2010). Ma et al (Ma et al, 2003) and Hu et al (Hu et al, 2011) reported a detection and identification method of *Monilinia* fungi based on species-specific microsatellites (Hu et al, 2011), (Ma et al, 2003). Identification methods based on amplified fragment length polymorphism (AFLP) are also reported (Gril et al, 2008), (Gril et al, 2010). In addition, molecular techniques have been developed for species identification on quiescent fruit infections of stone fruit (Côté et al, 2004), and for the early detection of infections in cherry fruit (Forster & Adaskaveg, 2000). In Banks et al (Banks et al, 1997), monoclonal antibodies are reported to be useful for identification and detection of *Monilinia* spp. in pome and stone fruit (Banks et al, 1997). Some of these approaches have set the basis for several studies about morphological and molecular diversity of *Monilinia* spp., describing the geographical distribution and host range of the

three main species of *Monilinia* that caused BR of stone and pome fruits, (Gell et al, 2007), (Petroczy & Palkovics, 2006), (De Cal et al, 2009).

### **2.3 Host range and distribution of *Monilinia* spp.**

*M. fructigena* is an economically important BR-agent that has been associated with European BR of pome fruits (Holst-Jensen et al, 1997a) (Byrde & Willetts, 1977). However, its occurrence in stone fruits has also been well documented in Europe (Larena et al, 2005) (Villarino et al, 2013), Brazil (Lichtemberg et al, 2014) and China (Zhu et al, 2011).

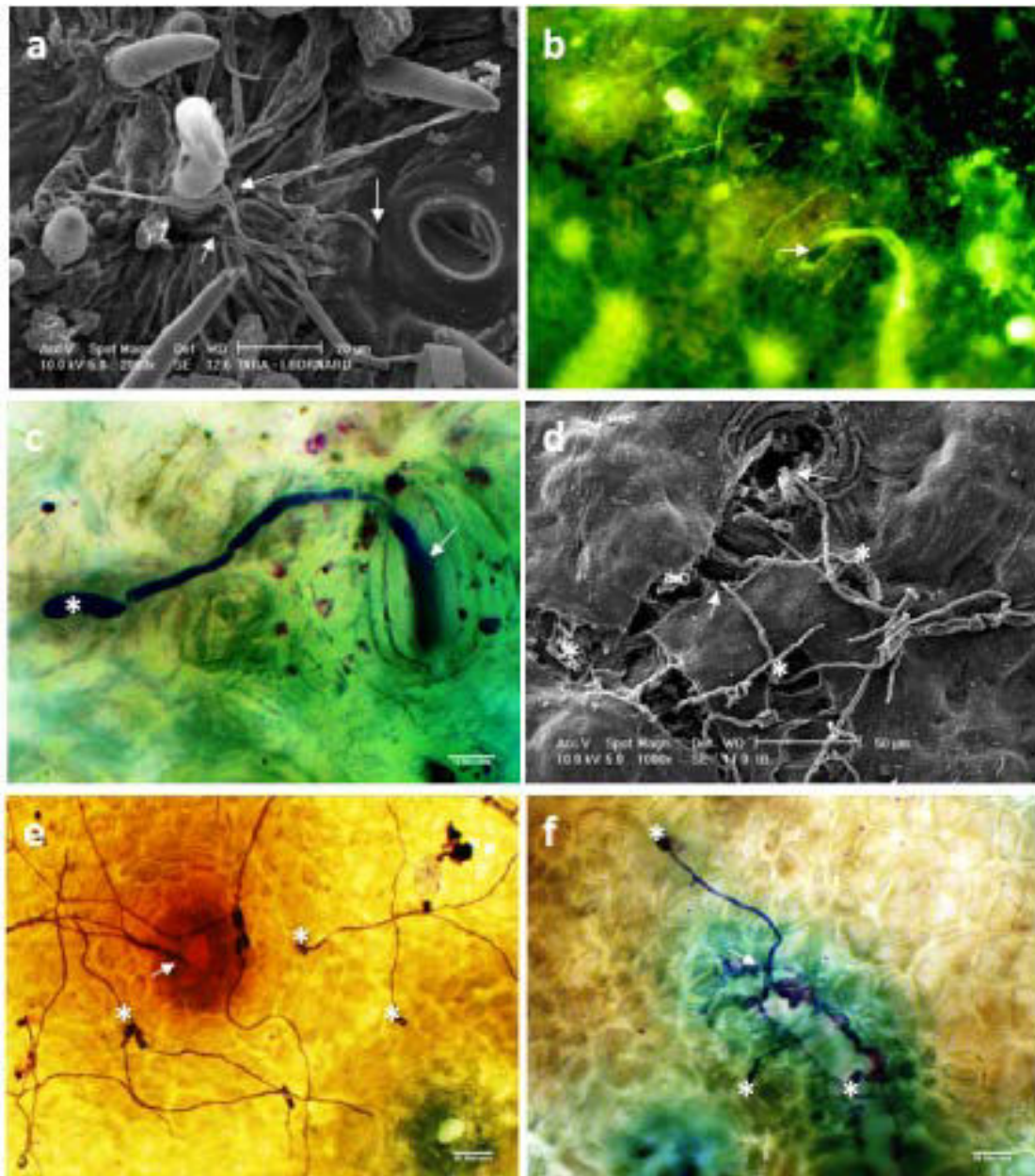
*M. laxa* has been historically associated with European blossom blight and BR of stone (Byrde & Willetts, 1977), (Villarino et al, 2013) and pome fruit (Muñoz et al, 2008), (Lesik, 2013). However in the last two decades it has been also reported in different regions of the world, including Brazil (Lichtemberg et al, 2014), (Souza et al, 2008), United States (Snyder & Jones, 1999), (Villani & Cox, 2010), (Cox et al, 2011), China (Zhu et al, 2011) and Iran (Nasrollanejad & Ghasemnezhad, 2009).

*M. fructicola* (G.Wint) is the most widely distributed species, occurring in Asia, North and South America, New Zealand and Australia (Fan et al, 2010), (Latorre et al, 2014). In Europe, it was a quarantine pathogen until early 2014, when it was removed from the European quarantine pest list due to its current spread in the following countries: France (Lichou J., 2002), Hungary (Petroczy & Palkovics, 2006), Switzerland (Bosshard et al, 2006), (Hilber-Bodmer et al, 2010) Germany (Grabke et al, 2011), Czech Republic (Duchoslavova et al, 2007), Slovenia (Munda & Marn, 2010), Italy (Pellegrino et al, 2009), (Martini et al, 2013) Austria (subsequently eradicated) (Jänsch et al, 2012), Poland (Poniatowska et al, 2013), Slovakia (Ondejková et al, 2010), Serbia and Spain (De Cal et al, 2009).

The low genetic diversity found in Spanish and French populations of *M. fructicola*, compared with American or New Zealand diversity, indicates few and recent introduction events of the pathogen to Europe (Villarino et al, 2012). In addition to its wide distribution, *M. fructicola* has been reported to infect other hosts such as Cornelian cherry (Beckerman & Creswell, 2014) and others that do not belong to Rosaceae family, for example grapes (Sholberg et al, 2003) and dragon fruit (Abd Ghani et al, 2011).

These three species share high levels of DNA similarities. *M. fructicola* and *M. fructigena* exhibited 97,5% sequences identity while *M. laxa* and *M. fructigena* displayed more than 99,1% for the *Cyt b* gene (Hily et al, 2011). In this way, we may expect that part of the knowledge acquired from one species may be extrapolated to the other members of *Monilinia* genus.

A fourth species, *M. polystroma* (also called 'Asiatic Brown Rot') is native of Japan, where it had been formerly confounded with *M. fructigena*. It was described as a new species after finding significant biological and morphological characteristics with respect to European isolates of *M.*



**Figure 3.** Sites of fungi penetration: A- scanning electron microscopy examination showing the development of fungi in apricot surface 8 hours post-infection (hpi). The fungi develops on the fruit surface, twists around trichomes (t) and moves to the stoma (s) direction and trichomes basis (arrows). B - fluorescence photomicrograph 24 hpi. In this image it is possible to see a hyphae entering through an open stomata (arrows). C and E- light microscopy images showing infection on the surface of a commercial nectarine 'Magique' at maturity, coloured with Toluidine blue, 0.5%, 15 (hpi). C- Beginning of spore germination (asterisks) and penetration through stomata aperture (arrow). D - electron microscopy image shows a strong concentration and germination of spores (asterisks) fungi around the fruit cracks of cv. 'Magique' cultivar 15 hpi. E- spore germination and development of mycelium in micro crack (m) direction. F – an infection of nectarine surface at maturity coloured with Toluidine blue, 0.5%, observed with light microscopy at 15 hpi. It is possible to note the distribution of spores (asterisks) and their germination. This image illustrates a chaotic germination of spores (asterisks) and the colonization of surface by hyphae. The arrow shows the penetration of hyphae in an epidermis aperture (o). In all images fruit were infected with a drop of  $10\ \mu\text{L}$  and  $10^5$  spore/ $\text{ml}^{-1}$  of conidia concentration.

*fructigena* (van Leeuwen et al, 2002). Molecular differences between European and Japanese isolates of *M. fructigena* were previously demonstrated, on the basis of the ITS region of ribosomal DNA (Fulton et al, 1999). *M. polystroma* has been reported to occur in pome and stone fruit orchards from China (Zhu & Guo, 2010), Poland (Poniatowska et al, 2013) and Hungary (Petroczy & Palkovics, 2009).

Two other less-distributed *Monilinia* species are described. *M. mumecola* was reported to infect *Prunus mume* in Japan (Harada et al, 2004), and be the causal agent of the BR of papaya in Hubei, China in 2009 (Hu et al, 2011), (Shao, 2009). Finally, *M. yunnanensis* has been recently designated as a new species causing BR in Chinese peach orchards and, based on the DNA sequence similarity analyses of marker genes, was found to be very close to *M. fructigena* (Hu et al, 2011); this species is also able to infect fruits of *Crataegus pinnatifida* (Zhao et al, 2013).

In summary, it is no longer relevant to affirm that the different BR-agents are distributed in specific regions. Indeed, all of the three main *Monilinia* species are present in almost all stone and pome fruit-producing countries (EPPO, 2016), likely due to open trade around the world. The worldwide distribution of *M. laxa* is very well illustrated in Rungjindamai et al (Rungjindamai et al, 2014). In the same way, the fact that *Monilinia* species have the ability to colonize fruit of virtually any *Prunus* or *Malus* hosts, suggests a relative wide host range of these agents. Few studies of host specificity in *Monilinia* spp. have been reported to date, among which the proteomic analysis conducted by Bregar et al (Bregar et al, 2012), showed a host-specific expression of some proteins between apple and apricot *M. laxa* isolates.

### **3 Penetration sites in relation to fruit growth**

As stated before, in this review we only discuss aspects of fruit infection. Different biologic mechanisms may be involved in pathogenesis of fruit and flowers by *Monilinia* spp., suggested by an absence of correlation between blossom bight occurrence and fruit rot impact, after artificial inoculation of *M. fructicola*, in Brazilian cultivars and selections of peach (Wagner Júnior et al, 2005). In fruit, *Monilinia* spp. has often been considered as an opportunistic fungi that may enter in the tissue only via naturally occurring entry points. Therefore, many studies have focused on these entrances or employed infection tests injuring the fruit first. Although in most of the cases the fungus penetrates using 'open doors', (Figure 3 F), most of the species may also be able to penetrate fruit through intact surface, after the establishment of latent or quiescent infections.

For example, the penetration of *M. fructicola* in immature apricot fruit was reported to occur through wounds, stomata, (Figure 3 B and C), intact cuticle or via trichoma bases, (Figure 3 A) (Wad & Cruickshank, 1992b). The same way in peach, hyphae infect fruits by either degrading the cuticle and epidermal tissue (Bostock et al, 1999) or directly entering through pre-existing skin microcracks

(Figure 3 D and E). Fungus incidence is greater if the fruit has small cracks or wounds (De Cal et al, 2013). It has been reported that *M. fructigena* infects fruit via wounds only, in contrast to *M. laxa* that may infect both healthy and wounded fruit (Xu et al, 2007). Indeed, infection may depend on which site is most frequently encountered by fungal germ tubes. Penetration site may also depend on the developmental stage of the fruit. For example, stomata are the preferred sites in the case of unripe peaches only. Curtis (Curtis, 1928) found that apricots were penetrated through cuticle and stomata, plums via stomata, and nectarines through the cuticle. Sharma and Kaul (Sharma & Kaul, 1990) described the penetration of apple under laboratory conditions by *M. fructigena* through lenticels.

### **3.1 Fruit susceptibility evolves along fruit development**

The stages of development of fruit are very important to understand the occurrence of BR, since the dramatic changes in fruit physiology and biochemical composition are in sync with changes in the susceptibility to BR infection (De Cal et al, 2013), (Wad & Cruickshank, 1992a), (Biggs & Northover, 1988).

The first stage starts after ovule fertilization, petal fall and ends when stone starts lignifying. In this stage the fruit is photosynthetically active, displays intense transpiration activity, and shows the highest nutrient content (Thomidis et al, 2007), resulting in a high susceptibility to BR, probably due, in part, to the fact that stomata are active, and offer an entrance opportunity to the pathogen (Curtis, 1928).

The second stage, also known as “pit hardening”, is the stage most resistant to infection by *Monilinia* spp. (De Cal et al, 2013), (Mari et al, 2003). This stage is characterized by intense metabolite activity of secondary compounds, like catechin, epicatechin and phenolic compounds, associated with the lignification of the endocarp, occurring in this stage. In order to find genes whose expression is involved in the synthesis of compounds conferring pathogen resistance, Guidarelli et al (Guidarelli et al, 2014), compared gene expression profiles obtained by microarray analysis of susceptible phase (stage S1) and resistant phase (S2) RNA samples from peel fruit, finding dramatic changes in the expression of phenylpropanoid and jasmonate-related genes, and thus supporting a potential role of these compounds in BR resistance along fruit development.

At the third stage, the highest cell expansion is observed and colour changes from greenish to yellow to red. This stage ends with physiological maturity. Stone fruits become increasingly susceptible to pathogens as they mature and ripen, enabling quiescent infections to become active and new infections to begin. Associated with this increased susceptibility, structural changes in the fruit surface take place, such as thinning and fracturing of the cuticle, changes in fruit surface

chemistry (e.g. production of sugars, decline of phenolic compounds and organic acids, etc.), structure and integrity of fruit mesocarp (Bostock et al, 1999).

Notably, various works in different *Prunus* species have observed a shift in the latent infection rate across the diverse stages of fruit development (Gell et al, 2008), (Northover & Cerkauskas, 1994), (Keske et al, 2011). However, the results vary among studies, probably due to differences in methodology and cultivars used in those studies. For instance, Lou and Michailides (Luo et al, 2001) observed that pit hardening of prunes presented the lowest rates of latent infections, differing from other works reporting a minimum rate of latent infections at the embryo growth stage (Gell et al, 2008), (Keske et al, 2011).

### **3.2 Infection by direct penetration of the cuticle**

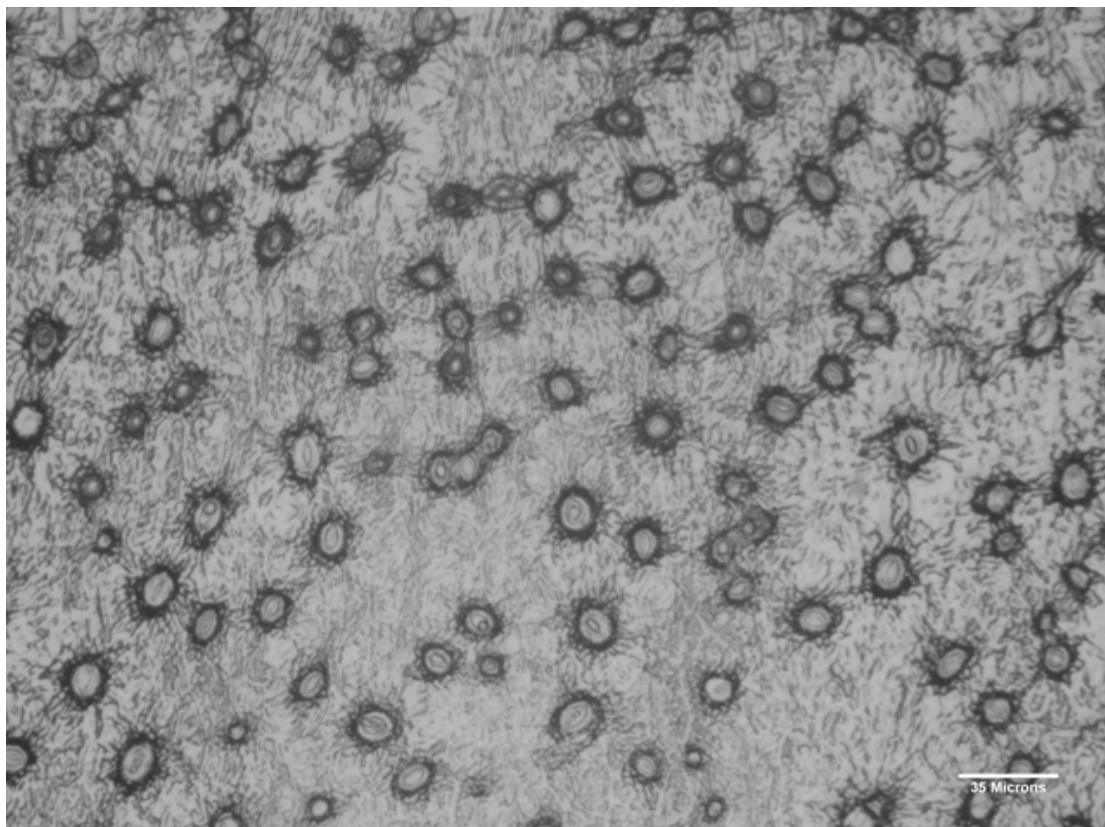
After conidial germination, *Monilinia* species are able to develop *appressoria* to establish a latent infection and ease the penetration of the intact cuticle when fruit maturity conditions allow colonization (Fourie & Holz, 2003). This structure allows adhesion of the pathogen to the surface of the host during infection (Lee & Bostock, 2006). Direct penetration of *Monilinia* spp. is enhanced by its production of cutinases (Bostock et al, 1999), whose redox-mediated over-expression results in an increased fungal virulence of *M. fructigena* in stone fruit (Lee et al, 2010). More details about the infection process are given in chapter 4.

### **3.3 Infection through the trichomes basis**

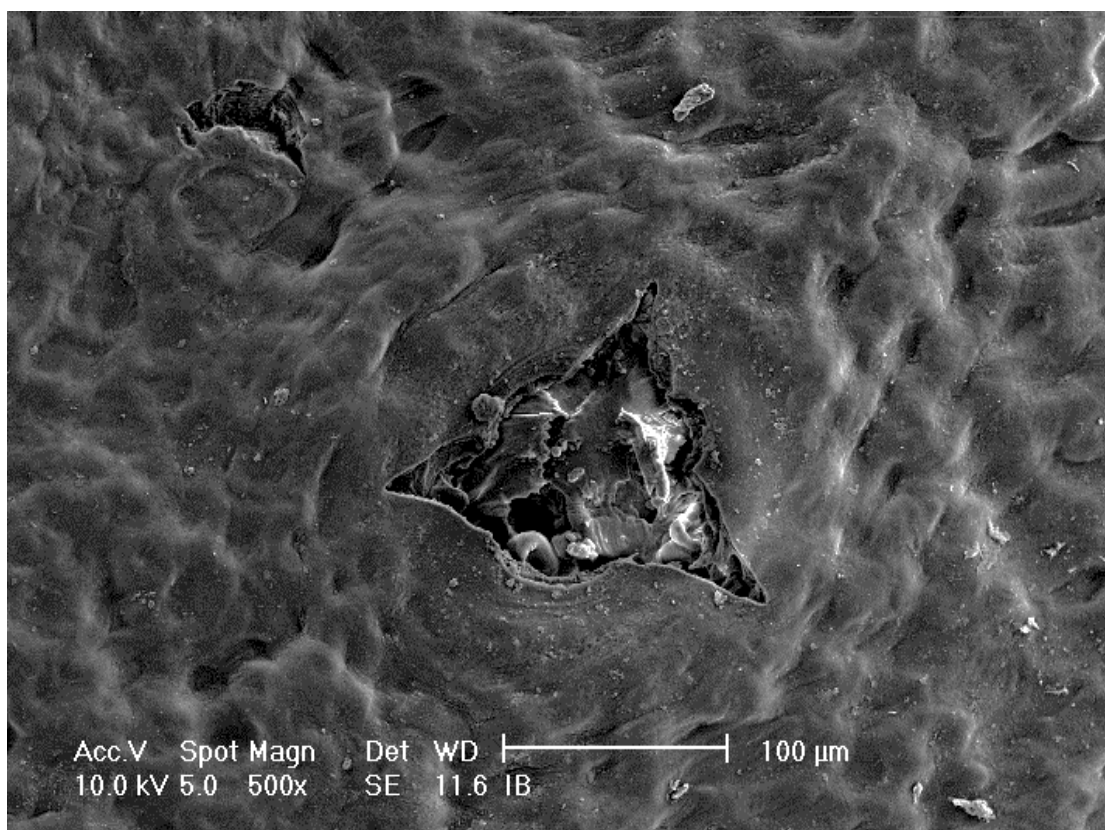
A dense layer of trichomes covers the surface of the peach fruit. The infection can occur in both pubescent and not-pubescent peach fruit. The role of trichomes in the infection remains controversial. Indeed, trichomes may protect the fruit in two ways: 1) Directly: exudates from trichome gland may act as fungicide and 2) Indirectly: the high density of trichomes could prevent the formation of “water film” important to spore germination. In contrast, trichome basis fracture can result in epidermis crack, resulting in points for fungal entrance (Silva et al, 2005), (Fernandez et al, 2011). Smith (Smith, 1936) showed that removing pubescence by means of brushing reduced the time of infection development, suggesting that the spores could reach fruit surface more directly. Other studies (Wad & Cruickshank, 1992b) affirmed that *M. fructicola* is able to penetrate apricots at hair bases. Similar results were found on mature peaches (Curtis, 1928), (Hall, 1971).

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





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



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

### **3.4 Infection through stomata**

The literature about stomata and their function on reproductive organs is limited especially for drupe fruits like peaches (Atkins et al, 1977). A majority of studies discuss their function and distribution in dry fruit like nuts, capsules and pod fruit (Jernsted & Curtis, 1979). They can occur in small numbers or are even restricted to certain parts of the fruit (Roth, 1977). The number of stomata per fruit is determined before petal fall and remains constant throughout fruit ontogeny (Blanke & Lenz, 1989). The morphology of the guard cells suggests that they have the same functions, as on leaves. In early stages, stomata provide aeration in the gas exchanges for the photosynthetic system; however, fruit stomata are only functional to a certain extent. Due to the development of the fruits, stomata can develop into lenticels and either close or remain open permanently (Roth, 1977).

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

Fungal invasion through stomatal apertures into the substomatal cavities was observed in apricots infected by *M. fructicola* under laboratory conditions (Wad & Cruickshank, 1992b). The authors reported that the fungus enters via the stomata and penetrates a guard cell through the thin walled region at the stomata pore. Close examination of serial radial or tangential sections showed that in most cases primary infection was through guard cells. However, in few cases the lesion centre did not coincide with stomata, and initial invasion was through wounds.

### **3.5 Infection through skin cracks and wounds**

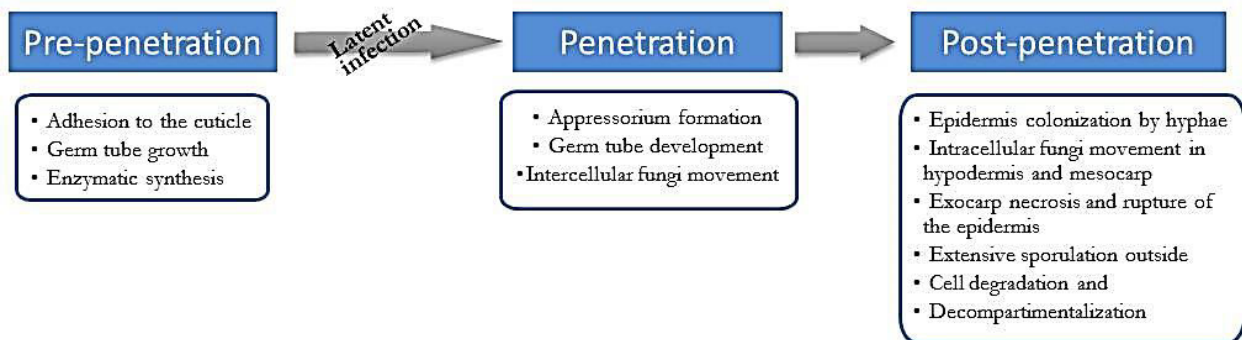
Cuticular crack is defined as the physical failure of the fruit skin, caused by forces of growth as turgor pressure within the fruit cells or hydration of fruit flesh acting on the skin (Milad & Shackel, 1992). Cuticular cracks on nectarine fruit occur during the final fruit growth stage (Gibert et al, 2007), (Gibert et al, 2009), (Gibert et al, 2010). Micro-cracks and cracks can develop on the surface of fruit when the growth speed of the internal cells is more rapid than epidermal cell growth. In this case, a time lag between fruit growth and cutin deposit can occur and provoke zones of weakness that may evolve into microcracks. Several factors contribute to fruit cracking, often in interactions, such as unbalanced water flux into and out of the fruit, maximal elastic limit of the cuticle, cuticle strain, and absence of cuticular membrane deposition. Observations of the fruit skin have shown that the cracks are frequently initiated around the lenticels (Brown & Considine, 1982), (Figure 3D, 3E and Figure 5).

Larger fruits can present high cuticular crack densities, which may represent more than 10% of the fruit surface area (Gibert et al, 2007).

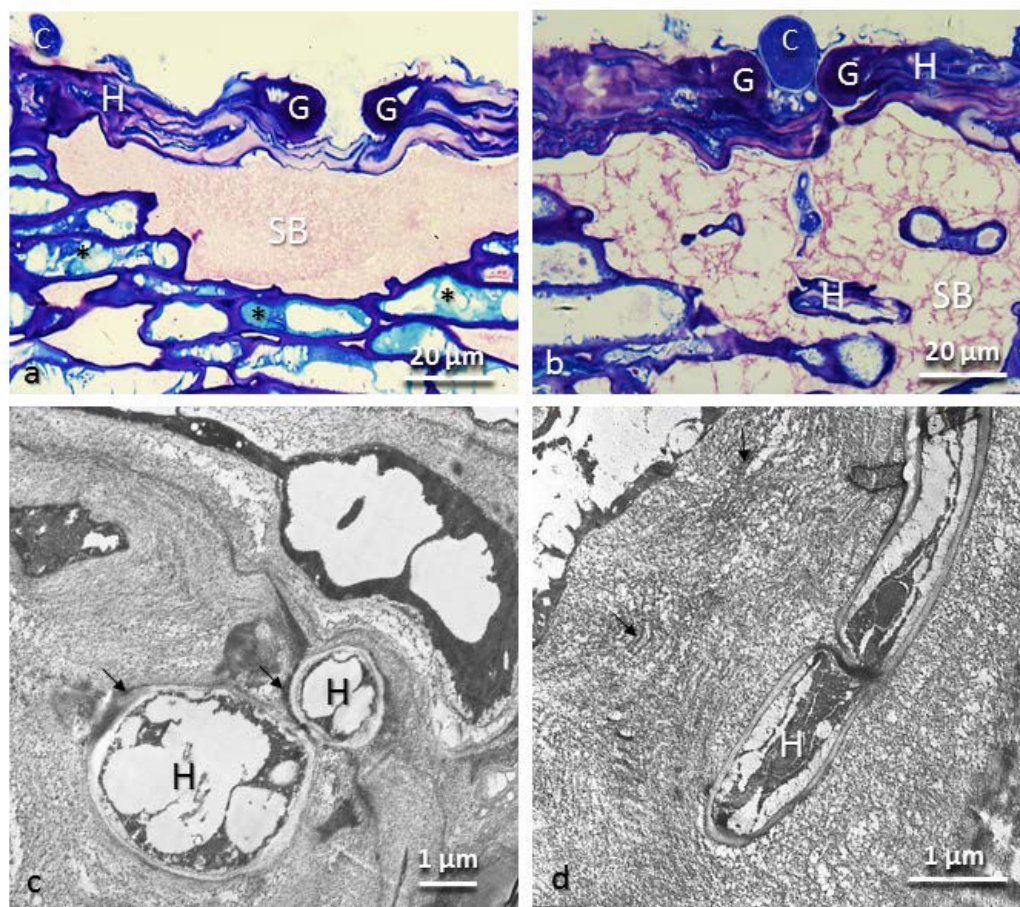
One of the first studies on *M. laxa* penetration in micro-cracks (Nguyen-The et al, 1989), observed a significant number of cracks and micro-cracks organized radially around lenticels and noticed that germinating conidia of *M. laxa* tended to accumulate in the micro-cracks in an anarchic pattern and without apparent direct attraction by micro-cracks, despite the fact that the germ tubes grew inside of them. However Borve et al (Borve et al, 2000), demonstrated a clear link between cracking and BR in cherries, by finding significant correlations between the cultivar-specific amount of micro-cracks and the resulting incidence of BR.

Skin wounding deprives the fruit of its main barrier to biotic stress agents, as demonstrated in several reports (Xu et al, 2007), (Hong et al, 1998), where BR infection rates obtained after infecting wounded regions of the fruit were significantly higher than infecting intact fruit regions. Effect of presence of skin barrier in BR resistance was investigated on apricot, peach and plum fruit, to find resistant genotypes (Pascal et al, 1998). Injured-fruit infection developed on all fruit with quite similar speed in all species. On the contrary, when uninjured fruit were infected, large variability was observed between genotypes of a same species and between species. These observations suggest that few resistant factors may be expressed at the flesh level and that resistance factors were no more efficient when the fruit was injured. However, Ogundiwin et al (Ogundiwin et al, 2008), explored larger genetic diversity by evaluating 81 peach genotypes by infection on wounded and unwounded fruit. The authors observed variability in both cases and suggested that BR resistance is associated with the pericarp or the mesocarp or both, depending on the genotype (Ogundiwin et al, 2008). Nonetheless, more recently the same group further explored the variability of infection reaction after wounding of a canning peach progeny (Martinez-Garcia et al, 2013), concluding that wounding the fruit generally abrogated any resistance to brown rot. Resistance factors at the level of the flesh (wounded fruit) may not provide total resistance to infection but may slightly act on the speed of lesion propagation. To further explore these potential factors of resistance, large trials considering a high replicate number on highly contrasted germplasm panels may be needed.

In conclusion, it is evident that stomata, lenticels, pores, cracks and microcracks offer preferential entry sites for *Monilinia* and make fungi colonization easier. Number of stomata, lenticels and pores may be under genetic control, but structure may be influenced by environment conditions. As for cracks and microcracks, genetic determinism has not been investigated, but studies have demonstrated the effect of cultural practices (e.g. irrigation and thinning) on their density (Gibert et al, 2007).



**Figure 6.** The process of *Monilinia* spp infection



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

## 4 Infection development

Infection is a term that implies the entry of an organism into a host and the subsequent establishment of a parasitic relationship (Byrde & Willetts, 1977). The process could be broadly divided in three stages: pre-penetration, penetration and post-penetration (Figure 6). The pre-penetration phase concerns the transport of the spores from the inoculum source to the organ host that will be infected. It will not be detailed here.

In general, fungi utilize diverse mechanisms to infect host tissue, which include i) chemical sensing and oriented growth in response to mechanical contact to optimally position infection structures, ii) the production of enzymes to degrade host surfaces, and iii) the formation of specialized structures such as *appressoria* (Mendgen et al, 1996). Initial events are adhesion to the cuticle and directed growth of the germ tube on the plant surface. At the penetration site, *appressoria* are often formed that may have melanised walls and develop high turgor pressure to support the penetration process. The penetration hypha accumulates components of the cytoskeleton in the tip and secretes a variety of cell wall-degrading enzymes in a highly regulated fashion in order to penetrate the cuticle and the plant cell wall. As cited in many articles and reviewed by Rungjindamai et al (Rungjindamai et al, 2014), the presence of moisture near the fruit is a crucial factor for spore germination and infection development.

### 4.1 Adhesion to the cuticle and germination

Conidia and ascospores, which are the main inoculum for BR infections, require free moisture for germination, which is obtained from films or droplets of water and from plant exudates that accumulate on the surface of the host or in damaged tissues (Bell & Wheeler, 1986). Germination of conidia takes about an hour in the presence of free water, while ascospores require 4 to 6 hours. However the germination process could sometimes last 60 hours, in the case of dried spores that need time to rehydrate and reactive the protoplast (Byrde & Willetts, 1977).

### 4.2 Latent infection

Infections may remain latent when microclimatic conditions and fruit growth stage are unfavourable (Byrde & Willetts, 1977), (Luo et al, 2001). Latent infection generally happens in immature fruit. A subcuticular infection begins, but growth of the pathogen quickly stops. These quiescent infections may be visible or nonvisible. Along fruit growth, *M. fructicola* expresses genes and proteins enabling later successful infection and colonization of the fruit (Lee et al, 2010). As the fruit matures, fungal growth restarts and BR develops (Rungjindamai et al, 2014).

The relationship between the numbers of conidia on the fruit surface and the incidence of latent infections in orchards or after harvest has been investigated for different fruit species (Xu et al,

2007), (Northover & Cerkauskas, 1994), (Luo et al, 2001)' (Fourie & Holz, 2003)' (Emery et al, 2000). A significant positive link has been reported for peaches (Gell et al, 2008). Therefore early identification of fungal infections is needed to determine pre- and post-harvest disease management practices, as well as postharvest shipping strategies. In order to choose targeted fungicide treatments, molecular methods to identify latent infection of *Monilinia spp.* have been developed (Forster & Adaskaveg, 2000).

### 4.3 *Appressorium* formation and hypha penetration

Formation of *appressorium* is induced by specific physical or chemical cues provided by the host plant. Irrespective of whether fungi use enzymes or force, or a combination of both to penetrate, *appressoria* need to adhere tightly to the plant surface. *Appressorium* differentiation can be stimulated in *C. gloeosporioides* by wax isolated from fruit of its host plant, avocado, but not by wax isolated from other plants (Kolattukudy et al, 1995). Careful analyses suggested that non-host wax contained inhibitors of *appressorium* development.

High pressure can be generated by turgor within the *appressorium* and possibly also by the cytoskeleton, and pushes the hypha to penetrate through the surface. Penetration is likely to be supported by enzymes that soften the host cell wall. To analyse the contribution of cell wall-degrading enzymes to the penetration process, Dumas et al (Dumas et al, 1999) used the endo-polygalacturonase promoter of *Colletotrichum lindemuthianum* (a necrotrophic fungus like *Monilinia spp.*), to control green fluorescent protein expression. These authors were able to show that the gene is expressed in *appressoria* prior to penetration. Finally, *Colletotrichum lindemuthianum*, as other necrotrophic fungi, required pectolytic enzymes not only for tissue maceration during *in-planta* growth, but also to assist forceful penetration.

*Appressorium* formation by *M. fructicola* on fruit surfaces has been related to BR incidence to fruit surface topography and hydrophobicity, as well as the presence of nutrients and fruit volatiles (Lee & Bostock, 2007), (Lee & Bostock, 2006). *Appressoria* were observed on the stomatal guard cell lips, and germ tubes apparently perceived particular topographical features to trigger differentiation of *appressoria*. Since *appressorium*-mediated penetration was observed both by natural openings (stomata) as well as by direct penetration of intact cuticle (through penetration pegs produced from *appressoria*), authors suggested that mechanisms may be diverse. In contrast, they did not observe *appressoria* on mature nectarine fruit. The authors suggested that *M. fructicola* restrains the formation of specialized infection structures such as *appressoria* to immature tissues and behaves as a saprophyte pathogen when nutrients are readily accessible, as in mature fruit. Also, a role of cAMP as well as calcium-calmodulin pathway was suggested in the formation of *appressorium* (Lee & Bostock, 2006).

#### **4.4 Appressoria melanization increase pathogenicity**

Melanins are brown-black pigments, biological macromolecules composed of various types of phenolic or indolic monomers that are produced by fungi and other organisms. Various fungi synthesize melanin from the oxidation of tyrosine. The extracellular dark pigments produced by fungi may be formed from various fungal phenols, usually named as heterogeneous melanins (Bell & Wheeler, 1986),(Butler & Day, 1998). The production of melanin by microorganisms has been associated with their virulence and the melanization of *appressoria* was considered necessary to different fungal pathogens for infection and disease development. Howard et al (Howard & Valent, 1996) proposed the importance of melanization for surface penetration. These authors exposed *appressoria* from the rice blast fungus *Magnaporthe grisea* to solutions of high osmotic pressure and observed no melanization and an inhibition of penetration of the leaves. They concluded that melanization is involved in the reduction of porosity of the *appressoria* wall. According to Dean (Dean, 1997), this causes the locking of cytosolic solutes efflux and leads to higher *appressoria* pressure.

Indeed, many fungal pathogens, such as *Venturia inaequalis*, *Magnaporthe gray*, *Pyricularia oryzae*, and *Colletotrichum legnarium*, need melanised *appressoria* to cause infection and disease development (Lee & Dean, 1993), (Butler & Day, 1998). De Cal and colleagues has reported that a melanin-deficient mutant strain of *M. laxa* (albino mutant) was no longer able to induce peach twig blight (Howard & Valent, 1996). They also observed that *M. laxa* treated with pyroquilon, an inhibitor of melanin biosynthesis, could not induce peach twig blight (De Cal & Melgarejo, 1994). Finally, they found that *in-vitro*, chlorogenic acid or pyroquilon added to the culture medium of *M. laxa* inhibited melanization of the colony (Villarino et al, 2011). They concluded that the ability of *M. laxa* to produce melanin is crucial for its pathogenicity.

Rehnstom and Free (Rehnstrom & Free, 1996) however, showed that melanin-deficient mutants of *M. fructicola* are able to infect nectarines, by producing lesions as large as those produced by the wild type. So they concluded that melanization is not required for the successful infection of host fruit. Nevertheless their presence could improve the success of development of fungi and increase their permanence in the field under adverse conditions.

#### **4.5 pH lowering regulates the expression of pathogenicity genes.**

Fungi are able to modify the host pH. Preliminary data on *M. laxa*, *M. fructicola*, and also *M. fructigena* indicate that they can reduce host pH during colonization of peach cvs. 'Big Top', 'Venus', and 'Tirrenia' by secreting gluconic acid (De Cal et al, 2013). Analysis of the acidification process in colonized fruit showed that gluconic acid was the main organic acid accumulated at the infection site

and under liquid-culture conditions. When compared to a nectarine cv. 'Big Top' and peach cv. 'Plácido' with differing sensitivities to *M. fructicola*, a 250% higher accumulation of gluconic acid was observed in the susceptible peach cultivar than in the less susceptible nectarine cultivar. Under liquid conditions, at pH 3.6–3.7, the relative expression of transcripts of *mfp2* and *mfp3*, encoding for two polygalacturonase genes of *M. fructicola*, increased 12-fold and 6-fold, respectively, suggesting the importance of acidification for the secretion of pathogenicity factors by *M. fructicola*. The authors also underlined the importance of acidification for the secretion of pathogenicity factors by *M. fructicola*, suggesting that ambient pH created by the pathogen is a regulatory cue that promotes pathogenicity expression. Specific genes contributing to pathogenicity may be expressed as a result of the environmental pH induced by the pathogen.

#### **4.6 Biochemical arsenal of *Monilinia* spp.**

Studies in the past decades ascertained the effects of fungicides on fungus enzymes in buffer extracts of mycelium of *M. laxa* (Calonge et al, 1969). Thus, they reported large groups of enzymes like catalases, peroxidases, glutamic dehydrogenases, esterases and alkaline phosphatases produced by this fungus.

The most important enzymes produced by *Monilinia* spp. may be the cutinases needed to penetrate the intact surface of fruit, (Bostock et al, 1999), (Lee et al, 2010). High levels of these enzymes may result from former activation, as in the necrotroph *Fusarium oxysporum* (Woloshuk & Kolattukudy, 1986). In the case of *M. fructicola*, gene expression of the cutinase *MfCUT1*, which is up-regulated in an oxidant environment, contributes directly to the virulence of the pathogen (Lee et al, 2010).

Cellulase has been found in all species of *Monilinia*, but its secretion seems to be very restricted. The cellulase secretion was detected in *M. laxa*, whereas for *M. fructigena* it was detected a trace of activity in extracts of rotted pear fruits. A very weak cellulase activity for *M. fructicola* in medium was found (Byrde & Willetts, 1977).

The polygalacturonic acid chain is attacked by three enzymes which are secreted by all three *Monilinia* spp., namely i) endo-polygalacturonase (EC 3.2.1.15), that hydrolytically attacks polygalacturonic acid, ii) pectin lyase or pectin methyl-trans-eliminase, (EC 4.2.2.10), that attacks a polygalacturonic acid of a high degree of esterification and iii) pectin esterase or pectin methylesterase (EC 3.1.1.11) which liberates the methoxyl groups from the carboxyl groups of the galacturonic acid. The optimum pH for each enzyme differs for each species (Byrde & Willetts, 1977). An important factor for the expression of *Monilinia* spp. polygalacturonases is the presence of calcium in the extracellular environment (Biggs et al, 1997). Recently, Chou et al (Chou et al, 2015) investigated five endopolygalacturonase (endo-PG) genes in *M. fructicola*. They were differentially



expressed during pathogenesis and in different culture media. *MfPG1* was the one mainly expressed. Gradziel and Wang (Gradziel & Wang, 1993) observed that an overexpression of *MfPG1* diminished virulence of the pathogen. Authors suggested that *MfPMG1* expression could be due to the activation of the plant defence by higher levels of reactive oxygen species (ROS) produced in this case.

Among enzymes degrading neutral sugars (arabinans and galactans) from the host cell wall,  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55) from *M. fructicola* was found to release monomeric arabinose from arabinans by hydrolysing the terminal bond<sup>127</sup>. This enzyme is localized in the hyphae of *M. fructigena* and may migrate to the plant plasmalemma and can be secreted by a process of reverse pinocytosis with involvement of multi vesicular bodies (Fuchs et al, 1965). Other enzymes that degrade neutral sugars have been found in *M. fructicola* as  $\beta$ -galactosidase, but have not been studied in details (Byrde & Willetts, 1977).

#### **4.7 Post-penetration**

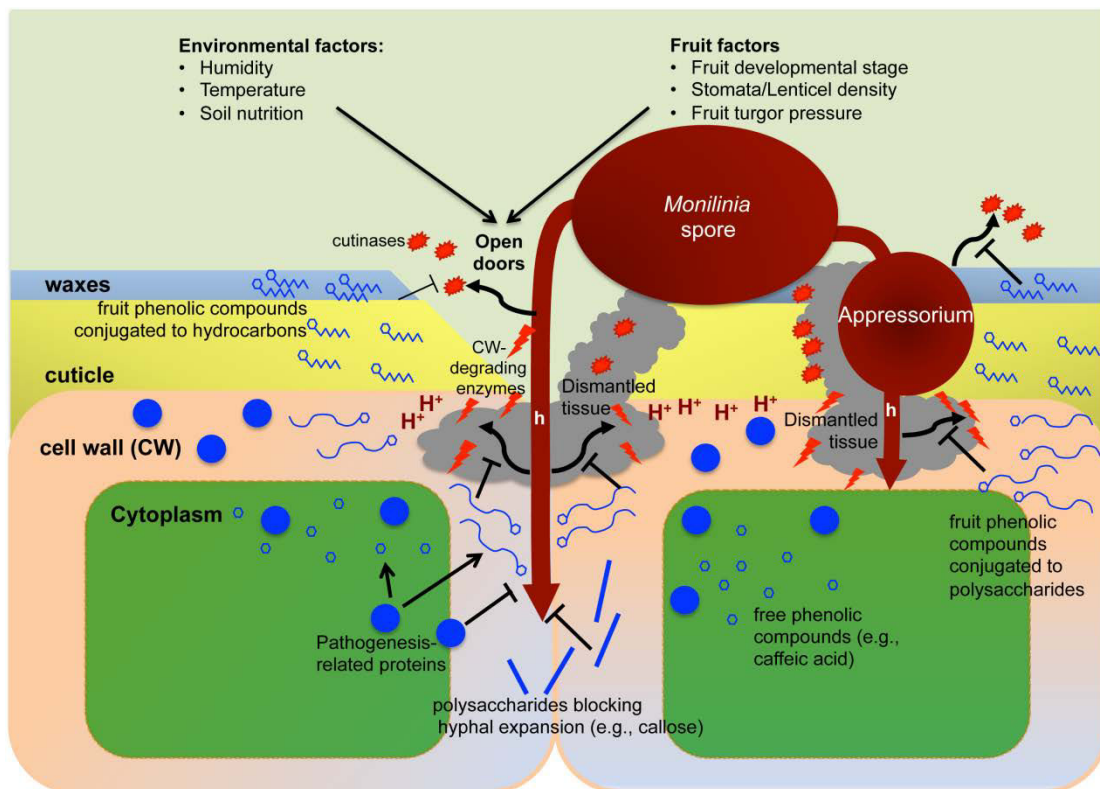
Once infection is established, the hyphae of the pathogen spread through the host tissues and bring about the symptoms such as the browning and softening of the tissue in fruit (Figure 7).

The spread of BR pathogens is generally intercellular. It could penetrate and permeate any part of the host. Investigations by Reinganum (Reinganum, 1964) showed a particular affinity of *M. laxa* for the middle lamella region. Transmission electron microscopy, of *M. fructigena* attacking pear fruit also confirmed that hyphae are generally intercellular, though in particular infections they become occasionally intracellular and the dead protoplasts are pushed across the cell lumen (Calonge et al, 1969).

Changes in the host plasmalemma could occur even if the membrane is intact. In infected tissues, its function could be drastically impaired as shown by conductivity measurements resulting in leakage of sugars and amino acids from cells. Subsequently, the pathogen has sources of carbon and nitrogen to use. Moreover if membranes of vacuoles, mitochondria, chloroplasts or other organelles have been damaged, their contents mix, following a process described as decompartmentalization (Byrde & Willetts, 1977).

Endopolygalacturonases and pectin esterases activities generate low molecular weight metabolites (Hall, 1971),(Pring et al, 1981). These secretions cause the collapse of the affected host cell (Lee & Bostock, 2006), (Pring et al, 1981),(Paynter & Jen, 1975). Willaman (Willaman, 1920), suggested that a hydrophilic gel of calcium pectate is formed from pectin degraded by *M. fructicola*. This gel may help the permanence of the fungus in the fruit mummy (Byrde & Willetts, 1977).

In fruit, the rate of increase in rot diameter depends on the combination of environment conditions, the host genotype, the pathogen species and the stages in fruit maturity (Calonge et al,



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

1969). After a few days, conidial pustules of the fungus burst through the fruit epidermis and cuticle. Apart from allowing the fungus to perpetuate itself, this bursting leads to the desiccation of the host tissues and often, ultimately to the formation of a mummified fruit. In the meantime, the pathogen develops a stroma of dense mycelium within the host (Byrde & Willetts, 1977).

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

## **5 Host factors for BR resistance/susceptibility in fruit**

Research has long tried to identify host factors contributing to BR resistance. Byrde and Willetts (Byrde & Willetts, 1977) listed some of them: flowering date, fruiting habit, gumming of wounds for cherries, duration of flowering for apricots, cork in lenticels, fiber and pentosan contents, parenchyma plugs in stomata, skin thickness, and texture on ripening for plums. However authors underlined the importance of caution since evidence is based on only few cultivars.

To date there is limited evidence on factors limiting BR in mesocarp and most research has shown that BR resistance relates to fruit epidermis (Bostock et al, 1999), (Lee & Bostock, 2007), (Gradziel & Wang, 1993), (Pascal et al, 1994), (Gradziel et al, 2003).

### **5.1 Constitutive components of BR resistance: plant cuticle, a multi-component barrier**

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

The first level is the epicuticular wax layer that covers the cuticle and is a complex mixture of very long saturated, unbranched chain aliphatics and n-alkanes, ranging in carbon number from 21 to 33, depending on the plant taxa (Baker, 1982), (Barthlott, 1990), (Bianchi, 1995). Waxes can form

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

However factors such as temperature, the health status of the plant and the chemical treatments may interfere with this process, (Silva et al, 2005). In their review, Reina-Pinto (Reina-Pinto & Yephremov, 2009) exposed various studies demonstrating that cuticular lipids play a role as messenger molecules in plant–pathogen interactions. For instance, Podila et al (Podila et al, 1993) showed that the germination and *appressorium* formation by *Colletotrichum gloeosporioides* in avocado is induced specifically by the surface waxes of this host, but not by waxes from other plants (Kolattukudy et al, 1995). The authors explained this effect by the longer-chain in fatty alcohols, the presence of terpenoid components and absence of inhibitors that allow the fungus to use the host surface wax to trigger germination and differentiation of infection structures. Some studies reported stimulatory effects of extracted cuticular waxes on the germination and differentiation of *Magnaporthe grisea*, *Metarhizium anisopliae* and *Puccinia graminis f.sp. tritici* (Hegde & Kolattukudy, 1997), (Inyang et al, 1999), (Reisige et al, 2006). Similarly, *Blumeria graminis f.sp. hordei* germination was more rapid and greater on the surfaces of intact than dewaxed barley (Zabka et al, 2008)

On the contrary, it was reported that cuticular waxes inhibit conidial germination of plant pathogens, such as *Podosphaera leucotricha* on certain varieties of apples (Calonge et al, 1969). This evidence suggests that the different constituents of waxes may play opposing roles for the pathogens. The extension of the scope of the results exposed above is limited since the quantity and composition of cuticular wax shows great variability among different plant species, different organs of an individual plant, and/or during the ontogeny of individual organs (Jetter et al, 2000). Unfortunately, with respect to *Monilinia* spp., there is a lack of information on the role of waxes in the fruit-fungi interactions and a direct translation of results from other plant-pathogen couples is not valuable. Further specific studies are therefore needed to decipher waxes role in *Monilinia* spp. infection.

The cuticle is the second barrier that the fungi need to cross. This structure consists of hydrocarbon polymers and cutin synthesized exclusively by the epidermal cells. For example, the cuticle of *Prunus persica* fruit has been characterized as a complex of structures with various protective purposes. In this species, cuticle is composed of 53% cutan, 27% waxes, 23% cutin and 1% hydroxycinnamic acid derivates; trichomes are covered with a thin cuticular layer containing 15% waxes and 19% cutin and filled by polysaccharide material (63%) containing hydroxycinnamic acid derivatives and flavonoids (Fernandez et al, 2011).

The cuticle is structurally diverse among species, but exhibits the organization of a composite material consisting of cutin, polyester that is partly covered and interspersed with waxes (epicuticular and intracuticular waxes) (Mettraux et al, 2014). However, a characterization of the fruit

surface of diverse varieties of *Prunus*, in order to determinate cultivar-specific skin features, has not been developed to date. Considering the cuticle as a structure of resistance to pathogen penetration deserves caution, seeing that its proprieties are dependent on qualitative and quantitative chemical composition. Indeed, a complex inter-relationship between the cuticular lipids and the fungus may occur, playing a molecular messenger role in interactions between plant and pathogen. As well as some components of epicuticular waxes, they can act as fungal pathogenicity activators or in contrast, inhibit the infection. Isaacson et al (Isaacson et al, 2009) demonstrated on tomato that cutin plays an important role in protecting tissues from necrotrophic infection by *Botrytis cinerea*. According to Kolattukudy et al (Kolattukudy et al, 1995), some pathogens sense plant surfaces thanks to cuticle monomers that may be produced by basal cutinase activity of fungal spores. Sensing of cutin monomers would then induce high levels of cutinase required for penetration.

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

The last barrier in the surface is the epidermis cell wall that could vary in composition and thickness. The major substance that reinforces the cell wall structure is the lignin. The process of lignification could improve the resistance of the cell wall against the action of degradation enzymes; block the diffusion of pathogen toxins and the diffusion of nutrients from the fruit, restringing the process of colonization. Sites around the infection point could also accumulate callose, suberin, tannin and pectin substances (Vance et al, 1980).

## **5.2 Phenolic acids and their redox-mediated role in fungal inhibition**

Early studies of peach phenolic compounds started from the observation that fruit from ‘Bolinha’ peach cultivar, known to be resistant to BR, displayed high levels of these compounds in their epidermis. This group of compounds became one of the most studied for BR resistance (Gradziel & Wang, 1993).

Among the phenolic compounds of the epidermis of peach fruit, chlorogenic and caffeic acids have high concentrations, especially in immature fruit and in fruit of peach genotypes, with a high level of resistance to *M. fructicola*. In cultures of *M. fructicola*, these phenolic acids did not suppress spore germination or mycelia growth but they inhibited cutinase activity. Likewise, the presence of caffeic acid in cultures prevented the appearance of two major cutinase isoforms. In addition, a series of cinnamic and benzoic acid derivatives also suppressed cutinase levels in culture (Villarino et al, 2013). These results led the authors to suggest that chlorogenic acid and related phenolics, in combination with other factors such as iron, could have a role in arresting *M. fructicola* in quiescent

infections (Hily et al, 2011). Furthermore, they may contribute to resistance by interference with the production of factors involved in degradation of host polymers. Subsequent studies *in vivo* confirmed the effects of caffeic acid, chlorogenic acid, or reduced glutathione on infection development. Adding those compounds in conidial suspensions of *M. fructicola* did not inhibit germination on flower petals and fruit, but inhibited *appressorium* formation from germinated conidia and subsequent BR lesion development (Lee & Bostock, 2007).

Further work conducted by the same group showed that antioxidant phenolic acids suppressed mRNA accumulation and enzyme activity of a cutinase (Wang et al, 2002). However, other antioxidant compounds also significantly attenuated *M. fructicola* cutinase production, indicating a general effect of antioxidants rather than a specific effect of a given phenolic compound (see section 5.3) (Lee et al, 2010), (Wang et al, 2002).

Villarino et al (Villarino et al, 2011) demonstrated that chlorogenic acid and its isomer, neochlorogenic acid, can interfere with the production of melanin in *M. laxa* without any effect on the growth and germination of the fungus (see section 4.4). (De Cal & Melgarejo, 1993). Even though these results are interesting, the role of the different phenolic compounds in limiting *Monilinia spp.* remains unsolved. Prusky and Lichter (Prusky & Lichter, 2008) have reviewed pathogen quiescence in post-harvest diseases and discussed how fruit factors such as high acidity and phenols in unripe fruits can contribute to disease resistance.

### **5.3 Active mechanisms in response to pathogen attack: defence proteins**

Although cuticle research has mainly focused on the analysis of cuticular lipids, cuticular proteins may also be of importance. They are referred to 'Lipid Transfer Proteins' (LTPs) and many have been shown to play an important role in plant defence (Reina-Pinto & Yephremov, 2009). They specifically inhibit pathogen and pest enzymes by forming complexes that block active sites or alter enzyme conformations, ultimately reducing enzyme function. They include defensins, amylase inhibitors, lectins, and proteinase inhibitors. Unlike simple chemicals such as terpenoids, phenolics, and alkaloids, proteins require a great deal of plant resources and energy to be synthesised consequently, many defensive proteins are only made in significant quantities after a pathogen or pest has attacked the plant. Once activated, however, defensive proteins and enzymes effectively inhibit fungi.

On defensins in particular, Nanni et al (Nanni et al, 2013) investigated the possible role of *Ppdfn1* in peach defence against fungal pathogens. *Ppdfn1* gene expression was analysed in peach tissues susceptible to *M. laxa*, such as flowers and fruit, and its induction upon pathogen infection was tested. They concluded that *Ppdfn1* displayed an antifungal activity through specific interactions with the membrane lipids of the fungi.

Plants also produce hydrolytic enzymes, such as chitinases, glucanases or lysozymes, in response to fungi attacks. Zemanek et al (Zemanek et al, 2002) showed increased levels of mRNAs encoded by  $\beta$ -1,3-glucanase gene following treatment of a peach cultivar with culture filtrates of the fungal pathogen *M. fructicola*.

The changes in the transcriptional level of genes coding to pathogenesis-related proteins (PR) has also been associated with the BR-infection process in European plum fruits (*Prunus domestica* L.). It is well known that some families of PR proteins are inducers of phenylpropanoid accumulation and other resistance effectors (Mur et al, 2004). El-kereamy et al (El-kereamy et al, 2009) described differential expression patterns of PR-10 coding gene among two European plum cultivars with contrasting BR-resistance phenotypes, as well as other transcripts coding to intermediary proteins in the signalling pathway of this PR. The authors observed that after *M. fructicola* artificial inoculation, transcripts of PR-10 and phospholipase D-alpha (PLD $\alpha$ , a cell membrane-phospholipid degrading enzyme, involved the signalling of stress responses) remained constitutively expressed in the resistant variety (cv. 'Violette'), while in the susceptible one (cv. 'Veeblue') these levels increased after pathogen attack. Hydrogen peroxide concentration in fruit tissues correlated with transcript pattern of these genes on both cultivars, with higher but steady levels of the compound in the resistant cultivar, suggesting an inhibitor role for the pathogen.

The same authors demonstrated the antifungal activity of PR-5, whose differential expression among plum cultivars was correlated with their BR-resistance. Activity was with a pattern similar to PR-10, *i.e.*, no significant change in PR-5 transcript levels after infection in resistant cultivars ('Violetta' and 'Stanley') and a rapid increase in susceptible genotypes ('Veeblue' and 'Victory'). Furthermore, the ectopic overexpression of this protein in *A. thaliana* transformants increased resistance to *Alternaria brassicicola*, as well as a higher induction of camalexin biosynthesis, and transcript abundance of genes coding to phenylalanine-ammonia lyase (PAL, a central point in phenylpropanoid and phytoalexin biosynthesis) and to three cytochrome P450 involved in the biosynthesis of some anti-fungal phenolics. (Wagner Júnior et al, 2011) Finally, the same authors (El-kereamy & Jayasankar, 2013) described a very similar expression pattern after *M. fructicola* infection in the gene coding to MYB3 transcription factor of European plums, suggesting an intermediary role of this transcription factor in the hormone-mediated defence responses that result in the induction of PR proteins. The study of the variability of these genes, which have effects in defence pathways, in *Prunus* germplasm collections has a crucial importance in the generation of knowledge for the development of more resistant varieties of fruit species.

## 5.4 ROS, oxidative stress and programmed cell death

The knowledge of virulence mechanisms in BR is still rudimentary; however recent research reported that ROS play dual roles in plant-host interactions. The production of ROS can either stimulate host resistance or enhance pathogen virulence. Chiu et al (Chiu et al, 2013) examined the regulation of the gene *MfCUT1* (that encodes the major cutinase of *M. fructicola*) by redox status. Authors reported that gene expression is down-regulated by caffeic acid (CA) and by the antioxidant glutathione (GSH) and up-regulated by a GSH synthesis inhibitor, the buthionine sulphoximine (BSO). These results indicate that changes in cellular redox status could impact virulence of BR and suggested that redox cycling is related to this regulation.

Liu et al (Liu et al, 2013) investigated the production of hydrogen peroxide, a major component of ROS in peach flower petals in response to *M. fructicola* and *Penicillium digitatum*, a non-host pathogen. During the interaction with the host, *M. fructicola* induced hydrogen peroxide accumulation in flower petals, high levels of protein carbonylation, lipid peroxidation and a significant reduction of hydrogen peroxide accumulation in tissues. They also observed a reduction in the incidence of BR with application of exogenous antioxidants. The presence of *M. fructicola* spores at the surface of intact flower petals induced gene expression and increased enzyme activity of NADPH oxidase, a membrane-bound enzyme complex important to generate ROS and cell wall peroxidase in host tissues. This resulted in the production of hydrogen peroxide while the same tissues inoculated with a non-host pathogen did not show significant responses (Liu et al, 2013). These results suggested that the antioxidant compounds can influence intracellular antioxidant levels in the pathogen, and that changes in the redox environment may influence both gene expression and the development of structures used by the pathogen to facilitate infection (Lee & Bostock, 2007).

In some cases the fruit can respond by death of cells around the point of infection, the formation of phellogen at the margin of twig lesions in stone and pome fruit trees (Zwigart, 1970), the suberisation of walls of surrounding living cells in fruit and the accumulation of phenolic compounds in cells up to 20 cells around the distant site of initial infection. Despite such responses aimed at limiting the spread of BR, growth of mycelium may continue although the activities of some enzymes are inhibited. Several penetrations within a small area would produce a greater and more obvious reaction by the host. The results obtained by Jenkins and Reinganum (Jenkins & Reinganum, 1965) with *Sclerotinia fructicola* on stone fruit, suggest that sometimes the host response to penetration permanently inactivates the fungus.

The diversity of studies and results published indicates a complex multifactor resistance that may involve different types of defence localized in different tissues (epidermis and mesocarp). They highlighted the involvement of constitutive factors (mechanical barrier), active compounds (waxes,



cutins, phenolic acids) as well as specific responses to the attack (proteins and enzymes, ROS). However, no generic model of fruit resistance to BR has been proposed.

## **6 Breeding for brown rot (BR) resistance**

Currently, commercial cultivars are more or less sensitive to BR. The peach cultivar known to have one of the highest level of resistance is the Brazilian cultivar 'Bolinha' (Gradziel et al, 1998), (dos Santos et al, 2012). Feliciano et al (Feliciano et al, 1987) investigated resistance in peach cultivars and found that 'Bolinha' had fruit with particularly small size and a thick cuticle with high phenolic content. This cultivar has been used as a donor of BR resistance in conventional breeding for canning and low-chill peach despite its poor fruit quality, high susceptibility to enzymatic browning, reduced fruit size, and high rate of pre-harvest fruit drop (Gradziel et al, 2003), (Topp et al, 2008), (Wagner Júnior et al, 2008), (Wagner Júnior et al, 2011). The case of 'Bolinha' demonstrates the challenge of breeding for BR, as characteristics associated with fruit resistance may conflict with commercial requirements. As mentioned before, Bostock et al (Bostock et al, 1999) suggested that cuticular characteristics may be involved in BR resistance. Many other fruit traits discussed in previous sections of this review may be implicated in host resistance to BR in stone fruit. However, the statistical and genetic correlations of those traits with the BR phenotype, as well as their genetic basis are poorly understood.

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

### **6.1 Genetic resources, breeding programs and phenotyping strategies**

As mentioned before, some traits associated with host resistance to BR are present in cultivars or accessions of poor commercial and productive quality. Identifying reliable sources of resistance to be introgressed in high fruit quality genetic backgrounds is one of the main objectives of such breeding programs. However, one of the first steps for the establishment of breeding programs or genetic studies for a given trait is the definition of a reliable measurement or phenotyping protocol, to compare afterwards the phenotypic variations among a population of genetically diverse individuals (cultivars, accessions or offspring from a cross), and then identify interesting breeding

materials in the basis of robust phenotypic data. In the case of assessment of cultivar-dependent BR impact on stone fruit, there is a lack of consensus in the employed experimental strategies and each laboratory has adopted a particular protocol, according to its experimental capacities and /or specific objectives.

## **6.2 Field-borne inoculum assessment**

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

In the Fruit Research Institute of Cacak (Serbia), preliminary evaluation of BR resistance of indigenous “vineyard” peach accession germplasm was made by the use of a six-level scale, which allowed the identification of 11 evaluated accessions showing higher resistance level (described as “Symptoms are not observed”) during three years, among a total of 75 genotypes evaluated (Paunovic & Paunovic, 1996). In the same research center, but in the sour cherry breeding program, (Radicevic et al, 2010), (Radicevic et al, 2012), a subjective scale from 1 to 9 (1 for no attack, 9 for very strong attack) was used to evaluate 11 advanced selections at the final step of the selection process, as well as 9 landraces from autochthonous germplasm (Bregar et al, 2012). Advanced selections showed relatively high levels of resistance (score between 2 and 3), but a slightly higher diversity was found in the local genotypes collection (from 1 to 4). Subjective scale scoring was also used in the sour cherry breeding program of the Institute of Plant Breeding in Dresden, Germany, as well as in the beginning of the peach breeding program aiming to develop cultivars adapted to humid and temperate climates at Embrapa in Pelotas, Brazil, from which mid- to high-resistant cultivars such as ‘Olympia’ and selection ‘Conserva 947’ have been generated (Raseira & Bonifacio, 2006).

## **6.3 Artificial infection assessment**

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



**Figure 9.** Development of brown rot 5-days post artificial infection in nectarines of cv. 'Summergrand' at maturity. Fruit were disinfected in water bath at 55°C for 40 seconds, put in acrylic plastic boxes and infected with one 10µL drop at  $10^5$  spores.ml<sup>-1</sup> of concentration deposited without wounding. Fruits were put in a chamber with controlled temperature (18°C) and 24°C respectively during dark (8 hours per day) and light (16 hours per day). High humidity was maintained in the closed boxes.

One of the first groups that started to use artificial inoculations of BR was at UC Davis, within the cling peach-breeding program, (Gradziel & Wang, 1993),(Gradziel, 2002), (Gradziel, 2012). Researchers considered the average rot diameter 72 hours after inoculation (10 µl drop of a conidial suspension of *M. fructicola* containing 10<sup>5</sup> spores/ml, on previously with diluted sodium hypochloride and ethanol-disinfected fruits), as specified elsewhere (Martinez-Garcia et al, 2013). In this way, a large phenotyping effort has been carried out to screen mature fruit for resistance to *M. fructicola* in over 4000 peach genotypes from very different origins: landraces, standard canning peach cultivars, advanced experimental selections with various pedigrees including some with 'Bolinha' heritage as well as some interspecific hybrids generated in order to introgress BR resistance from almonds. The material selected with this protocol has been useful also for studies of genetic dissection of the BR resistance trait in segregating populations (see "QTLs for BR resistance sections").

As mentioned in other sections of this review, Pascal et al (Pascal et al, 1994) evaluated two screening tests for resistance to *M. laxa* in apricots (7 accessions), peaches (12 accessions) and diploid plums (7 accessions of *P. salicina*, *P. cerasifera* and interspecific hybrids between them) at INRA, Avignon, France. The tests consisted of artificial inoculation of uninjured and artificially injured fruit. Each fruit was inoculated with a 20 µl droplet containing conidia of *M. laxa* at a concentration of (10<sup>6</sup> spores/ml). Percentage of infected fruits and rot diameter progression were recorded, Figure 9. The authors observed no correlation between the BR resistance rankings from the uninjured and injured tests. Accordingly, they suggested that epidermal resistance and flesh resistance were not linked processes. This work also highlighted high variability of lesion progression within the uninjured test and very similar rot spread within the injured test, suggesting that no resistance expressed at the flesh levels in the tested material. In INRA-Avignon, a breeding program focused on pest resistance (including resistance to BR by *M. laxa*) has generated very interesting materials, such as introgression of *Prunus davidiana* resistance to peach materials (Pascal et al, 1998).

Material from the breeding program of Embrapa-Pelotas (Brazil) has also been screened with artificial inoculation, and BR resistance results on these breeding materials have been reported (dos Santos et al, 2012). BR screenings were made by monitoring the percentage of infected fruits 72 and 96 h after spraying a solution (containing 10<sup>5</sup> spores/ml) over intact harvested fruits. The authors observed a significant genetic component when comparing some selections and cultivars. Interesting selections such as 'Conserva 1798', 'Conserva 1596', 'Conserva 1218' and 'Cascata 1493' were identified (Wagner Júnior et al, 2008). Authors evaluated three crosses ('Conserva 672' x 'Maciel', 'Conserva 672' x 'A.334' and 'Leonense' x 'Bolinha') by drop-inoculations. Broad-sense heritability was estimated to be around 80%. Twelve seedlings from these three progenies were determined to be of equal or better resistance than the 'Bolinha' cultivar.

Resistance to *Monilinia* spp. in peach cultivars for the fresh market has been an important objective in the breeding program of University of Milan (formerly at University of Bologna, Italy). Offspring from crosses between melting flesh peaches were selected (Bassi et al, 1998), (Bassi & Rizzo, 2003). In these works, mature fruits were artificially sprayed with a suspension of *M. laxa* ( $10^5$  spores/ml), and the disease impact was registered as percentage of infected fruits after 5 days of incubation at 25°C and 95-100% relative humidity. Several parental combinations were analysed. The 'Contender' x 'Elegant Lady' F1 population presented the most interesting results with individuals presenting higher levels of resistance than the resistant parent (cv. 'Contender'). Besides generating pre-breeding materials, this population has been useful in the genetic dissection of BR resistant-related traits (Pacheco et al, 2014). This group is currently developing new phenotyping strategies, based on *in planta* spray of conidial suspension of *M. laxa*, aimed at increasing the capacity of sample analysis in breeding programs bearing high numbers of seedlings, obtaining promising results for scoring BR-resistance phenotype (Pacheco et al, 2015).

Studies of BR resistance evaluations in apricots by artificial inoculum have been reported mostly from two breeding programs. At the Regional council for agriculture of Rome (CRA-FRU, Italy), several apricot accessions showing high BR resistance have been evaluated by artificial inoculation procedures consisting in fruit disinfection (diluted sodium hypochlorite and ethanol), inoculation with a drop of *M. laxa* conidial suspension ( $10^5$  spores/ml) in two points near peduncle cavity, incubation for 7 days at 22°C and registration of affected fruit percentage. Among the evaluated crosses, the authors found remarkable levels of BR resistance: selections such as '485GII37', '493C12III61' and '493 C12 VI 1' (open pollinations of cultivars 'Don Gaetano', 'Fiammetta' and 'Boreale', respectively) showed 0-10% of infected fruits; while 'Don Gaetano' F2 seedlings like '493C11VIII8' or '493C11VIII26' showed very high infection rates (more than 50%). Based on the observed segregations, the authors concluded that BR resistance on the analysed crosses behave as a quantitative trait (Côté et al, 2004), (Nicotra et al, 2006), (Conte et al, 2010).

Walter et al (Walter et al, 2004), tested several methods to evaluate BR in 'Sundrop' and 9 accessions from the 'Clutha' series ('Sundrop' x 'Moorpark'), bred in HortResearch at Clyde research orchard (Alexandra, New Zealand). In this study, the authors analysed some infection parameters for 3 seasons: lesion area (artificial drop infections with *M. fructicola* and *M. laxa* spore suspensions in wounded and intact fruits), spore count on lesions, storage rot (natural orchard infection at room temperature and high humidity) and cuticle thickness. The authors determined that the most robust method to evaluate BR resistance in apricot was measuring lesion area on wounded, artificially infected fruits 72h after inoculation. However they recommended combining more than one method for the evaluation of the material. Remarkably, the accession 'Clutha 14/107' showed significantly highest value of resistance to *M. fructicola* (measured as the mean of lesion area obtained in 3

seasons), the lowest quantity of produced spores per mm<sup>2</sup> lesion, a storage rot rate of less than 5% and one of the highest cuticle thickness.

BR resistance was screened in several released cultivars and advanced selections from the sweet cherry breeding program at the Pacific Agri-Food Research Centre, British Columbia, Canada (Kappel & Sholberg, 2008). During 4 years, a total of 36 genotypes were submitted to artificial inoculation (25-50 fruits triplicates per accession; ethanol and sodium hypochlorite fruit disinfection; spray of 10<sup>4</sup> spores/ml; incubation at 13°C and 95-97% relative humidity; BR impact assessment after 8 and 11 days after inoculation). Based on the difference in the percentage of rotten fruits between each genotype and the overall population mean, they established three resistance categories: more resistant than overall mean, close to mean and less resistant than mean. Although they identified some cultivars showing a high resistance level in two of four years (cultivars 'Staccato'<sup>TM</sup>, 'Stardust' and 'Sweetheart'), the authors stated that the observed resistance level was not enough to avoid fungicide applications in plants of these accessions and confirmed the results of Brown and Wilcox (Brown & Wilcox, 1989), demonstrating that there are no sources of high level genetic resistance to BR in sweet cherry materials.

Although it is difficult to find reliable sources of resistance in stone fruit species, seasonally consistent differences in the tested materials have been observed in all the works presented in this section. The existence of these differences indicates that exploring wider germplasm and using these sources to introgress resistance in cultivars of high fruit quality could result in new selections with improved BR resistance (Gradziel, 2002). The 'BR resistant' cultivars and selections found up to now still have too low resistance levels to allow the suppression of fungicide application; however, the most resistant could already be cropped under integrated pest control strategies, suited to minimize exogenous chemical input in the orchard.

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

## **6.4 QTL of resistance**

In order to generate new cultivars with less necessity of fungicide inputs, the identification of genes or loci associated with resistance to BR would allow progressing in the incorporation of favourable alleles in breeding programs. In addition to functional studies seeking to understand the

interactions between the pathogen and its host, genetic studies have been conducted to identify genomic regions associated with BR resistance. Although possible mechanisms of resistance can be inferred from these studies, their principal objective is the discovery and further incorporation of resistance alleles into breeding materials with the use of linked markers. Indeed, high-throughput molecular genetic tools and a high-quality genome sequence have been developed recently for peaches, (Verde et al, 2013) and can now be exploited to radically improve the efficiency of disease resistance breeding in peaches, as well as in other *Prunus* species. Indeed, as commented before, breeding programs aimed at enhancing BR resistance have been impaired by time-consuming procedures for assessing this trait on field-grown segregating trees.

Therefore, an important objective is the generation of new tools for the early selection of seedlings with enhanced BR resistance. Marker-assisted selection is a valuable strategy for these purposes, as it allows the early selection of seedlings bearing favourable alleles at marker loci genetically linked to genomic regions that control the trait of interest. Considering that fruit resistance to BR may be a multifactor system and that each different cultivar may hold only a little part of these factors, dealing at the same time with different sources of resistance may lead to confusion rather than to better understanding. Therefore, association studies have not been engaged and the first studies seeking for QTL of resistance had focused on bi-parental progenies stemming from a cross between a susceptible parent and a potential donor of resistance. This approach may represent the first compulsory step to identify genome regions controlling resistance. Hopefully, the comparison of detected loci between crosses may help identifying different factors of resistance coming from different donors. The final step would then be the combination of these different factors in elite genotypes to confer higher resistance.

To date, two studies exploring genomic regions linked to BR-resistance have been published, both using peach host species. Martinez-García et al (Martinez-Garcia et al, 2013), performed a QTL analysis using *M. fructicola*-resistance phenotypic data of 73 seedlings from the Pop-DF progeny ('Dr. Davis' x 'F8, 1-42'), with parental accessions derived from canning peach and peach-almond back-crossing in the UC Davis breeding program (Martinez-Garcia et al, 2013). A linkage map composed by 1037 SNPs segregating through the population, was used for Interval Mapping-QTL analysis. The study revealed three QTL, two of them in LG1 and one in the LG4 of *Prunus* genome. The genomic region of one of the QTL in LG1 was significantly correlated with three years of phenotypic evaluation. The region included two potential candidate genes, coding for PAMP-triggered immunity, and effector-triggered immunity (ETI) proteins. SNP markers of this region are promising tools to enhance efficiency of breeding programs using similar genetic background.

The second genomic study based on QTL analysis was performed using 80 melting-fleshed F1 individuals from the 'Contender' x 'Elegant Lady' cross, genotyped with a set of 89 markers (63 SSR

and 26 SNP) and phenotyped for two seasons with artificial infections of *M. fructigena*, in the presence and absence of an artificial wound. The aim was to find genetic markers associated with skin and flesh resistance to BR (Pacheco et al, 2014). In this material, maturity date of seedlings correlated negatively with their BR resistance (late-maturing individuals appeared as less resistant); however, using a Multiple QTL model including maturity date as a covariate phenotype, significant genotype-phenotype associations were found between skin resistance and both M1a and EPPISF032 SSR markers (located in the LG2 and 4 of *Prunus* genome, respectively). Additionally flesh resistance was correlated with SNPs located in LG3 of peach genome, confirming the independence between genetically controlled mechanisms for skin and flesh resistance (Willaman, 1920).

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

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

## 7 Conclusion

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

In this review we have collected the information available in historic and contemporary literature about the elements involved in the interaction between *Monilinia* spp. and *Prunus* fruit. We conclude that host specificity is not a strict condition for disease impact and infection development and that one of the main causes for the success of pathogen colonisation is the relatively high presence of “open doors” in some *Prunus* fruit’ epidermis, especially in peaches, cherries and plums. In the last decade, many works have identified and validated some important elements of the fungal infection and host resistance processes; nevertheless, the scientific community has not assembled these elements to generate a precise BR resistance model that



explains the phenotypic diversity among *Prunus* species and their varieties. Finally, the significant influence that environment has in the infection process has been a persistent constraint that hampers a clear identification of such elements, but has to be considered in the generation of new varieties.

These elements constitute valuable information and are useful in the design of new phenotyping approaches for breeding, as well as to test new alternative methods for BR control at the pre- and post-harvest stages. BR-resistant breeds and sustainable pathogen control strategies are being developed and validated (Rungjindamai et al, 2014),(Martini & Mari, 2014). In the meantime, stone and pome fruit growers have the difficult task to combat damages caused by BR with lower quantities of synthetic fungicides, as recommended (or imposed) by IPM regulations and initiatives, and by adopting agronomical strategies and practices to eliminate natural inoculum sources.

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## Chapitre 2 : Etude de la probabilité d'infection au cours du développement du fruit, en lien avec les caractéristiques structurales et biochimiques du fruit

La cuticule recouvrant la surface externe de l'épiderme des fruits est la première barrière de protection contre les stress abiotiques et biotiques. Certaines des propriétés les plus importantes de l'épiderme proviennent de la composition de la cuticule. Dans ce travail, la dynamique d'accumulation de ces composés secondaires à la surface de fruits de pêche a été étudiée pour trois cultivars (*Persica Prunus*). A cet effet, des triterpénoïdes, des composés phénoliques et des cires cuticulaires ont été analysés à différents stades du développement du fruit par chromatographie en phase gazeuse (GC) et chromatographie liquide à haute performance (HPLC). De nouveaux composés, des dérivés de triterpénoïdes, ont été identifiés et les profils au cours du temps des différents composés ont été comparés. Nous avons essayé d'établir des relations entre les changements de ces composés et les caractéristiques du fruit qui démontrent également une dynamique temporelle marquée le long du développement des fruits, telles que la conductance cuticulaire et la probabilité d'infection par la moniliose. Un ensemble de 35 composés différents ont été analysés par GC, regroupés en 8 classes: triterpènes, alcanes, acides gras, alcools gras, aldéhydes gras, composés phénoliques, phytostérols et non identifiés. A notre connaissance, ceci est la première fois que les acides p-Coumaroyl-dihydroxy-URS-12-en-28-oïque et 3 $\beta$ -p-Coumaroyloxy-URS-12-en-28-oïque, des dérivés de triterpénoïdes, sont identifiés à la surface de pêches. Même si un lien temporel simple peut être observé entre les différents processus intervenant dans le développement de fruits, de bonnes relations ont été observées entre certaines cires et la conductance cuticulaire, ce qui pourrait expliquer en partie la réduction de la conductance de la cuticule au cours du développement du fruit. Le lien entre la probabilité d'infection par la moniliose et les composés de surface a également été étudié avec des résultats intéressants, à savoir des inhibiteurs et inducteurs potentiels ont été identifiés. Cependant, plus d'analyses sont nécessaires pour explorer l'effet potentiel de ces composés sur l'activité fongique.

# Profiling of triterpenoids, phenolics, and cuticular waxes from fruit surface during peach development

## Abstract

The cuticle covering the outer surface of epidermal fruit is the first protective barrier against abiotic and biotic environmental stresses. Some of the most important properties of epidermis come from the cuticle composition. In this work, the developmental dynamics of secondary compounds of peach fruit surface was investigated for three *Prunus persica*. For this purpose, triterpenoids, phenolics, and cuticular waxes were analyzed at different stages of fruit development by gas chromatography (GC) and High-performance liquid chromatography (HPLC) analyses. New compounds, derivatives from triterpenoids, were identified and the profiles along time of the different compounds were compared. Besides, we tried to establish relationships between the changes of these compounds and characteristics of the fruit that also displayed marked temporal dynamics along fruit development as fruit cuticular conductance and brown rot infection probability. From GC analyses, a set of 35 different compounds were analyzed, grouped into 8 classes: triterpenes, alkanes, fatty acids, fatty alcohols, fatty aldehydes, phenolics, phytosterols and unidentified. To the best of our knowledge, this is the first time that p-Coumaroyl-dihydroxy-urs-12-en-28-oic acids and 3 $\beta$ -p-Coumaroyloxy-urs-12-en-28-oic acids, derivatives of triterpenoids, were identified on peach fruit surface. Even though a simple temporal link might be observed between fruit developmental processes, good relationships were observed between some waxes and cuticular conductance, which could explain in part the reduction of cuticular conductance during fruit development. The link between brown rot infection probability and surface compounds was also investigated with interesting results, i.e. potential inhibitor and inductor compounds were found. However this investigation deserves more analyses to prove the relationships with the fungal activity.

## 1 Introduction

Stone fruits are important in agricultural economies of many countries. With a global production of 21.6 kilotons in 2013 (FAOSTAT), the production of peaches and nectarines is one of the ten largest fruit production in the world (Statista). In addition, peach is the third most important deciduous fruit crop in the world, and the second in European Union (EU) after apple. In Europe, peach production corresponds around of 20 % worldwide production the most important producing countries are Italy, Spain, Greece, Turkey and France, respectively (FAOSTAT). The vast majority of

peach production is consumed fresh and fruit, highly perishable, can only be stored for a few days. In addition, post-harvest diseases can cause severe losses in peaches. Monetary losses are especially high because all production, harvest, and packing costs have already been invested in the fruit.

Brown rot (BR) is the most important in pre and post-harvest disease of peaches. Caused by the fungus *Monilinia spp.* it can provoke as much as 30 to 40% losses of the crop. No other alternative than chemical treatment is available, hence fungicide applications are required until pre-harvest. Such applications are damaging the environment and may let residues in fruits. According to Martini and Mari, (Martini & Mari, 2014) the worldwide yearly value of *Monilinia* losses is 1.7 thousand million Euro.

In fruit, *Monilinia spp.* have often been considered as opportunistic fungi that may enter in the tissue only via naturally occurring entry points, even if direct penetration by degradation of cuticle and epidermal tissue has been observed (de Oliveira Lino et al, 2016). A relevant study about the effect of the presence of skin barrier in BR resistance was reported by (Pascal et al, 1994). Injured-fruit infection developed on all fruit with quite similar speed in all species. On the contrary, when uninjured fruit were infected, large variability was observed between genotypes of the same species and between species. These observations suggest that resistant factors may be expressed at the peel level and that resistance factors were no more efficient when the fruit was injured.

The cuticle covering the outer surface of epidermal cells is the first protective barrier against abiotic and biotic environmental stresses (Chassot et al, 2008). Indeed, this barrier forms a protective coating to prevent desiccation of the plant organs due to uncontrolled non-stomatal water loss and it is the first line of defense against infection by plant pathogen. In the case of fleshy fruits, the cuticle is an important factor reducing transpiration and contributing to shelf life and post-harvest storability (Szakiel et al, 2012).

Some of the most important properties of epidermis come from the cuticle composition. The cuticle is structurally diverse among species; biochemically this structure consists in cutin, a polyester that is partly covered and interspersed with waxes (epicuticular and intracuticular waxes), (Jetter et al, 2000), (Serrano et al, 2014). Recent discoveries and unresolved questions in the field of fleshy fruit cuticle structure, formation, and function are summarized in (Martin & Rose, 2014) review. Waxes are both embedded in the cutin (intracuticular) and form a continuous layer on its top (epicuticular). They are complex mixtures of lipids, mostly composed of long-chain acyl molecules including alkanes, aldehydes, ketones, alcohols, and esters. They are often associated with cyclic compounds, such as triterpenoids and sterols (Jetter et al, 2008); (Martin & Rose, 2014). It has been reported that triterpenoids are located almost exclusively in the intracuticular wax compartment (Jetter & Schäffer, 2001), (Szakiel et al, 2012).

The chemical composition of these waxes influences the morphology, arrangement and microstructure of the plant surface; which determines the relative adhesion of water, pesticides and fungal spores. On the other hand, many pathogens probe the chemical nature of their substrate and some wax constituents may behave as allelochemicals by influencing fungal development (Flaishman et al, 1995), (Hegde & Kolattukudy, 1997), (Serrano et al, 2014). The influence of epicuticular wax on *Peltaster fructicola* and *Leptodontidium elatius* growth, epiphyte fungi causing sooty blotch disease in apple, was investigated by (Belding et al, 2000). The major components of epicuticular wax did not act as substrates or inhibitors of sooty blotch growth.

A wide group of phytochemicals found in the wax is the group of triterpenes (Lv et al, 2016; Peschel et al, 2007; Szakiel et al, 2012). These phytochemicals exert numerous biological effects and display various pharmacological activities. Indeed, triterpenoids display various pharmacological effects such as anti-inflammatory antiulcer, antibacterial, antiviral (including anti-HIV), hepatoprotective, immunomodulatory, hypolipidemic and cholesterol-lowering, antiatherosclerotic, wound healing, anticoagulant and anticarcinogenic properties, with relative low toxicity (Bednarek & Osbourn, 2009), (Szakiel et al, 2012).

Screening plant material in the search for triterpenoid-rich plant tissues, (Szakiel et al, 2012) identified fruit peel and especially fruit cuticular waxes as promising and highly available sources. Plants employ triterpenoid metabolites for a variety of basic functions and especially for more specialized chemical interactions as protection in the abiotic and biotic environment (Tholl, 2015). They have been considered functional compounds in the defense, but the defense functions of each specific triterpene are not yet clarified. Oleanolic acid (OA), and ursolic acid (UA) are highly sought-after pentacyclic triterpenoids because of their wide spectrum of biological activities. Inoculation with *Penicillium expansum* induced, only in few cases, a significant reduction in OA content. The authors suggested that triterpene synthesis might be influenced by this pathogen and the reduction in OA content could be linked of its use for defense. As for a protective effect of these compounds against pathogens, *in vitro* tests have shown a marked antifungal activity of three triterpene compounds extracted from *Curtisia dentata* leaves (Shai et al, 2008). However, further *in vivo* studies are needed to conclude on their antifungal activity.

Phenolic compounds are an important class of secondary metabolites widely found in fruit, mostly represented by flavonoids and phenolic acids. The growing interest in these substances is mainly due to their antioxidant potential and the association between their consumption and the prevention of some diseases (Haminiuk et al, 2012). They can be classified into water-soluble compounds (phenolic acids, phenylpropanoids, flavonoids and quinones) and water-insoluble compounds (condense tannins, lignins and cell-wall bound hydroxycinnamic acids), (Haminiuk et al, 2012).

Another main characteristic of phenols is their antibacterial and fungicidal feature. For example chlorogenic, neochlorogenic and caffeic acids were shown as inhibitory compounds against the brown rot fungi *M. laxa* and *M. fructicola* in peach fruit. No direct toxicity to the pathogen was observed, but rather an interference with fungal cutinase production (Bostock et al, 1999) or melanin production (Villarino et al, 2011).

Thereby, this highlights that the cuticle has a biological barrier function which limits entry of pathogens. In waxes many compounds with potential fungicide activity could be found embedded and deposited on the external surface, suggesting that the cuticle is not simply a physical barrier, but also a dynamic defense component with signaling circuits and effector molecules (Commenil et al, 1997), (Reina-Pinto & Yephremov, 2009), (Serrano et al, 2014), (Lara et al, 2014), (Belge et al, 2014a).

In this context, the present study intends to provide a picture of the developmental dynamics of compounds from peach fruit surface. For this purpose, triterpenoids, phenolics, and cuticular waxes from fruit surface of three cultivars were analyzed at different stages of fruit development. New compounds, derivatives from triterpenoids, were identified and the profiles along time of the different compounds were compared. Besides, we tried to establish relationships between the changes of these compounds and other characteristics of the fruit that also display marked temporal dynamics along fruit development. In this way, fruit cuticular conductance and brown rot infection probability were recorded during fruit development and links with compound dynamics were sought.

To the best of our knowledge, this is the first study focusing on the characterization of the cuticular waxes of peach fruits during their development. In addition, it provides the identification of new secondary compounds of the fruit surface.

## **2 Material and Methods**

### **2.1 Plant material**

Summergrand, Zephir and Magique (*Prunus persica*) nectarine cultivars were grown in an experimental orchard located at INRA Research Center of Avignon following standard cultural practices and chemical spray programs, while no fungicide treatments against *Monilinia* spp. were performed.

### **2.2 Fruit sampling**

Fruits without visible wounds or rot and homogeneous in size were harvested at seven dates during fruit development in 2012 for Summergrand and Zéphir, midseason harvest cultivars, and at five dates in 2015 for Summergrand, Zéphir, and Magique, an early harvest cultivar. All the cultivars used in this study were nectarines, Summergrand is yellow fleshed and Zéphir and Magique are

white fleshed. At each harvest, fruit mass and the three dimensions of fruit (cheek, suture and height diameters) were measured. For biochemical analyses, five lots for each cultivar were analyzed in 2012 and 2015. The number of fruit per lot depended on the maturity stages ranging from five fruit at the beginning of fruit development to only one fruit at maturity. For infection tests, 20 fruits per cultivar were infected at each harvest during fruit development in both years.

### **2.3 Extraction**

Fruits were washed with reverse osmosis water and dried. They were then immersed in chloroform for 30 seconds with agitation, and then the extract was filtrated. In 2012 the extract was divided in two parts for analyses of secondary compounds and waxes respectively. For the HPLC analyses of secondary compounds an aliquot was taken and the internal standard (taxifolin in methanol solution) was added. The extract was concentrated in a rotary evaporator and the last milliliter was evaporated in argon gas for obtain a dry residue. The residue was solubilized in methanol, filtrated in a membrane filtered at 45 µm and then analyzed. For the GC-MS analyses of waxes an internal standard was added to extract, Docosane (Sigma-Aldrich, Saint-Quentin Fallavier, France Aldrich) and then the extract was concentrated in a rotary evaporator. The last milliliter was evaporated in argon gas for obtain a dry residue. This residue was weighed; the bottle was sealed and to GC-MS analysis.

### **2.4 Analysis of wax compounds**

Extracts for waxes compounds were analyzed as described by (Bourdenx et al, 2011). The samples were analyzed by gas chromatography with a Hewlett-Packard 5890 series II equipped with a flame ionization detector and a 30-m, 0.32-mm HP-1 capillary column with helium as the carrier gas. Quantification was based on flame ionization detector peak areas and the internal standard. The total amount of cuticular wax was expressed per unit of fruit surface area.

### **2.5 HPLC Analysis**

The quantitative analyses were performed with a High Performance Liquid Chromatography system -SHIMADZU- Prominence equipped with a reversed phase C18 column (MERCK Superspher RP18 endcapped) coupled with a photodiode array detector. The mobile phase was a mixture of solvent A; ultrapure water (Millipore Synergy-UV) acidified at pH 2.6 with orthophosphoric acid at 85% (Prolabo Normapur) et filtered with membrane Millipore Durapore HVLP 04700 0.45µm and B; Methanol (Prolabo Hypersolv Chromanorm).

The linear gradient elution from 35 to 80% B in 28 min was employed, followed by 15 min isocratic elution with 80% B, linear gradient to 90% B in 5 min, 10 min isocratic elution with 90% B, linear gradient to 100% B in 5 min, 5 min isocratic elution with 100% B, and column equilibration for



22 min with initial conditions. The column temperature was set to 30°C and the flow rate was 0.7 mL/min. The chromatograms were analyzed at 210 nm for triterpenoids detection, 315 nm and 330 nm for phenolic derivatives detection.

Identification of phenolic compounds and triterpenoids was achieved by using High Performance Liquid Chromatography coupled with Electrospray Ionization - Mass Spectrometry (HPLC/ESI-MS) using a Waters ACQUITY UPLC chromatograph (Waters, Milford, MA) coupled to an UV-vis diode-array detector and a HCT ultra ion trap mass spectrometer equipped with an electrospray ionization source (Bruker Daltonics, Bremen, Germany). Mass detection was conducted in the negative electrospray ionization mode from m/z 100 to 1000. MS conditions were as follows: capillary voltage of 2 kV, nitrogen flow rate at 10 L/min; desolvation temperature at 365 °C and nebulization pressure at 50 psi.

The compounds were characterized according to their UV and mass spectra, and retention time, co-chromatographies were performed with known standards when available. For quantitative analysis, a calibration curve was obtained by injection of known concentrations of standard compounds (Extrasynthèse, Genay, France): betulinic acid, ursolic acid, oleanolic acid. For derivatives of p coumaroyl acid, p coumaric acid was used as standard (Sigma-Aldrich, Saint-Quentin Fallavier, France). The results were expressed in µg per cm<sup>2</sup> of fruit surface.

## **2.6 Cuticular conductance**

Fruit conductance to water vapor was estimated from total fruit transpiration measurements in four years (2012, 2013, 2014 and 2015). Data were obtained from 5, 10 or 20 fruits, depending of available number of fruits in the orchard. Freshly harvested fruits were measured (cheek, suture and height diameters) and placed in a ventilated chamber. Temperature and relative humidity of the chamber were continuously registered (Sefram Log 1520; St Etienne, France). Each fruit was weighed hourly for about 7h. The method to calculate hourly surface conductance from transpirational water loss and to deduce fruit surface conductance is detailed in (Gibert et al, 2005).

## **2.7 *Monilinia* susceptibility**

The isolate of *Monilinia laxa* (MI3), was obtained from a mono-spore from an apricot fruit mummy on March 25th 2011, was maintained on stored it at -20°C kept in a glycerol solution in aliquots of 45 µl and multiplied by transplanting aliquots of 5 µl on a petri dish containing V8 agar modified (V8A; 200 ml V8 juice, 1g CaCO<sub>3</sub>, 2 g glucose, 2 g yeast extract, 40 g agar, 1 L distilled water) and incubated at 25°C and with 12 h dark and 12 h light cycle for 15 days. Conidial suspensions were prepared before each infection test by washing the colonies with sterile distilled water with one drop of Tween 80 for each petri dish, the number of spores of a 1/10th dilution of the mother solution on a Malassez cell under microscope was counted to estimate and adjust the spore concentration of the

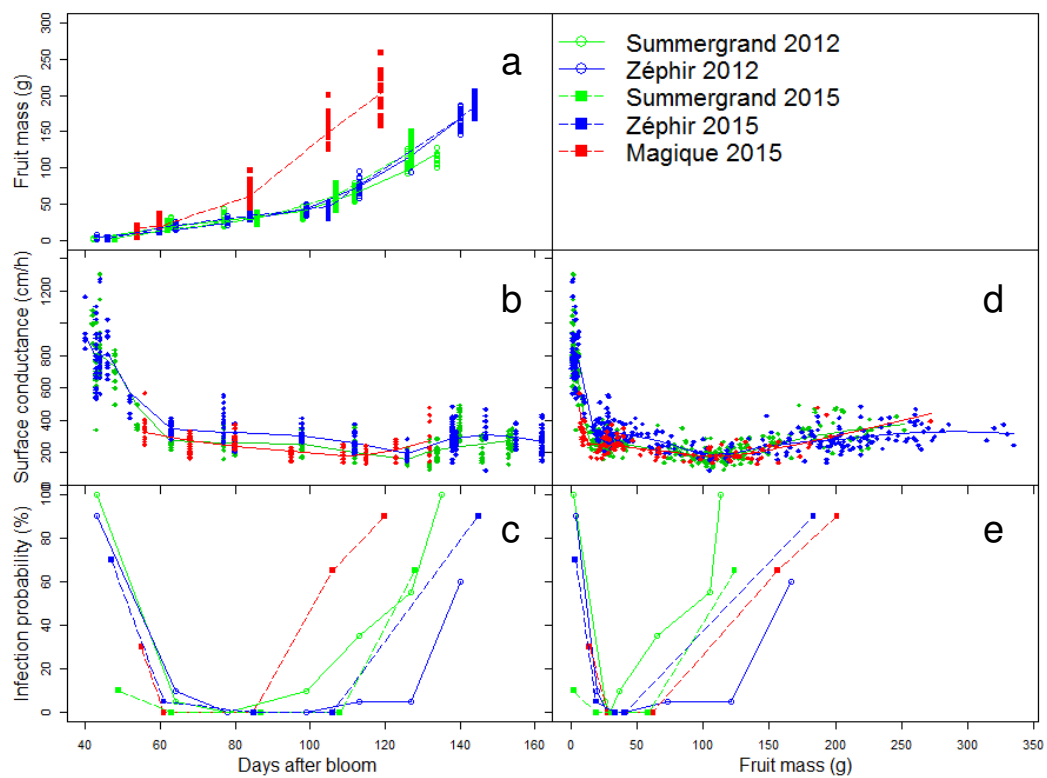


Figure1: Evolution of the fruit fresh mass (a), surface conductance (b) and Infection probability (c) during fruit development in (DAB) and relationship between fruit fresh mass and surface conductance (d), and infection probability (e) .

suspension at  $10^5$  conidia.mL<sup>-1</sup>. The viability and the probability of germination were verified for each suspension on a PDA.

Before infection, in the lab, fruits were measured and disinfected in water bath at 55°C for 40 seconds, then, put in acrylic plastic boxes placed in a growth chamber and deposited a 10µL drop of *M. laxa* suspension at  $10^5$  conidia.mL<sup>-1</sup>. The high humidity was guaranteed in the closed boxes by adding cups of water. The infection status was recorded (i.e. healthy or infected) of each fruit during 6 days.

## 2.8 Statistical analysis

Data were analyzed using the R software v.3.1.2 (Team, 2014).

To detect significant differences between the genotypes and the effect of the time on the course profile of the compounds, a generalized linear mixed-effects model (GLMM) was used as described in (Bugaud et al, 2012). The lmer function in the 'lmer4' library was used. A complete model including the DAB effect, the genotype effect, as well as quadratic and cubic terms of the DAB, was compared to a model including the DAB, quadratic and cubic for the DAB, and to another similar model including only the genotype. For surface compounds only, the effect of the years was added to the complete model and tested. A comparison of the nested models was performed with a likelihood ratio test using the ANOVA function in the R software.

A principal component analysis (PCA) was performed to summarize the large datasets of wax and surface compounds and to analyze how the genotype affects the diversity of waxes and surface compounds during fruit development. The trajectory during fruit development of fruit composition from Zéphir 2012 dataset was traced on the plan of the first two principal components. The wax and surface compounds were grouped by a clustering analysis.

## 3 Results

### 3.1 Evolution of fruit characteristics during development

Peach fruit development is marked by different stages principally differentiated by cell multiplication, endocarp formation and cell elongation, which give a special shape to the fruit mass curve. In parallel, fruit surface also evolves which results in marked modifications of fruit water permeability. At last, the fruit behavior in response to fungal attack also changes with fruit development.

### 3.2 Fruit growth

Little effect of year was observed for Summergrand and Zéphir between the 2 years considered whereas a cultivar effect expressed in flowering and maturity timings, growth duration, and final fruit size (Fig. 1a). Magique was an early harvest cultivar that arrived at maturity stage

around 118 DAB while Summergrand and Zephir arrived at maturity stage in 2015 10 and 27 days after, respectively. Despite short growth duration, Magique fruit reached similar mass than Zephir ones.

### **3.3 Fruit surface conductance**

The permeability to water of the fruit was examined through the survey of total fruit water loss. Multi-year data were grouped for each cultivar to draw evolution curves of fruit surface conductance along time (DAB) (Fig. 1b). The three cultivars followed a very similar trend characterized by a strong drop in the early stage of development, followed by a quasi-constant evolution afterwards. This feature is all the more visible when plotting surface conductance against fruit mass (Fig. 1d). Surface conductance of very immature green fruit, 12 g was very high and rapidly declined with a small increase in fruit mass. Surface conductance remained low during fruit growth and increased slightly at the approach of maturity, especially for large fruit of Summergrand and Magique cultivars. This profile of surface conductance curve was very similar for all cultivars. Year effect was not significant (data not shown).

### **3.4 Fruit susceptibility to *M. laxa***

The follow up of infection susceptibility of fruit to *M. laxa* in laboratory conditions drew a curve with a very pronounced form marked by three successive stages (Fig. 1c). The three cultivars followed this trend and year effect was not pronounced, especially when considering infection probability in relation with fruit mass rather than DAB (Fig. 1e). Early data we collected from small fruit highlighted a very sensible and short first stage that ended up around 60 DAB when fruit fresh mass around 30 g. Low infection probability for Summergrand at this stage in 2015 may be explained by a first harvest occurring too late, despite the fruit were quite small still.

This sensitive stage corresponded well with the stage of high surface conductance described above (Fig. 1b, d). It was followed by a stage of high resistance, the infection probability being null for all cultivars. This stage occurred during endocarp lignification at a time when mass growth was slow. The last stage was characterized by a continuous increase in infection probability ending up at very high values at maturity. The start of increase was dependent on the cultivar and year whereas the rate of increase appeared quite constant. All these changes of fruit characteristics occurring during fruit development may be accompanied by changing in fruit surface composition.

### **3.5 Identification and characterization of fruit cuticular compounds**

In 2012 the same extracts were used for wax analyses with CGP method and surface compounds with HPLC. HPLC analyses were realized in addition to CGP analyses, in order to investigate the compounds of secondary metabolism present on the peach fruit surface. Gas

µg/cm <sup>2</sup> DAB	Summergrand																				
	40			61			78			96			110			124			134		
	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd
<b>Triterpenes</b>	<b>4.27</b>	<b>75.34</b>	<b>108.49</b>	<b>14.58</b>	94.08	<b>612.12</b>	<b>43.03</b>	94.30	<b>1173.94</b>	<b>67.76</b>	93.97	<b>589.68</b>	<b>31.23</b>	87.52	<b>215.01</b>	<b>22.03</b>	83.00	<b>621.21</b>	<b>15.69</b>	77.24	<b>284.30</b>
Oleanoic acid	<b>0.80</b>	14.18	25.47	<b>3.27</b>	21.06	124.52	<b>12.85</b>	28.16	331.69	<b>22.52</b>	31.22	172.74	<b>8.61</b>	24.13	59.27	<b>5.96</b>	22.47	139.93	<b>3.56</b>	17.53	168.23
Ursolic acid	<b>3.47</b>	61.16	93.05	<b>11.32</b>	73.02	487.78	<b>30.18</b>	66.14	843.52	<b>45.25</b>	62.74	425.98	<b>22.62</b>	63.40	155.74	<b>16.07</b>	60.53	481.99	<b>12.13</b>	59.71	136.95
<b>Alkanes</b>	<b>0.49</b>	8.70	<b>11.38</b>	<b>0.07</b>	0.45	<b>1.51</b>	<b>0.22</b>	0.49	<b>2.21</b>	<b>0.35</b>	0.48	<b>3.85</b>	<b>1.82</b>	5.10	<b>12.54</b>	<b>2.00</b>	7.52	<b>27.50</b>	<b>2.71</b>	13.34	<b>25.47</b>
C19 nonadecane	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.02</b>	0.04	1.19	<b>0.02</b>	0.02	0.68	<b>0.03</b>	0.08	0.20	<b>0.01</b>	0.04	0.86	<b>0.01</b>	0.03	0.34
C20 eicosane	<b>0.11</b>	1.94	3.29	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00
C21 heneicosane	<b>0.03</b>	0.56	1.60	<b>0.01</b>	0.05	0.31	<b>0.01</b>	0.02	0.11	<b>0.01</b>	0.02	0.11	<b>0.01</b>	0.02	0.05	<b>0.01</b>	0.04	0.27	<b>0.02</b>	0.09	0.57
C23 tricosane	<b>0.02</b>	0.40	0.54	<b>0.01</b>	0.04	0.18	<b>0.01</b>	0.03	0.15	<b>0.02</b>	0.03	0.36	<b>0.12</b>	0.33	0.82	<b>0.24</b>	0.91	3.58	<b>0.46</b>	2.28	11.09
C24 tetracosane	<b>0.05</b>	0.89	1.45	<b>0.00</b>	0.03	0.16	<b>0.01</b>	0.02	0.18	<b>0.02</b>	0.02	0.25	<b>0.03</b>	0.08	0.20	<b>0.03</b>	0.10	1.18	<b>0.05</b>	0.25	1.62
C25 pentacosane	<b>0.12</b>	2.14	5.78	<b>0.03</b>	0.18	0.65	<b>0.07</b>	0.16	0.67	<b>0.12</b>	0.17	1.78	<b>0.43</b>	1.21	2.97	<b>0.47</b>	1.76	5.80	<b>0.71</b>	3.50	9.25
C27 heptacosane	<b>0.11</b>	1.88	2.59	<b>0.01</b>	0.08	0.84	<b>0.04</b>	0.08	2.00	<b>0.04</b>	0.06	0.67	<b>0.44</b>	1.25	3.06	<b>0.48</b>	1.80	6.86	<b>0.62</b>	3.04	4.08
C29 nonacosane	<b>0.03</b>	0.51	0.68	<b>0.01</b>	0.05	0.24	<b>0.05</b>	0.10	0.80	<b>0.09</b>	0.13	1.71	<b>0.59</b>	1.66	4.09	<b>0.66</b>	2.47	10.79	<b>0.72</b>	3.56	8.41
C31 hentriacontane	<b>0.02</b>	0.37	0.69	<b>0.00</b>	0.03	0.14	<b>0.02</b>	0.04	1.24	<b>0.03</b>	0.04	1.43	<b>0.17</b>	0.47	1.15	<b>0.11</b>	0.42	1.34	<b>0.12</b>	0.59	1.23
<b>Fatty acids</b>	<b>0.25</b>	4.36	<b>7.35</b>	<b>0.13</b>	0.84	<b>3.85</b>	<b>0.50</b>	1.10	<b>16.60</b>	<b>0.49</b>	0.68	<b>10.72</b>	<b>0.85</b>	2.37	<b>5.82</b>	<b>1.08</b>	4.06	<b>38.86</b>	<b>0.70</b>	3.43	<b>24.19</b>
C16 hexadecanoic acid, Palmitic acid	<b>0.06</b>	1.06	1.48	<b>0.05</b>	0.29	1.25	<b>0.16</b>	0.35	6.46	<b>0.10</b>	0.14	2.32	<b>0.16</b>	0.44	1.07	<b>0.13</b>	0.47	4.49	<b>0.08</b>	0.41	2.82
C18:0 octadecanoic acid, Stearic acid	<b>0.04</b>	0.67	1.15	<b>0.02</b>	0.11	0.82	<b>0.06</b>	0.14	2.57	<b>0.05</b>	0.07	0.66	<b>0.12</b>	0.32	0.80	<b>0.08</b>	0.30	3.83	<b>0.05</b>	0.25	2.42
C18:1	<b>0.02</b>	0.38	0.77	<b>0.00</b>	0.03	0.16	<b>0.01</b>	0.02	0.23	<b>0.01</b>	0.01	0.57	<b>0.02</b>	0.06	0.14	<b>0.03</b>	0.12	0.97	<b>0.04</b>	0.21	1.84
C18:2	<b>0.05</b>	0.97	1.74	<b>0.02</b>	0.12	0.69	<b>0.09</b>	0.19	3.04	<b>0.09</b>	0.12	1.30	<b>0.18</b>	0.50	1.24	<b>0.21</b>	0.78	9.09	<b>0.06</b>	0.31	2.47
C20 eicosanoic acid - Arachidic acid	<b>0.02</b>	0.42	1.37	<b>0.00</b>	0.03	0.21	<b>0.01</b>	0.02	0.19	<b>0.02</b>	0.02	0.22	<b>0.02</b>	0.05	0.13	<b>0.03</b>	0.12	1.68	<b>0.01</b>	0.07	0.62
C22 docosanoic acid - Behenic acid	<b>0.03</b>	0.58	1.42	<b>0.02</b>	0.15	0.59	<b>0.07</b>	0.16	1.21	<b>0.11</b>	0.16	5.46	<b>0.04</b>	0.10	0.25	<b>0.03</b>	0.11	2.37	<b>0.13</b>	0.63	7.01
C24 tetracosanoic acid - Lignoceric acid	<b>0.02</b>	0.29	0.70	<b>0.01</b>	0.04	0.39	<b>0.04</b>	0.08	3.41	<b>0.03</b>	0.04	1.29	<b>0.15</b>	0.41	1.00	<b>0.24</b>	0.91	9.63	<b>0.15</b>	0.76	5.02
C26 hexacosanoic acid - Cerotic acid	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.03	0.16	<b>0.03</b>	0.06	0.44	<b>0.05</b>	0.07	2.92	<b>0.15</b>	0.42	1.03	<b>0.21</b>	0.79	7.61	<b>0.13</b>	0.65	2.99
C28 octacosanoic acid - Montanic acid	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.04	0.24	<b>0.03</b>	0.07	1.33	<b>0.03</b>	0.04	0.85	<b>0.02</b>	0.07	0.16	<b>0.12</b>	0.45	4.56	<b>0.03</b>	0.15	0.72
<b>Fatty alcohols</b>	<b>0.23</b>	4.04	<b>6.77</b>	<b>0.13</b>	0.81	<b>3.42</b>	<b>0.44</b>	0.96	<b>6.88</b>	<b>0.99</b>	1.37	<b>8.87</b>	<b>0.47</b>	1.33	<b>3.26</b>	<b>0.43</b>	1.61	<b>11.07</b>	<b>0.52</b>	2.54	<b>4.28</b>
C18 octadecanol	<b>0.03</b>	0.60	2.38	<b>0.01</b>	0.04	0.20	<b>0.01</b>	0.02	0.15	<b>0.01</b>	0.02	0.38	<b>0.04</b>	0.11	0.27	<b>0.01</b>	0.03	0.32	<b>0.02</b>	0.08	0.41
C20 eicosanol	<b>0.04</b>	0.65	1.33	<b>0.00</b>	0.03	0.19	<b>0.01</b>	0.02	0.10	<b>0.01</b>	0.02	0.55	<b>0.01</b>	0.02	0.05	<b>0.01</b>	0.03	0.48	<b>0.02</b>	0.09	0.86
C22 docosanol	<b>0.03</b>	0.47	1.16	<b>0.01</b>	0.06	0.24	<b>0.02</b>	0.04	0.25	<b>0.09</b>	0.13	7.55	<b>0.01</b>	0.03	0.09	<b>0.02</b>	0.06	0.67	<b>0.02</b>	0.08	0.60
C23 tricosanol	<b>0.05</b>	0.97	2.48	<b>0.00</b>	0.02	0.13	<b>0.00</b>	0.01	0.39	<b>0.01</b>	0.01	0.22	<b>0.01</b>	0.01	0.04	<b>0.01</b>	0.04	0.45	<b>0.04</b>	0.19	1.81
C24 tetracosanol	<b>0.08</b>	1.36	1.88	<b>0.02</b>	0.13	0.51	<b>0.04</b>	0.10	0.67	<b>0.07</b>	0.10	0.92	<b>0.03</b>	0.10	0.24	<b>0.05</b>	0.17	1.52	<b>0.09</b>	0.42	1.22
C26 hexacosanol	<b>0.00</b>	0.00	0.00	<b>0.05</b>	0.34	1.63	<b>0.17</b>	0.38	1.99	<b>0.33</b>	0.46	3.67	<b>0.14</b>	0.40	0.99	<b>0.12</b>	0.46	4.30	<b>0.14</b>	0.71	1.38
C28 octacosanol	<b>0.00</b>	0.00	0.00	<b>0.02</b>	0.12	0.85	<b>0.09</b>	0.19	3.10	<b>0.25</b>	0.35	3.32	<b>0.13</b>	0.36	0.89	<b>0.12</b>	0.44	3.02	<b>0.12</b>	0.58	0.83
C30 triacontanol	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.07	0.18	<b>0.09</b>	0.20	1.02	<b>0.20</b>	0.28	3.03	<b>0.10</b>	0.29	0.71	<b>0.10</b>	0.37	1.86	<b>0.08</b>	0.40	0.85
<b>Fatty aldehydes</b>	<b>0.00</b>	0.00	<b>0.00</b>	<b>0.01</b>	0.05	<b>0.34</b>	<b>0.11</b>	0.24	<b>3.58</b>	<b>0.11</b>	0.15	<b>3.07</b>	<b>0.13</b>	0.37	<b>0.92</b>	<b>0.12</b>	0.46	<b>2.85</b>	<b>0.13</b>	0.62	<b>4.04</b>
C18 octadecanal	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.06</b>	0.12	3.39	<b>0.02</b>	0.03	1.42	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.03	0.31	<b>0.01</b>	0.05	0.66
C22 docosanal	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.02	0.53	<b>0.01</b>	0.03	0.17
C24 tetracosanal	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.02</b>	0.06	0.15	<b>0.04</b>	0.14	0.91	<b>0.03</b>	0.16	0.89
C30 triacontanal	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.05	0.34	<b>0.05</b>	0.12	2.51	<b>0.09</b>	0.12	2.17	<b>0.11</b>	0.31	0.77	<b>0.07</b>	0.28	2.62	<b>0.08</b>	0.38	2.83
<b>Phenolics</b>	<b>0.01</b>	0.21	<b>0.38</b>	<b>0.01</b>	0.05	<b>0.23</b>	<b>0.05</b>	0.12	<b>1.20</b>	<b>0.08</b>	0.12	<b>0.74</b>	<b>0.02</b>	0.04	<b>0.11</b>	<b>0.02</b>	0.06	<b>0.96</b>	<b>0.00</b>	0.02	<b>0.11</b>
Cinnamic acid	<b>0.01</b>	0.21	0.38	<b>0.01</b>	0.05	0.23	<b>0.05</b>	0.12	1.20	<b>0.08</b>	0.12	0.74	<b>0.02</b>	0.04	0.11	<b>0.02</b>	0.06	0.96	<b>0.00</b>	0.02	0.11
<b>Phytosterols</b>	<b>0.27</b>	4.67	<b>8.99</b>	<b>0.07</b>	0.45	<b>2.11</b>	<b>0.39</b>	0.86	<b>4.94</b>	<b>0.42</b>	0.58	<b>3.96</b>	<b>0.20</b>	0.55	<b>1.35</b>	<b>0.19</b>	0.70	<b>7.29</b>	<b>0.21</b>	1.03	<b>5.07</b>
Stigmasta-3,-5-diene	<b>0.19</b>	3.30	5.47	<b>0.01</b>	0.05	0.14	<b>0.03</b>	0.06	0.51	<b>0.02</b>	0.03	0.51	<b>0.08</b>	0.23	0.56	<b>0.03</b>	0.13	0.47	<b>0.05</b>	0.22	0.76
β-Sitosterol	<b>0.08</b>	1.37	4.07	<b>0.06</b>	0.40	1.98	<b>0.36</b>	0.80	4.65	<b>0.40</b>	0.55	3.68	<b>0.11</b>	0.32	0.78	<b>0.15</b>	0.57	6.95	<b>0.16</b>	0.80	5.62
<b>Unidentified</b>	<b>0.15</b>	2.67	<b>4.13</b>	<b>0.51</b>	3.27	<b>17.80</b>	<b>0.88</b>	1.94	<b>51.74</b>	<b>1.91</b>	2.65	<b>9.05</b>	<b>0.97</b>	2.71	<b>6.65</b>	<b>0.68</b>	2.58	<b>29.69</b>	<b>0.36</b>	1.78	<b>5.46</b>
<b>Total wax</b>	<b>5.67</b>			<b>15.50</b>			<b>45.63</b>			<b>72.12</b>			<b>35.68</b>			<b>26.54</b>			<b>20.32</b>		<b>352.91</b>

Table 1. Wax constituents (mean and relative percent) identified in cuticle of Summergrand cultivar during peach development.

$\mu\text{g}/\text{cm}^2$	Zephir																				
	42		63			80			98			112			126			140			
DAB	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd
<b>Triterpenes</b>	<b>6.04</b>	82.35	<b>118.30</b>	<b>30.52</b>	93.75	<b>1029.02</b>	<b>44.90</b>	91.79	<b>747.91</b>	<b>64.77</b>	92.96	<b>2606.09</b>	<b>27.60</b>	89.75	<b>597.32</b>	<b>28.46</b>	85.35	<b>567.09</b>	<b>22.56</b>	75.02	<b>192.27</b>
Oleanoic acid	<b>0.28</b>	3.84	3.87	<b>7.39</b>	22.70	232.09	<b>13.21</b>	26.99	154.96	<b>22.41</b>	32.16	799.80	<b>9.72</b>	31.62	228.98	<b>9.10</b>	27.30	178.26	<b>6.94</b>	23.08	75.74
Ursolic acid	<b>5.76</b>	78.51	115.91	<b>23.13</b>	71.05	797.51	<b>31.70</b>	64.80	623.98	<b>42.36</b>	60.80	1809.54	<b>17.88</b>	58.13	368.43	<b>19.36</b>	58.05	389.44	<b>15.62</b>	51.93	117.18
<b>Alkanes</b>	<b>0.43</b>	5.89	<b>8.30</b>	<b>0.12</b>	0.36	<b>2.04</b>	<b>0.71</b>	1.46	<b>20.04</b>	<b>0.54</b>	0.77	<b>15.22</b>	<b>1.25</b>	4.08	<b>12.44</b>	<b>2.35</b>	7.05	<b>35.42</b>	<b>3.88</b>	12.90	<b>66.01</b>
C19 nonadecane	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.03	0.44	<b>0.01</b>	0.02	0.93	<b>0.01</b>	0.02	0.05	<b>0.01</b>	0.03	0.43	<b>0.01</b>	0.03	0.27
C20 eicosane	<b>0.04</b>	0.52	0.21	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00
C21 henicicosane	<b>0.02</b>	0.33	1.18	<b>0.01</b>	0.04	0.37	<b>0.01</b>	0.03	0.50	<b>0.01</b>	0.02	0.50	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.02	0.23	<b>0.02</b>	0.06	0.40
C23 tricosane	<b>0.02</b>	0.22	0.27	<b>0.01</b>	0.02	0.27	<b>0.08</b>	0.16	4.31	<b>0.04</b>	0.06	1.90	<b>0.09</b>	0.30	1.16	<b>0.27</b>	0.80	6.97	<b>0.65</b>	2.16	15.81
C24 tetracosane	<b>0.02</b>	0.30	1.19	<b>0.01</b>	0.02	0.15	<b>0.02</b>	0.04	0.37	<b>0.02</b>	0.03	0.59	<b>0.02</b>	0.06	0.23	<b>0.04</b>	0.11	0.62	<b>0.06</b>	0.21	0.74
C25 pentacosane	<b>0.15</b>	2.00	7.48	<b>0.05</b>	0.15	0.82	<b>0.29</b>	0.60	9.72	<b>0.21</b>	0.30	7.15	<b>0.35</b>	1.14	6.13	<b>0.66</b>	1.96	12.76	<b>1.05</b>	3.50	20.47
C27 heptacosane	<b>0.13</b>	1.76	2.03	<b>0.02</b>	0.05	0.59	<b>0.03</b>	0.06	1.23	<b>0.04</b>	0.06	2.07	<b>0.38</b>	1.24	3.23	<b>0.62</b>	1.85	8.78	<b>0.97</b>	3.24	17.69
C29 nonacosane	<b>0.03</b>	0.39	0.48	<b>0.02</b>	0.05	0.45	<b>0.22</b>	0.46	4.69	<b>0.17</b>	0.24	5.26	<b>0.35</b>	1.15	2.44	<b>0.66</b>	1.99	7.79	<b>1.01</b>	3.35	15.46
C31 hentriacontane	<b>0.03</b>	0.38	0.54	<b>0.01</b>	0.03	0.44	<b>0.04</b>	0.09	1.66	<b>0.03</b>	0.05	1.16	<b>0.05</b>	0.17	0.69	<b>0.09</b>	0.27	0.84	<b>0.10</b>	0.34	1.72
<b>Fatty acids</b>	<b>0.09</b>	1.26	<b>1.27</b>	<b>0.31</b>	0.94	<b>9.91</b>	<b>0.79</b>	1.61	<b>13.30</b>	<b>0.74</b>	1.06	<b>17.62</b>	<b>0.31</b>	1.01	<b>11.21</b>	<b>0.69</b>	2.07	<b>9.23</b>	<b>1.57</b>	5.24	<b>50.06</b>
C16 hexadecanoic acid, Palmitic acid	<b>0.04</b>	0.57	0.99	<b>0.10</b>	0.30	3.81	<b>0.13</b>	0.26	2.89	<b>0.13</b>	0.18	4.24	<b>0.05</b>	0.16	1.08	<b>0.09</b>	0.28	1.62	<b>0.16</b>	0.53	6.64
C18:0 octadecanoic acid, Stearic acid	<b>0.01</b>	0.18	0.54	<b>0.04</b>	0.12	1.57	<b>0.07</b>	0.15	1.42	<b>0.07</b>	0.10	1.52	<b>0.02</b>	0.07	0.51	<b>0.04</b>	0.12	0.84	<b>0.08</b>	0.28	3.10
C18:1	<b>0.02</b>	0.25	0.15	<b>0.01</b>	0.03	0.24	<b>0.01</b>	0.03	0.20	<b>0.01</b>	0.02	0.83	<b>0.01</b>	0.03	0.20	<b>0.02</b>	0.06	0.44	<b>0.05</b>	0.16	1.51
C18:2	<b>0.02</b>	0.26	0.30	<b>0.05</b>	0.17	1.87	<b>0.14</b>	0.29	2.31	<b>0.10</b>	0.14	2.96	<b>0.05</b>	0.15	1.39	<b>0.11</b>	0.34	4.87	<b>0.13</b>	0.43	7.34
C20 eicosanoic acid - Arachidic acid	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.03	0.31	<b>0.02</b>	0.04	0.44	<b>0.02</b>	0.03	0.44	<b>0.01</b>	0.02	0.10	<b>0.01</b>	0.04	0.43	<b>0.04</b>	0.12	1.79
C22 docosanoic acid - Behenic acid	<b>0.00</b>	0.00	0.00	<b>0.04</b>	0.12	0.94	<b>0.27</b>	0.55	6.37	<b>0.22</b>	0.31	6.06	<b>0.01</b>	0.05	0.96	<b>0.05</b>	0.14	3.66	<b>0.14</b>	0.46	10.13
C24 tetracosanoic acid - Lignoceric acid	<b>0.00</b>	0.00	0.00	<b>0.03</b>	0.08	0.54	<b>0.05</b>	0.11	1.75	<b>0.05</b>	0.07	1.78	<b>0.05</b>	0.17	1.74	<b>0.16</b>	0.49	2.35	<b>0.42</b>	1.39	14.79
C26 hexacosanoic acid - Cerotic acid	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.04	0.62	<b>0.07</b>	0.15	2.48	<b>0.10</b>	0.15	2.99	<b>0.07</b>	0.24	2.00	<b>0.15</b>	0.45	6.73	<b>0.41</b>	1.35	13.92
C28 octacosanoic acid - Montanic acid	<b>0.00</b>	0.00	0.00	<b>0.02</b>	0.05	0.54	<b>0.02</b>	0.04	0.26	<b>0.04</b>	0.06	1.96	<b>0.04</b>	0.12	3.26	<b>0.05</b>	0.15	2.48	<b>0.16</b>	0.52	8.53
<b>Fatty alcohols</b>	<b>0.20</b>	2.72	<b>3.03</b>	<b>0.30</b>	0.92	<b>5.24</b>	<b>0.65</b>	1.33	<b>9.48</b>	<b>1.15</b>	1.65	<b>29.22</b>	<b>0.53</b>	1.71	<b>9.32</b>	<b>0.53</b>	1.58	<b>6.85</b>	<b>0.79</b>	2.64	<b>10.70</b>
C18 octadecanol	<b>0.02</b>	0.32	0.30	<b>0.01</b>	0.02	0.36	<b>0.03</b>	0.06	1.48	<b>0.02</b>	0.02	0.56	<b>0.01</b>	0.02	0.05	<b>0.01</b>	0.03	0.19	<b>0.02</b>	0.05	1.14
C20 eicosanol	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.03	0.44	<b>0.01</b>	0.03	0.33	<b>0.01</b>	0.02	0.65	<b>0.01</b>	0.02	0.11	<b>0.01</b>	0.02	0.11	<b>0.02</b>	0.05	0.40
C22 docosanol	<b>0.05</b>	0.74	3.77	<b>0.02</b>	0.06	0.41	<b>0.02</b>	0.04	0.52	<b>0.05</b>	0.07	3.06	<b>0.01</b>	0.04	0.64	<b>0.02</b>	0.05	0.49	<b>0.03</b>	0.10	1.87
C23 tricosanol	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.02	0.17	<b>0.01</b>	0.01	0.13	<b>0.01</b>	0.02	0.42	<b>0.00</b>	0.01	0.31	<b>0.01</b>	0.03	0.23	<b>0.04</b>	0.13	1.13
C24 tetracosanol	<b>0.12</b>	1.66	1.23	<b>0.04</b>	0.12	1.86	<b>0.05</b>	0.10	1.16	<b>0.09</b>	0.12	2.30	<b>0.05</b>	0.16	0.56	<b>0.05</b>	0.15	0.71	<b>0.11</b>	0.36	1.33
C26 hexacosanol	<b>0.00</b>	0.00	0.00	<b>0.15</b>	0.45	3.20	<b>0.20</b>	0.41	3.82	<b>0.39</b>	0.56	10.16	<b>0.16</b>	0.54	2.85	<b>0.17</b>	0.52	2.81	<b>0.25</b>	0.85	2.82
C28 octacosanol	<b>0.00</b>	0.00	0.00	<b>0.06</b>	0.18	1.45	<b>0.18</b>	0.36	2.17	<b>0.32</b>	0.45	7.42	<b>0.13</b>	0.43	2.27	<b>0.14</b>	0.42	1.90	<b>0.20</b>	0.66	1.67
C30 triaccontanol	<b>0.00</b>	0.00	0.00	<b>0.02</b>	0.05	0.51	<b>0.15</b>	0.32	1.96	<b>0.27</b>	0.38	5.69	<b>0.15</b>	0.50	2.83	<b>0.12</b>	0.37	1.80	<b>0.13</b>	0.45	1.21
<b>Fatty aldehydes</b>	<b>0.00</b>	0.00	<b>0.00</b>	<b>0.02</b>	0.06	<b>0.56</b>	<b>0.11</b>	0.22	<b>1.09</b>	<b>0.17</b>	0.24	<b>5.39</b>	<b>0.10</b>	0.34	<b>1.61</b>	<b>0.13</b>	0.40	<b>2.78</b>	<b>0.17</b>	0.57	<b>4.22</b>
C18 octadecanal	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.03</b>	0.06	0.94	<b>0.03</b>	0.04	1.47	<b>0.01</b>	0.02	0.10	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.04	0.42
C22 docosanal	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.01</b>	0.02	0.16
C24 tetracosanal	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.00</b>	0.00	0.00	<b>0.02</b>	0.06	0.17	<b>0.03</b>	0.08	0.97	<b>0.05</b>	0.16	0.83
C30 triaccontanal	<b>0.00</b>	0.00	0.00	<b>0.02</b>	0.06	0.56	<b>0.07</b>	0.15	1.25	<b>0.14</b>	0.20	4.36	<b>0.08</b>	0.26	1.34	<b>0.11</b>	0.32	3.03	<b>0.10</b>	0.35	3.19
<b>Phenolics</b>	<b>0.00</b>	0.00	<b>0.00</b>	<b>0.02</b>	0.07	<b>0.78</b>	<b>0.11</b>	0.23	<b>6.14</b>	<b>0.10</b>	0.15	<b>1.60</b>	<b>0.01</b>	0.02	<b>0.15</b>	<b>0.01</b>	0.04	<b>0.93</b>	<b>0.02</b>	0.05	<b>1.07</b>
Cinnamic acid	<b>0.00</b>	0.00	0.00	<b>0.02</b>	0.07	0.78	<b>0.11</b>	0.23	6.14	<b>0.10</b>	0.15	1.60	<b>0.01</b>	0.02	0.15	<b>0.01</b>	0.04	0.93	<b>0.02</b>	0.05	1.07
<b>Phytosterols</b>	<b>0.40</b>	5.46	<b>6.71</b>	<b>0.18</b>	0.54	<b>3.81</b>	<b>0.43</b>	0.88	<b>23.32</b>	<b>0.51</b>	0.73	<b>13.86</b>	<b>0.11</b>	0.35	<b>2.38</b>	<b>0.14</b>	0.40	<b>1.74</b>	<b>0.19</b>	0.65	<b>3.25</b>
Stigmasta-3,-5-diene	<b>0.32</b>	4.35	5.02	<b>0.01</b>	0.05	0.23	<b>0.04</b>	0.07	1.16	<b>0.04</b>	0.05	1.01	<b>0.02</b>	0.07	0.77	<b>0.03</b>	0.09	0.82	<b>0.04</b>	0.13	0.42
$\beta$ -Sitosterol	<b>0.08</b>	1.11	3.90	<b>0.16</b>	0.50	3.58	<b>0.40</b>	0.81	23.77	<b>0.47</b>	0.67	12.96	<b>0.09</b>	0.28	1.61	<b>0.11</b>	0.32	1.10	<b>0.15</b>	0.52	3.26
<b>Unidentified</b>	<b>0.17</b>	2.32	<b>0.93</b>	<b>1.09</b>	3.36	<b>27.64</b>	<b>1.22</b>	2.49	<b>23.36</b>	<b>1.70</b>	2.44	<b>73.48</b>	<b>0.84</b>	2.74	<b>21.87</b>	<b>1.03</b>	3.09	<b>32.19</b>	<b>0.88</b>	2.93	<b>20.49</b>
<b>Total_wax</b>	<b>7.34</b>			<b>32.55</b>			<b>48.92</b>			<b>69.67</b>			<b>30.75</b>			<b>33.35</b>			<b>30.07</b>		

Table 2. Wax constituents (mean and relative percent) identified in cuticle of Zéphir cultivar during peach development.

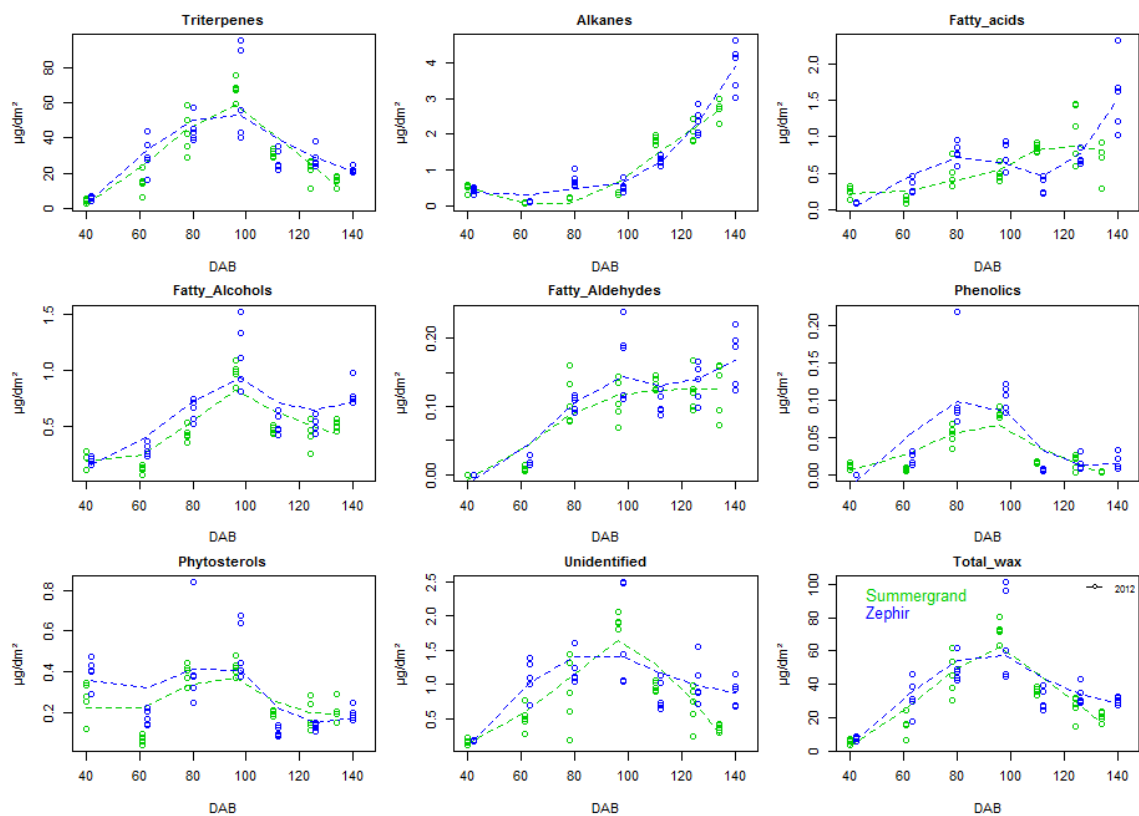


Figure 2. Changes in amounts of wax compounds summed by classes and by cultivar during fruit development in days after bloom (DAB)

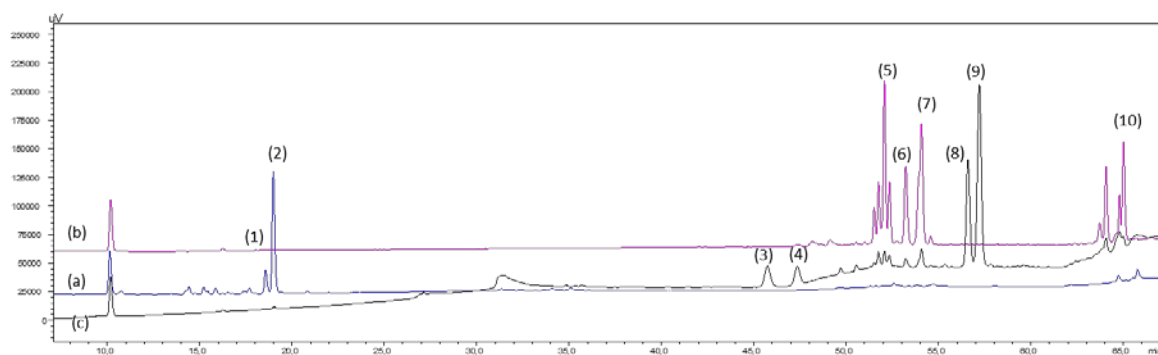


Figure 3: HPLC chromatograms of the peach surface compounds in Zephir cultivar. Peak identities are given in Table 3.

Table 3 HPLC-DAD and ESI-MS characteristics of compounds identified in peach surface.

chromatography (van Leeuwen et al) analyses were performed to characterize the diversity of compounds immersed in wax.

### **3.6 Cuticular wax composition**

With CG wax analysis 36 different compounds were detected in 2012 for Summergrand and Zéphir and grouped into 8 classes: triterpenes, alkanes, fatty acids, fatty alcohols, fatty aldehydes, phenolics, phytosterols and unidentified (Tables 1 and 2). At the first harvest for Summergrand (41 DAB), the main wax class in quantitative terms was the triterpenes, followed by alkanes, phytosterols, fatty acids, fatty alcohols, unidentified compounds and phenolics. No fatty aldehydes were present at first harvest. For Zephyr same results were obtained, apart those fatty acids were present in smaller amounts and that phenolics were absent. At maturity (last harvest), triterpenoid acids remained predominant for both cultivars, followed by alkanes, fatty acids, fatty alcohols and/or unidentified compounds, depending on the cultivar. Phytosterols and fatty aldehydes were present in lower amounts and phenolics in very small amounts.

During fruit development, the amounts of compounds from the different classes were very similar between the two cultivars. All along fruit development, the triterpenoid acids were the predominant class for both cultivars. However, slight differences in composition inside each classes were noticed. For example, at the first harvest Summergrand extracts were richer in fatty acids than Zephyr extracts, both in total quantity and number of different compounds (0.25 and 0.09  $\mu\text{g}\cdot\text{cm}^{-2}$  and seven and four different compounds, respectively for Summergrand and Zéphir). On the contrary, the general trends were slight higher levels of total waxes for Zephyr than Summergrand at almost all harvest dates (Fig. 2).

Looking at the dynamics of the wax compounds, they were very similar between the two cultivars (Fig. 2). Alkanes, fatty aldehydes and fatty acids increased during fruit development, while triterpenes, phenolics alcohols and unidentified compounds showing bell curves with maximum amounts between 80 and 100 DAB, depending on the wax classes and cultivars. In contrast, phytosterols and fatty acids dynamics were not significantly different from constant (tests with generalized linear mixed model by GLMM). The decrease observed at the end of fruit development for many compounds is due to a synthesis stop while fruit continues to growth which provokes a dilution effect.

### **3.7 Identification of secondary compounds**

With the objective to investigate the secondary compounds present at the peach surface, HPLC analyses were performed on same extracts used for wax analyses in 2012 on Summergrand and Zéphir cultivars and repeated in 2015 with technical improvements and addition of Magique cultivar. In total ten compounds were identified in both years. The peach extracts were a mixture of



N° Pic	Compound	Abrev	RT HPLC	$\lambda_{max}$	MS( <i>m/z</i> )	MS <sup>2</sup> ( <i>m/z</i> )	MS <sup>3</sup> ( <i>m/z</i> )
1	<i>p</i> -Coumaroylpentacetyldihexoside	pcdhpa1	18,5	311	697	655, <b>637</b> , 613, 595, 391	[637]: <b>595</b> , 577, 553, 535, 331, 287, 215
2	<i>p</i> -Coumaroylpentacetyldihexoside	pcdhpa2	19,0	315	697	655, <b>637</b> , 613, 595, 391	[637]: <b>595</b> , 577, 553, 535, 331, 287, 215
3	Dihydroxy-urs-12-en-28-oic acid <sup>b</sup>	Ter1	45,5	198	471	425, <b>407</b>	[408]: <b>439</b> , 422, 390, 254, 205
4	Dihydroxy-urs-12-en-28-oic acid <sup>b</sup>	Ter2	47,0	198	471	<b>407</b>	[407]: <b>390</b> , 352, 307, 277
5	<i>p</i> -Coumaroyldihydroxy-urs-12-en-28-oic acid <sup>b</sup>	der1	52,5	198/312	617	573- <b>497</b> -453	[497]: 453-407-391
6	<i>p</i> -Coumaroyldihydroxy-urs-12-en-28-oic acid <sup>b</sup>	der2	53,0	198/310	617	<b>573</b> , 497	[573]: 529
7	<i>p</i> -Coumaroyldihydroxy-urs-12-en-28-oic acid <sup>b</sup>	der3	54,0	198/308	617	573, <b>497</b>	[497]: 453, <b>407</b> , 391
8	3 $\beta$ -hydroxyolean-12-en-28-oic acid (oleanolic acid) <sup>a</sup>	a_o	56,5	198	455	<b>407</b>	[407]: 439, 421, 407, 391, <b>377</b>
9	3 $\beta$ -Hydroxy-urs-12-en-28-oic acid (ursolic acid) <sup>a</sup>	a_u	57,0	198	455	<b>407</b>	[407]: 439, 421, 407, 391, <b>377</b>
10	3 $\beta$ - <i>p</i> -Coumaroyloxy-urs-12-en-28-oic acid <sup>b</sup>	der5	65,0	198/312	601	555, <b>437</b>	[437]: traces 391, 367

Abbreviations: (RT) is the retention time in HPLC (min). ( $\lambda_{max}$ ) and (sh) are the wavelengths for the maximum absorption and for the shoulder in the UV-vis spectrum, respectively. (MS) gives the parent ion for [M-H]<sup>-</sup> (*m/z*), MS<sup>2</sup> and MS<sup>3</sup> the fragmentations of the parent ion and the given selected ion, respectively. The major fragment ion is in bold. <sup>a</sup>structure confirmed by standard. <sup>b</sup>ursolic acid or oleanolic acid as the principal structure.

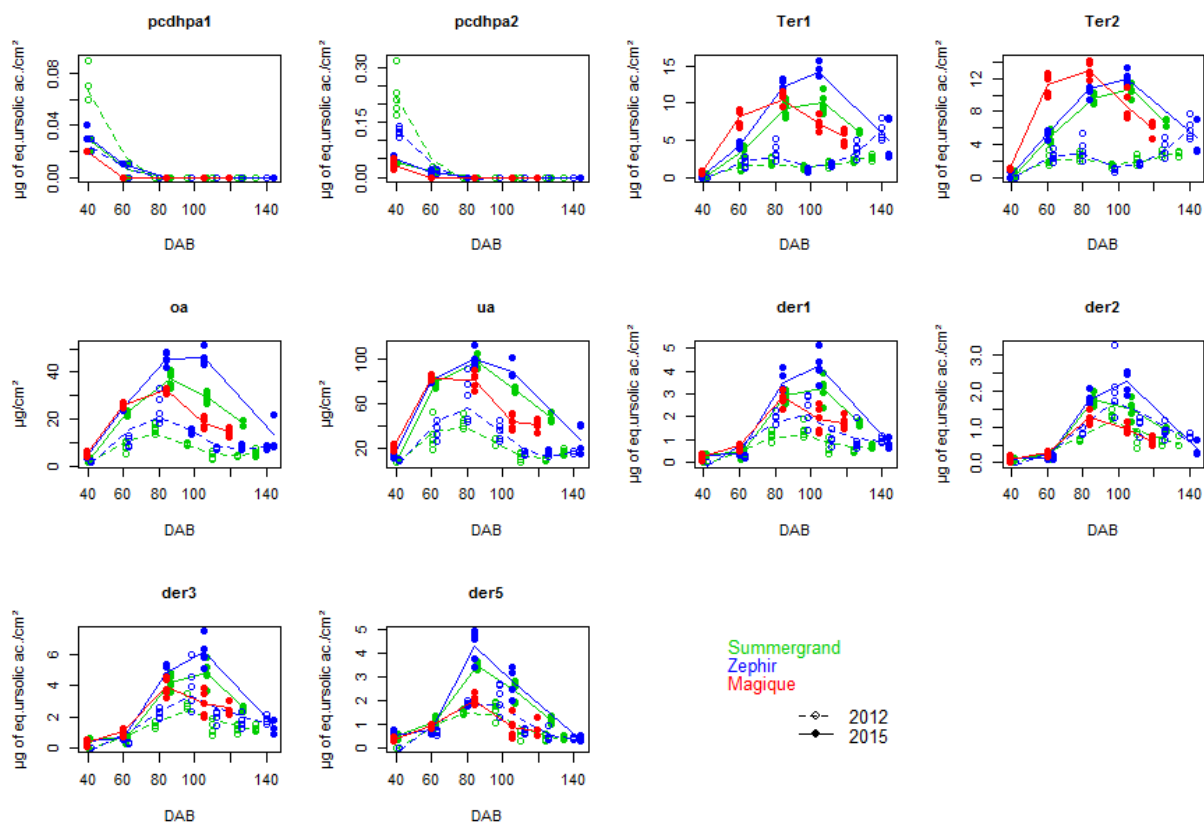


Figure 4. Evolution of ten peach surface compounds during fruit development (DAB) in 2012 and 2015 for 3 cultivars.

triterpenoids, phenolics and derivatives of triterpenoids. The major surface compounds were separated by HPLC and detected at 210 nm, 315 nm and 330 nm (Fig. 3). The compounds were characterized by comparing their absorption spectra and their retention times with trade references and mass spectrometry substances (Table 3).

Two coumaric acid derivatives (1, 2) were identified by ESI-MS in the form *p*-coumaroylpentacetyldihexosides. Their structure assignment was based on the double loss in fragmentation of 42 amu typical of the acetyl group and its  $\lambda_{\max}$  at 314 nm. Similar *p*-coumaroyldi- and triacetyldihexosides were found in leaves, stems and fruits of bilberry (Bujor et al, 2016). These phenolics were different from the phenolics detected in the GC analysis. Triterpenoids oleanolic acid (8) and ursolic acid (9) were identified based on their respective standards and as described recently by (Belge et al, 2014b) in the wax of peach. These compounds were the same compounds as the ones identified by GC in the wax samples and were predominant in quantity. As a matter of fact, the precursors hydroxyursolic acid and hydroxyoleanolic acid (3, 4) were also identified. The presence of vicinal *trans* hydroxyl groups in ursolic and oleanolic derivatives was assigned by NMR in apple peel (He & Liu, 2007).

About ten *p*-coumaric acid were evidenced in two groups on the chromatogram. The more important ones were identified by ESI-MS and quantified. The three compounds (5, 6, 7) may be the respective *cis* and *trans* stereoisomers of *p*-coumaroyl-dihydroxy-urs-12-en-28-oic acid and *p*-coumaroyl-dihydroxy-olean-12-en-28-oic acid. The position of the acyl group was not further determined. However, (He & Liu, 2007) and (McGhie et al, 2012) reported the presence of  $3\beta$ -*cis*- and  $3\beta$ -*trans*-*p*-coumaroyloxy-2 $\alpha$ -hydroxyurs-12-en-28-oic acid and  $3\beta$ -*cis*- and  $3\beta$ -*trans*-*p*-coumaroyloxy-2 $\alpha$ -hydroxyolean-12-en-28-oic acid in apple peels. Finally, another derivative of triterpenoid was found: a *p*-coumaroyl derivative of ursolic acid or oleanolic acid (10) was identified based on the maximal absorption at  $\lambda_{\max}$  at 310 nm as well as a parent ion at  $m/z$  601 and a major fragment ion at  $m/z$  437 (loss of *p*-coumaric acid) in MS<sup>2</sup>.

These compounds were not detected in the GC analysis. The two triterpenoids prevailed in terms of amounts, followed by the hydroxyursolic and hydroxyoleanolic acids, next by the 4 derivatives and finally the two phenolics. Zephir displayed higher amounts of compounds than the two other cultivars, apart for the phenolics that were higher for Summergrand.

In general higher levels of surface compounds were observed in 2015 than in 2012, especially for the hydroxyursolic and hydroxyoleanolic acids, the triterpenoids and the *p*-Coumaroyl-dihydroxy-urs-12-en-28-oic acid (Ter1, Ter2, oa, ua, der1). However, same patterns were observed whatever the cultivars and years. Indeed, the profiles of surface compounds along time grouped in 2 classes depending on the compounds (Fig. 4). Most of them followed bell curves, except phenolic compounds (pcdhpa 1 and 2) that showed a peak at the first harvest, then severely dropped and

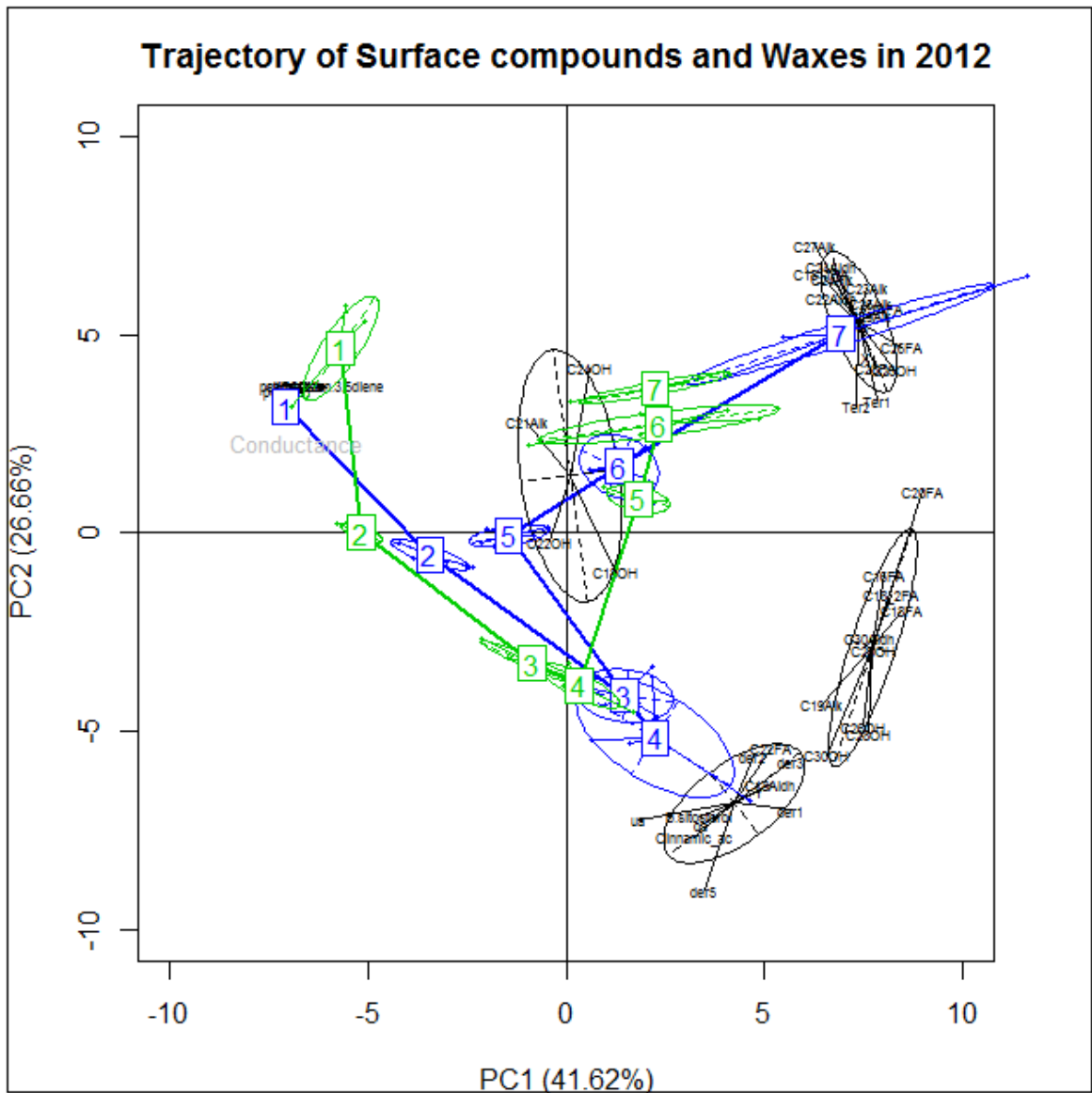


Figure 5: Principal component analysis (PCA) of the wax and surface compounds. Correlations of wax and surface compounds grouped by black ellipses (clustering) for the first two principal components of a PCA analysis performed on Zephyr 2012 dataset. The trajectories during fruit development of Summergrand (in green) and Zephyr (in blue) are drawn on the two-first principal components. Summergrand was plotted as inactive data on the PCA . Numbers refer to the harvest number. Surface conductance was added as supplementary data in light gray.

disappeared between 60 and 80 DAB. Curves for Magique cultivar were shifted to the left because of its earlier maturity date.

Triterpenoid compounds (oa, au) reached their maximum around 80 DAB for Summergrand and Zephir, while the hydroxyursolic and hydroxyoleanolic acids and the three derivatives der1, der2 and der3 (7, 8, 9) displayed a peak later on, at around 100 DAB, indicating that the three derivatives may be formed from the association of the triterpenoids and hydroxyursolic and hydroxyoleanolic acids.

### **3.8 Developmental variations of wax and surface compounds**

An analysis of the wax and surface compounds using principal component analysis (PCA) for surface compounds during fruit development helped in tracking the developmental variations (Fig. 5). The PCA was carried out on Zephir 2012 dataset and the principal components (PC) scores were plotted on the first two axes, which explained 68% of the variation. A clustering on the compounds ended up into 5 groups that well explained the two first components and trajectories. The first component (PC1) essentially opposed compounds present at the first harvest to the others. The second component (PC2) dissociated the compounds majority in the middle of fruit development from the ones present at the maturity stage that were principally wax compounds. The group of compounds, only waxes (C21Alk, C18OH, C22OH, C24OH), located in the middle of the plan had constant levels during fruit development.

Data from Summergrand 2012 dataset was plotted as inactive data on the first plan, which means that they were passively positioned on the axis defined by Zephir 2012 dataset. The trajectories of the two cultivars were very similar with an amplitude of the course higher for Zephir than for Summergrand, indicating larger amounts of compounds for the former.

### **3.9 Relationships between wax and surface compounds and fruit characteristics**

The evolution of cuticular conductance during fruit development in relation with wax and surface compounds was investigated. Among all the relationships observed, five were retained because they brought potential explanations to the spectacular drop of cuticular conductance in the beginning of fruit development. They were the sum of hydroxyursolic and hydroxyoleanolic acids (Ter1 and Ter2), the sum of derivatives (Der1, Der2, Der3, Der5), the sum of non-identified wax compounds, the sum of aldehyde wax and the sum of triterpenes (Fig. 6). Indeed, cuticular conductance displayed high levels for low amounts of these compounds. When these compounds exceeded a certain amount then cuticular conductance dropped down and stayed low. In addition, this behavior was quite common to all cultivars and years, with small variations in the levels of the thresholds.

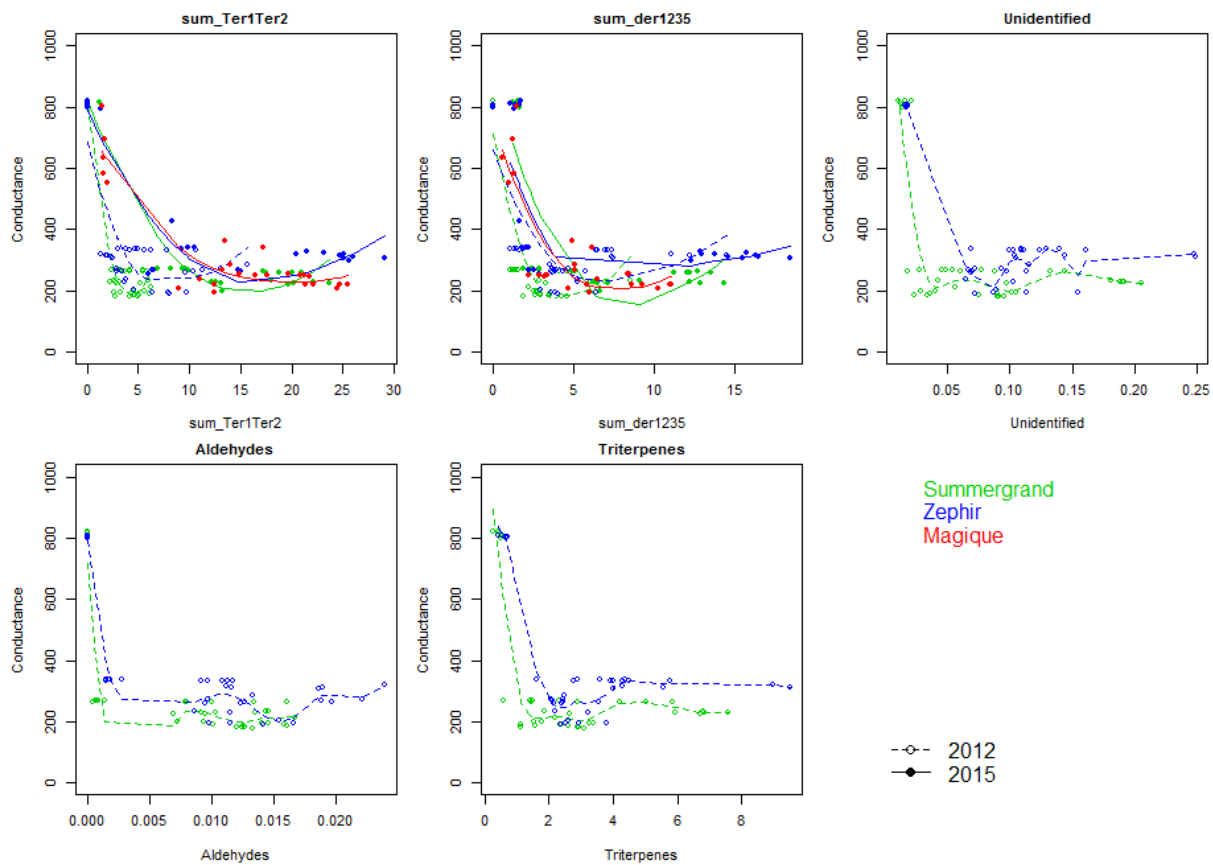


Figure 6: link between surface conductance and surface compounds.

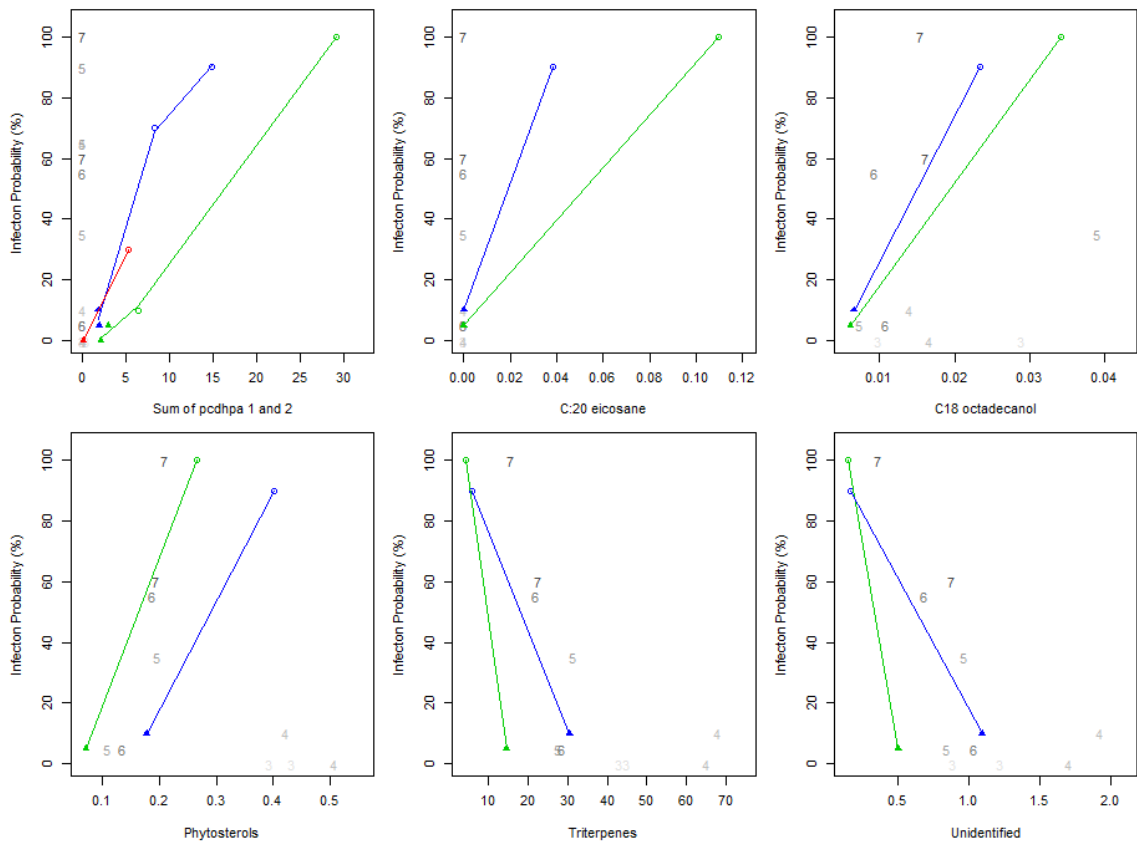


Figure 7 A: Relationship between infection probability to *Monilia laxa*, surface compounds and wax at immature stages.

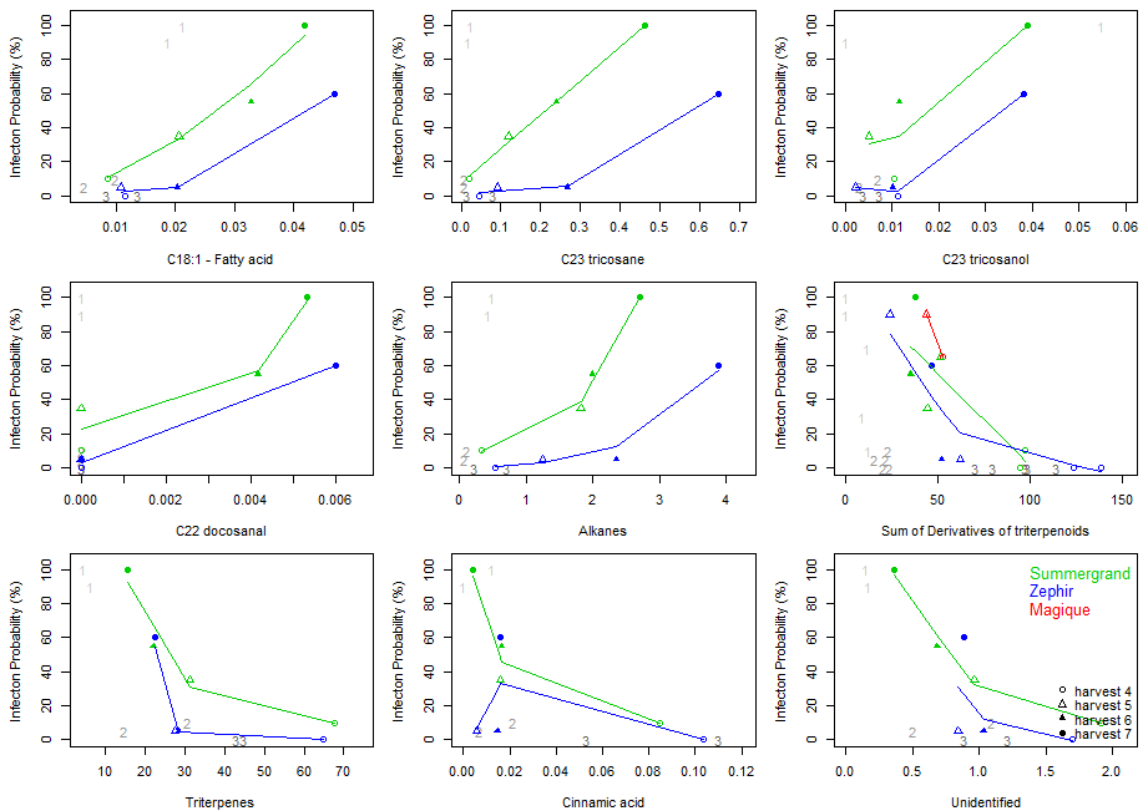


Figure 7 B: Relationship between infection probability to *Monilia laxa*, surface compounds and wax at maturity stages.

This may only be due to temporal coincidence between the evolution of cuticular conductance and the dynamics of accumulation of the compounds. However the waterproofing effect of wax in plant surface is well known in the literature and the wax compounds found here could reduce water permeability of fruit and explain part of the drop of cuticular conductance in early fruit development.

### **3.10 Do wax and surface compounds correlate with *Monilia laxa* infection probability?**

Peach brown rot is a fungal disease which first develops on the fruit surface. As detailed above, the infection probability intensely evolves during fruit development. It is renowned as a complex disease involving many factors including biochemical and physical fruit barriers. Since the dynamics of infection probability was characterized by two susceptible stages at the beginning and the end of fruit development and that fruit characteristics dramatically evolved from immature green to mature fruit, factors influencing resistance and susceptibility may be different at these two stages. Therefore, the two susceptible stages were analyzed separately.

At the very early stage, between 41 and 64 DAB (data from the 2 first harvests only), a positive relationship was found between infection probability and the sum of pcdhpa1 and 2 and some wax compounds (the alkane C20 eicosane, the fatty alcohol C18 octodecanol and the phytosterols) (Figure 7A). On the contrary, a negative relationship was found with the unidentified wax compounds and Triterpenes. At the end of fruit development, after 97 DAB (data from the harvests 4 to 7 in 2012 and 4 and 5 in 2015) a positive relationship was found between infection probability and some wax compounds, namely the fatty acid C18:1, the alkane C23 tricosane, the fatty alcohol C23 tricosanol, the fatty aldehyde C22 docosanal and the sum of alkanes. In contrast, a negative relationship was found between the sum of derivatives of triterpenoids, the triterpenoids and the cinnamic acid (Figure 7B).

## **4 Discussion**

All plant organs exposed to the atmosphere are covered with layers that reduce water losses and may also be involved in the defense of the plant. Regarding fruit in particular, crucial aspects of cuticle formation especially composition of the different layers and their evolution during fruit development hence remained unknown. Indeed interesting information about wax compounds of fruit can be found in the literature (McGhie et al, 2012), (Belge et al, 2014b), (Jetter & Schäffer, 2001), (Peschel et al, 2007), but little is known about the process of wax deposition during fruit development.

In this study, we investigated wax and surface compounds of two cultivars during fruit development. Surface compounds can be extracted by either mechanical methods, as film adhesive



that proved high selectivity for epicuticular waxes, or by using chloroform extraction which enables to release intracuticular material as well (Jetter & Schäffer, 2001). In this study, the latter procedure was used, consisting in soaking fruit in chloroform. These procedures enable removing wax only without removing the epidermis compounds as catechin for example. This explains why catechin was not found in our study, proving that only surface compounds were extracted.

#### 4.1 New compounds detected in fruit surface

The extraction procedure used enabled to extract higher levels of triterpenoids than in epicuticular wax extracts since triterpenoids are located almost exclusively in the intracuticular wax compartment (Jetter & Schäffer, 2001), (Szakiel et al, 2012), (Wang et al, 2014). In addition, the analysis by HPLC allowed to the best of our knowledge, described for the first time as cuticular compounds, embedded in waxes with free terpenoids, described for the first time in peach, *i.e.* derivatives of triterpenoids and phenolics. We found eight classes of wax compounds: triterpenes, alkanes, fatty acids, fatty alcohols, fatty aldehydes, phenolics, phytosterols (stigmasta-3,-5 diene and  $\beta$ -Sitosterol) and unidentified compounds.

The oleanolic acid (oa) and the ursolic acid (ua), detected in our experimental conditions were previously identified in peach cultivars by (Belge et al, 2014b). and in sweet cherry at maturity stage (Peschel et al, 2007). Triterpene acids were also detected by (McGhie et al, 2012) in apple peels and tentatively identified as ursolic or oleanolic acid derivatives containing hydroxyl, oxo, and coumaroyl oxo groups. The triterpenes have been identified as major constituents on fruit surface. They can represent up to 50 to 75,6% of the total wax mass in sweet cherry, and between 40 and 50% in peaches, depending on the cultivar, and on the number of compounds identified (Peschel et al, 2007), (Belge et al, 2014a). The results of the present study are in agreement with the literature, since triterpenes represented at maturity stage 75% and 77% of total wax for Zéphir and Summergrand cultivars respectively, followed by alkanes, fatty acids and fatty alcohols.

Relatively low differences were found between the quantity of triterpenoids detected with CG and by HPLC analyses. The content for these compounds were slightly underestimated in CG analyses compared with HPLC results. The order of appearance for these compounds during peach fruit development could be explained by their process of biosynthesis. The phenolic compounds, pcdhpa 1 and 2 are probably derivatives of sucrose as hexose. They are present very early and disappear quickly. The triterpenoids are free compounds without phenol groups. They appear relatively early in the process. The derivatives of triterpenoids appear when the triterpenoids are at their maximal content and accumulate while triterpenoid contents decrease. Concurrently, almost all phenolic compounds disappear. These parallel timing, together with the chemical structure of the derivatives of triterpenoids, *i.e.* they possess a p-coumaroyl group and a dihydroxy or monohydroxy ursolic or

oleanolic acid, tends to suggest that there is a successive process of synthesis of the wax constituents at the fruit surface.

#### **4.2 Variations with cultivars and between years**

M Bain & Mcg Mcbean (1969) investigated by electron microscopy the development of the cuticular wax layer in prune plums. They reported that little variations occur in the surface of the wax layer throughout the growing season but changes in the fine structure of the surface wax were followed as the temperature was increased from 49 to 63°C to simulate conditions of dehydration.

Lv et al (2016) studied oleanolic and ursolic acids, commonly found in the cuticular wax in apple peel, reported variations between years and in sun-exposed sides for all cultivars. This information reveals how the effects of temperature and solar radiations can change the quantity and quality of wax and the fruit surface compounds. The differences between years of surface compounds could be explain by the fact that a small variation between the mass and the day of harvest for the genotypes and the differences of environment conditions between the years, as temperature, (M Bain & Mcg Mcbean, 1969), an effect of cultivars and technical improvements for better.

#### **4.3 Evolution of wax and surface compounds during development**

The surface compounds were not degraded during fruit development. They were only diluted because of the high rate of growth and the stop of compound synthesis. In sweet cherry cultivars *Prunus avium* an decrease in wax mass per unit fruit surface area accounted for a decrease in triterpenes, while alkanes and alcohols on a unit area basis remained approximately constant throughout stage III (Peschel et al, 2007).

The composition of wax and cutin was studied by GC-MS between 22 and 85 days after full bloom by (Peschel et al, 2007) the results demonstrated that mass of wax and cutin per unit of surface area markedly decreased after 40 DAB, of sweet cherry fruit development. This decrease mirrored the increase in fruit surface area indicating that, on a whole fruit basis, mass of wax and cutin must have remained essentially constant relative to the marked increase in surface area. The authors affirm that the decrease of compositional wax was not a mere dilution effect caused by surface area expansion, but resulted from specific changes of individual constituents, their results are in agreement with results found in the present study.

#### **4.4 Role of wax and surface compounds in preventing fruit gas exchanges**

There is circumstantial evidence that the most effective part of the cuticular transpiration barrier resides in waxes, (Schönherr, 1976). Among the different constituents of wax, negative relationships with surface conductance were found for the aldehydes, triterpenes and unidentified

compounds at the early stage when surface conductance drops dramatically. Among these different groups of wax constituents, aldehydes only may play an effective role in reducing water permeability of the fruit. Indeed, high levels of triterpenoids in cuticular wax are generally not positively correlated with water impermeability (Szakiel et al, 2012). However the relationship observed in our experiment would suggest that a very small amount of aldehyde deposit ( $0.02\mu\text{g}/\text{cm}^2$ ) is sufficient to procure a very high efficiency in limiting water loss. In parallel to the drop of cuticular conductance, a drop of dark respiration rate and  $\text{CO}_2$  exchanges have been reported by (Pavel & DeJong) in this same early period of fruit development. In addition to wax deposit, stomatal regulation may play a role in controlling water loss and gas exchanges but the literature is very poor concerning this aspect on very immature green fruit.

#### **4.5 Potential effect of wax and surface compounds on brown rot infection**

The diversity of class of compounds in fruit peel and the quantity of compounds could be one of the factors responsible for the cultivar variability of resistance to some diseases. In particular, the potential fungicide activity of some phenolic compounds from fruit surface has been investigated (Bostock et al, 1999). The effect of chlorogenic and caffeic acids from fruit surface on the reduction of *M. fructicola* infection was reported to be due to the suppression of the fungal production of cutinase (Bostock et al, 1999) (Villarino et al, 2011) explored the effect of the chlorogenic acid and its isomer, neochlorogenic acid from peel of peach cultivars *M. laxa*. They reported a role of these two compounds in decreasing brown rot infection by limiting fungal melanin biosynthesis. Their presence in high levels in immature fruit is concomitant with a low susceptibility to brown rot infection at this stage. These phenolic compounds are the major phenolic acids in the epidermis and subtending cell layers of peach fruit but are not present in the outer fruit surface as analyzed in the present study.

The phenolic compounds detected in this study (pcdhpa 1 and 2) are reported here for the first time in peach surface. Contrary to chlorogenic and caffeic acids, they were positively linked to infection probability. Their effective role of induction of *M. laxa* infection in early stages of fruit development needs to be tested since a simple temporal concomitance may also explain the positive relationship observed. Besides phenolic compounds, infection probability appeared to be linked to other compounds, either in positive or negative relationships.

The effect of extracts of leaf containing C28 aldehyde octacosanal (octacosanal), C26 and C30-aldehydes also present, as inductors of appressoria formation in the fungi *Puccinia graminis* f.sp. *tritici* was reported by (Reisige et al, 2006). These compounds seem to be involved in the pathogen system of plant recognition (Tsuba et al, 2002). (Podila et al, 1993), studying chemical signals from avocado surface, reported that C24 fatty alcohols very long-chain of fatty alcohols allow the fungus *Colletotrichum gloeosporioides* to use the host surface wax to trigger germination and differentiation

of infection structures in the pathogen. In our results a positive relationship was found between the infection probability to *M. laxa* and alkanes (C20 eicosane), fatty alcohol (C18 octadecanol) at immature stage and fatty aldehyde (C23 tricosane) and fatty alcohol (C23 tricosanol) at mature stage.

Further investigations on the fungicide activity of surface compounds are needed to ascertain the role of the different compounds displaying negative or positive relationships with infection probability. One way to proceed would be to perform *in vitro* tests to establish the effect of each individual compound on the germination, growth and/or sporulation of the fungus. Methodological development may be necessary because of the highly hydrophobic feature of many surface compounds. Another “strategy” could be to explore variations of these compounds in a large collection of genotypes so as to increase the power of statistical tests to explore correlations between the infection probability and compound levels. These results open new research perspectives to progress in the search for fruit resistance to brown rot that were stagnating for a while now.

## 5 Conclusion

In this work, peach fruits surface was characterized during their development at point of biochemical compounds and waxes with CGP and HPLC analysis. The evolutions have to be the same shape with little differences due to cultivar, fruit mass and year. To the best of our knowledge it was the first time that new compounds from derivatives of triterpenoids was identified on peach fruit surface, p-Coumaroyl-dihydroxy-urs-12-en-28-oic acid and 3 $\beta$ -p-Coumaroyloxy-urs-12-en-28-oic acid.

We attempt to put in evidence the physical changes in fruit surface with the evolution of these compounds and waxes during stages of fruit development as the mass of fruits, cuticular conductance and even the susceptibility to brown rot. An effect of the time could be involved in some linked found here, however good relationship was found for some waxes, that could explain in part the reduction of cuticular conductance in fruit development but did not explain the strong fall in early stages.

The link between infection probability and surface compounds was investigated with interesting results, potential inhibitors and inductors compounds were found, however this investigation deserves more analysis to prove the relationship with fungi activity. The HPLC and CGP, analyses on peach fruit surface are good methods for study secondary metabolites on fruit surface. This method will be used in further *in vitro* investigations for search potential compounds with direct effects on defense of pathogens, including *Monilinia laxa*.

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### **Chapitre 3: Exploration des caractéristiques physiques du fruit immature en relation avec la sensibilité à *M.laxa***

*Monilinia laxa* est un des champignons responsables de la pourriture brune des fruits à noyau. Au stade I du développement du fruit, les jeunes fruits verts sont généralement sensibles. Le but de cette étude était d'étudier la relation entre les caractéristiques physiques du jeune fruit et la sensibilité à *M. laxa* au stade I. Une centaine de génotypes issus d'une population interspécifique de pêcher, appelé BC2, a été caractérisée par des infections au laboratoire, un suivi de transpiration cuticulaire et, plus innovant, un comptage de stomates (uniquement pour les nectarines). Les infections ont révélé des symptômes 'tache claire', jamais décrits dans la littérature, observés pour la majorité des génotypes. Ces expériences ont donné lieu à l'estimation de la progression de l'infection. Le nombre et la densité de stomates se sont avérés n'avoir aucun effet sur la probabilité d'infection bien que les stomates aient longtemps été suggérés comme un facteur principal de la sensibilité à la pourriture brune. Au contraire la conductance de la surface était positivement corrélée au niveau d'infection. Par conséquent, la sensibilité à la pourriture brune au stade I résulte certainement d'une absence ou d'un dépôt très partiel de la cuticule. Des co-localisations ont été observées entre des QTLs de probabilité d'infection et de conductance de la cuticule ainsi qu'une co-localisation entre le seul de QTL de nombre de stomates et des QTLs de progression d'infection. Cette étude fournit des conclusions expérimentales sur le rôle des caractéristiques du fruit. Cette information ouvre la voie à la compréhension globale des interactions fruit-pathogène au cours du développement du fruit.



# Why young peach fruit are susceptible to *Monilinia laxa* ?

## Abstract

*Monilinia laxa* is a fungus causing brown rot of stone fruit. In the first stage of fruit development, young green fruits are generally sensitive. The aim of this study was to investigate the relationship between the physical characteristics of young fruit and susceptibility to *M. laxa* in stage I. We characterized a hundred individuals of an interspecific peach progeny, called BC2, through laboratory infections, a transpiration monitoring and, more innovative, stomata counts (only for nectarines). The infections revealed 'clear spot' symptoms, ever described in the literature, for a majority of genotypes. These experiments resulted in the estimation of the progression of the infection. The number and density of stomata had no effect on the probability of infection although stomata have long been suggested as a main factor of susceptibility to brown rot at stage I. Contrariwise the surface conductance was positively correlated to the infection level. Hence, susceptibility to brown rot at stage I certainly results from absence or very partial deposit of cuticle. QTL have been identified for variables describing the infection, related to the conductance of the cuticle and regulating the number of stomata. This study provides experimental conclusions on the role of fruit characteristics. This information paves the path to global understanding of fruit-pathogen interactions during fruit development.

**Keywords:** *P. persica*, brown rot, green fruit, stomata, surface conductance, quantitative genetics, QTL

## 1 Introduction

*Monilinia* species have often been considered as opportunistic fungi as they enter the fruit mainly through injuries, either visible (insect or birds bites, injuries due to wind, ...) or microscopic (cuticular cracks or microcracks...) and also through stomata (see (Lino et al, 2016) for review). Thus, it appears that fruit resistance comes mainly from the epidermis which no longer plays its role of barrier when open doors are present. Consistently, (Pascal et al, 1994) observed a large variability of response to infection of unwounded fruit between cultivars. By contrast, they did not show significant differences between cultivars for fruit infections with injury. Therefore they concluded on the major role of integrity of the epidermis in protecting fruit against *M. laxa*. According to Arnoux (Arnoux & Marboutie, 1979), the risk of contamination by *M. laxa* can be formulated as the product of the amount of inoculum and the surface of entry points (micro-cracks cuticular surface) in given

climatic conditions (Gibert et al, 2009). However, it is important to mention that these fungi are also able to secrete enzymes such as cutinases, allowing penetration of the intact cuticle, first barrier blocking the progression of penetrating hyphae (Macedo & Pio, 2005).

The flesh of the fruit is protected by the skin (epicarp) covered with a cuticle including cutins and waxes. When the fruit has a very rapid growth, it sometimes occurs a gap between the growth of the flesh and the extension of the cuticle (deposit cutine), causing the appearance of micro-cracks to the surface of the fruit (Peschel et al, 2007). (Nguyen-The et al, 1989) observed that the mycelium of *M. laxa* was developing through cuticular cracks, regarded as microscopic preferred entrance channels for the fungus. This occurs mainly during the stage III of fruit growth.

In addition to microcracks, stomata can also act as entry points for the infection. (Wad & Cruickshank, 1992) demonstrated that *M. fructicola* infecting apricots in laboratory can enter the cell guard by the thin wall of the stomata. Then the fungal invasion develops through the substomatal cavities. Regarding fruit stomata, literature is limited especially for stone fruit like peach (Atkins et al, 1977). However it is well known that the stomata number is determined very early in the organogenesis. For fruit the stomata number is determined before petal fall and then remains constant throughout the whole fruit ontogeny (Blanke & Lenz, 1989). In the early stages, stomata allow gas exchange for photosynthesis (Roth, 1977). Then they lose this feature, close permanently or remain open continuously, and can develop into lenticels. At maturity stage, the number of stomata at the surface of a peach fruit is low and is not decisive for the susceptibility to pathogens. However, in the case of young fruit, the high density of stomata could be one of the main factors that explain the susceptibility to pathogens at stage I.

Young peach fruit (stage I) and fruit in the maturation stage (stage III) are susceptible to *Monilinia* spp. By contrast, at the intermediate stage corresponding to pit hardening (stage II), fruit are generally resistant (Byrde & Willetts, 1977). The first stage starts after the ovule fertilization and ends up when stone starts lignifying. In this early stage the fruit is photosynthetically active, displays intense transpiration activity and high density of stomata. The second stage, also known as "pit hardening", is the most resistant to infection by *M. spp* (Mari et al, 2003). This stage is characterized by intense metabolite activity of secondary compounds, like catechin, epicatechin and phenolic compounds, associated to the lignification of the endocarp. At the third stage, stone fruit become increasingly susceptible to pathogens as they mature and ripen (Gradziel & Wang, 1993). Associated with this increased susceptibility, structural changes in the fruit surface take place, such as changes in fruit surface chemistry (e.g. production of sugars, decline of phenolic compounds and organic acids, etc) and thinning and fracturing of the cuticle (Bostock et al, 1999). Although at this stage the number of stomata could be insignificant compared to the number of micro-cracks, it may play an indirect role by developing into lenticels that act as stress concentrators and increase probability of

rupture of the cuticular membrane (Brown & Considine, 1982). (Nguyen-The et al, 1989) observed on nectarines a significant number of cracks and micro-cracks organized radially around lenticels and noticed that conidia of *M. laxa* tended to accumulate in the micro-cracks. (Borve et al, 2000) demonstrated a clear link between cracking and BR in cherries, by finding significant correlations between the cultivar-specific amount of micro-cracks and the resulting incidence of Brown Rot. In this context, we focused on the poorly known early phase of the fruit growth (stage I) to decipher whether physical fruit characteristics were involved in young fruit susceptibility to brown rot. A dual objective was pursued: i) explore the relationship between the susceptibility of the fruit to *M. laxa* and physical characteristics of the fruit, within a mapping population, ii) study the genetic control of these variables. To answer these questions, young fruits of a hundred individuals of a mapping population were characterized by three variables: i) stomatal density calculated from the number of stomata estimated for each genotype, ii) fruit surface conductance calculated by monitoring transpiratory losses, iii) susceptibility to *M. laxa* through infections on laboratory. The data acquired during this phenotyping were then used to identify QTL loci controlling the different variables.

## **2 Materials and methods**

### **2.1 Vegetal material**

The breeding population was derived from clone P1908 of *Prunus davidiana* (D) as follows (Pascal et al, 1998). In the first generation, P1908 was crossed with *Prunus persica* cv. 'Summergrand' (S). A F1 progeny was obtained. In the second generation, one F1 hybrid resistant to powdery mildew was back-crossed to S to produce a BC1 progeny. In the third generation, BC1 individuals were used to pollinate *P. persica* cv. 'Zéphir' (Z) to derive the breeding population (BC2). S and Z are respectively yellow and white nectarine cultivars with large tasty fruit.

The study was conducted at the INRA Research Centre of Avignon (southern France). BC2 genotypes and the three parents were planted in 2001 in an orchard with one tree per genotype. The trees were 17 years old in 2015. All the genotypes were grafted onto GF305 seedling rootstocks and were grown under normal irrigation, fertilization and pest-control conditions. All trees were homogeneously pruned and thinned.

### **2.2 Fruit sampling**

Fruit were harvested in spring 2015, in the first stage of development (small green fruit). Two sets of samples were harvested for each genotype at 2 different dates. The early flowering and maturing genotypes were harvested first. In order to phenotype the entire population at a rate of about 24 genotypes per day, six working days were necessary. Thus the first series of measurements

took place from 23<sup>rd</sup> to 30<sup>th</sup> April. The same genotypes were harvested a second time (2nd series: 4<sup>th</sup> to 12<sup>th</sup> May) 12 days after the 1<sup>st</sup> harvest in the same order to assess response differences depending on the status of the fruit development. For each genotype and each of the two harvests, 20 fruit were harvested for infection tests and 10 fruit for transpiration measurements. To estimate the number of stomata, 3 fruits per genotype were harvested during the 2<sup>nd</sup> series of sampling only. At each harvest date fruit were weighed and the 3 diameters (fruit height, between cheeks diameter and the distance from the suture to the opposite side) were measured using a caliper. Fruit were considered similar to ellipsoid and their surface was estimated using the formula:

$$4 \pi \left( \frac{(ab)^p + (ac)^p + (bc)^p}{3} \right)^{\frac{1}{p}}$$

with  $p = 1,6075$  and  $a$ ,  $b$  and  $c$  representing the three radius.

### **2.3 *Monilinia laxa***

The strain of *Monilinia laxa* used for infections was M13. It was isolated from an apricot fruit mummy on March 25th 2011, and cultured in the laboratory INRA Gotheron (Drôme). A mono-spore strain was isolated and stored at -20°C. This fungus was bred on V8 agar medium at a temperature of 21°C ± 2°C (16 hour day and 8 hour night) and kept in a glycerol solution in aliquots of 45µl, stored at -20°C. It was multiplied by transplanting aliquots on petri dishes containing V8 agar, medium based on vegetable juice, two weeks before an experiment that required a spore suspension. The petri dishes closed with parafilm were incubated at 21°C ± 2°C and 16 hours of light.

### **2.4 Infection tests**

A spore suspension of *M. laxa* was prepared before each infection test from a culture dish of 15 days. 5mL of sterile distilled water was added on the culture medium, the agar surface was scraped with a sterile spatula which allowed taking off the spores and the mycelium. The resulting mixture was transferred into a vial containing small glass beads and wherein a drop of Tween was added. The vial was vortexed for approximately 30 seconds to separate the spores from the mycelium. Thereafter, the entire suspension was sampled using a syringe and passed through a filter of 30 microns of porosity to obtain a suspension with a minimum of hyphae and a maximum of spores. To estimate the spore concentration of the suspension, the number of spores of a 1/10th dilution of the mother solution was counted on a Malassez cell under a microscope.

For each suspension spore viability was verified. Five to seven separate drops of 5µL of the final suspension were deposited on a PDA culture medium which transparency facilitates microscopic observation. Seeded Petri dishes were closed and placed at room temperature. After 24h, the

germination rate was calculated for each drop by microscopic counting of the spores germinated or not. A spore was considered germinated when it had a germ tube larger than the spore.

Fruit were numbered and placed in a crystal plastic box. Each fruit was infected by a drop of 10µL of spore suspension (concentration of 100 spores/µl) deposited on the fruit intact surface (at the cheek), without wounded the fruit. In order to maintain sufficient humidity in the boxes, water cups were added to favor the development of the fungus. An observation was made every day and during five days to record fruit infection or not and to measure the diameter of the lesion if any. From this monitoring the maximum speed of progression of the lesion and the time elapsed between the drop deposit and the first observation of an infection spot (DelaiMin) were calculated for each genotype.

To take into account a probable phenology shift between genotypes, the maximum of infection probability between the 2 harvests for each genotype was computed and used in further data analyses.

## 2.5 Fruit surface conductance assessment

For the calculation of the permeation coefficient of water vapour through the fruit surface (surface conductance),  $\rho$ , monitoring of fruit mass loss was performed.

For each genotype, 10 freshly harvested fruits were numbered, weighed and their three diameters were measured. The fruit pedicel was covered with varnish to prevent water loss from this zone. Fruit were then placed in a ventilated chamber. Temperature and relative humidity were continuously measured (SEFRAM LOG 1520). Each fruit was weighed every 90 minutes for about 7h. Fruit surface was approximated as an ellipsoidal surface computed from the three fruit diameters (see above).

The hourly surface conductance ( $g(h), cm\ h^{-1}$ ) of each fruit was computed following the method described by (Gibert et al, 2005) as:

$$g(h) = \frac{T_f(h)}{S_f \times \frac{M_w}{R \times Temp} \times P^*(H_f - H_a)}$$

where  $h$  is time since sampling ( $h$ ),  $T_f$  is the transpiration per unit time ( $g\ h^{-1}$ ) which is equal to the weight loss,  $S_f$  is the fruit surface area at  $h = 0\ (cm^2)$ ,  $M_w$  is the molecular mass of water ( $18\ g\ mol^{-1}$ ),  $R$  is the gas constant ( $83\ cm^3\ bar\ mol^{-1}K^{-1}$ ),  $Temp$  the temperature ( $K$ ),  $P^*$  is the saturation vapour pressure ( $bar$ ) [depending on temperature following the equation of Fishman and Génard (1998):  $P^* = 0,008048 * \exp(0.0547 * (Temp - 273.15))$ ],  $H_f$  is the relative humidity within the fruit (assumed to be equal to 100%), and  $H_a$  is the relative humidity of the atmosphere.

During the measuring time, the hourly surface conductance per fruit could either be relatively constant or decrease due to the light drying out of the fruit. To compute the finest unique value of

hourly surface conductance for a given fruit, the relationship between  $g(h)$  and the relative water loss ( $RWL_h$ ) was explored.  $RWL_h$  was defined as:

$$RWL_h = \frac{(M_0 - M_h)}{M_0}$$

where  $M_0$  is the initial fruit fresh mass and  $M_h$  is the fruit fresh mass at time  $h$ . When the linear relationship was significant ( $P < 0.05$ ), fruit surface conductance was taken as equal to the y-axis intercept of the linear relationship ( $RWL_h=0$ ), corresponding to the maximal value observed, otherwise it was taken as equal to the mean of the hourly conductance over the observations (Gibert et al, 2005).

A total of 149 and 129 genotypes were monitored for fruit mass loss at the two harvest series respectively; 127 genotypes were common to both series. Fruit total surface conductance was computed for each fruit by multiplying the hourly surface conductance by the surface of the corresponding fruit.

## 2.6 Stomata number estimation

A preliminary experiment was performed to set up a protocol suitable to estimate the mean number of stomata per fruit for the genotypes of the whole population. It has been realized on 4 fruits from F169 and C222 genotypes with masses ranging from 2 to 4.2 g. Prints from the entire fruit were sampled by defining transects from the peduncle to the style. Each transect was divided into 4 to 5 zones. Five photos were taken in each zone (two at the top right and left, two at the bottom right and left and one in the center) (*Annexe 1. Supplementary Fig S1*). The number of stomata was calculated according to two methods: i) counting all stomata from all transects and zones under the microscope (with the naked eye), ii) counting stomata on all the pictures taken throughout the zones and analyzed with ImageJ software - win64 (*Annexe 1. Supplementary Fig S2*). Results obtained are presented in Supplementary data (*Annexe 1*). They allowed setting a protocol to phenotype a large number of genotypes, nectarines only, on the basis of young fruit.

Measurements of number of stomata for each genotype were conducted once, during the 2<sup>nd</sup> series of harvests on 121 nectarines genotypes. The footprint of the fruit surface was made on the equator ring from the suture to the suture, with a hardener varnish (ECRINAL ASEPTA laboratories). After hardening of the varnish, the imprint was removed with an adhesive tape and disposed on a slide. With a microscope equipped with a camera, five pictures were taken per imprint distributed along the imprint. The number of stomata present on each photo was counted manually using the ImageJ-win64 software. A total of 1815 photos taken on 363 fruit were analyzed. For each genotype, the average number of stomata  $n$  was calculated from 3 fruits:

$$n = \frac{1}{3} \sum_{i=1}^3 \left( \frac{\sum_{j=1}^5 \text{Number of stomata on picture } j \text{ of fruit } i}{\text{Surface of the 5 pictures}} \times \text{Surface of the fruit } i \right)$$

The average number of stomata  $n$  calculated in this way for each genotype was considered as a constant during fruit development. On the contrary the stomatal density decreased during fruit growth. For any fruit of a given genotype harvested at any time during fruit growth, the stomatal density was estimated by dividing the number of stomata  $n$  of the genotype by the surface of the fruit considered.

## 2.7 Genetic linkage map construction and QTL analysis

Genotyping of the BC2 population was performed using the International Peach SNP Consortium (IPSC) 9K peach SNP array v1 (Illumina Inc. San Diego, CA, USA), which was (Verde et al, 2012). SNPs showing severe segregation distortion ( $\chi^2$  test,  $p < 10^{-6}$ ) and more than 1% of missing data were excluded.

Two different genetic linkage maps of the BC2 progeny were developed from the dataset of the 9k SNP array by filtering on the basis of parental alleles, one monitoring the polymorphism between D and *P. persica* 'Summergrand' (S) genomes and the second tracking the heterozygosity of Z. The SNP dataset was combined with the previous mapping dataset used in Illa et al. (2009). For the SNP map tracking the polymorphism between D and S genomes, we only kept markers with D nucleotide being different from both S and Z nucleotides ('DvsS'). The map built on 163 individuals is 420 cM long with 340 independent SNP markers and a density of 1.24 cM between markers. The second map, monitoring Zephyr heterozygosity, was based on the SNP having a specific nucleotide for one of the two alleles of Z ('SNP\_Z'). The map developed on 111 individuals included 117 independent SNP markers, covered 389 cM with a density of 3.3 cM between markers. Linkage group 2 was almost missing possibly due to very high homozygosity of Zephyr. Details on DNA extraction, SNP genotyping and map construction are available on (Desnoues et al, 2016).

The QTL research was carried QTLs by using R/qtl (Broman, 2003) implemented in R (R Development Core Team (2014)). For all traits, a nonparametric test (model 'np' of function 'scanone') was used based on the method described by (Kruglyak & Lander, 1995) to search phenotype-marker associations without assuming a normal distribution of phenotypic data. Regarding the traits that follow a normal distribution, or those that could be transformed to follow a normal distribution, QTL detection was performed by marker regression ('mr' method) (Soller et al, 1976). Given that we found correlations between traits and that we observed co-locations of QTL between traits, for example between QTL for infection probability and QTL for fruit mass, the QTL analysis was also performed using covariates, for traits that follow a normal distribution only. Considering the particular characteristics of the BC2 progeny, the analysis was performed only at the

genetic markers (no interval mapping), and individuals with missing genotypes were discarded. The likelihood value for presence of a QTL was expressed as a LOD score. Permutation tests were used to estimate genome wide significance thresholds. However, a less stringent threshold, namely a linkage-group-wide significance level of 5%, was used to detect suggestive QTLs, i.e. QTLs that may be associated with phenotype but are not strongly supported statistically.

## 2.8 Projection of QTL on a physical map

In addition, an integrated physical map was built for each of the eight linkage groups. Markers were placed on the map according to their positions in the peach genome sequence v2.0 ([http://www.rosaceae.org/species/prunus\\_persica/genome\\_v2.0.a1](http://www.rosaceae.org/species/prunus_persica/genome_v2.0.a1)). BioMercator software v4.2 (Arcade et al., 2004) was used to make links between each of the genetic maps and the SNP-based physical map mentioned above, and QTLs detected on 'DvsS' and 'SNP\_Z' maps were projected together on the physical map. Mapchart 2.2 software (Voorrips, 2002) was used to generate map figures.

## 2.9 Statistical analysis

To explore the effects of different factors on the variables of interest, linear regression models were tested using the package 'lme4' of R. In the case of average data by genotype, simple fixed effects models were compared using the 'lm' function. When repetitions were available for genotypes, mixed effects models were defined using the 'lmer' function to take into account the variability associated with the genotypes (random part). The effect of each factor was tested by comparison (ANOVA) of nested models with and without the factor tested.

The 'loess' function, allowing adjustment by local polynomial regression, was used to add trends of the relationships between two variables on graphs.

# 3 Results

## 3.1 Physical characteristics of young fruit of the population

Young fruits of a hundred individuals of a mapping population were studied to characterize two physical characteristics potentially linked to susceptibility to *M. spp.* infections: stomata density and surface conductance.

The density of stomata of a fruit is the relevant variable to relate to infection susceptibility. It can be decomposed into two components: the total number of stomata of the fruit and the surface of the fruit. The latter constantly changes during fruit development whereas the former can be considered as a genetic constant as far as it varies between genotypes. Consequently, the average stomata number per genotype was estimated from 3 young fruit of the 121 nectarine genotypes of the BC2 population.



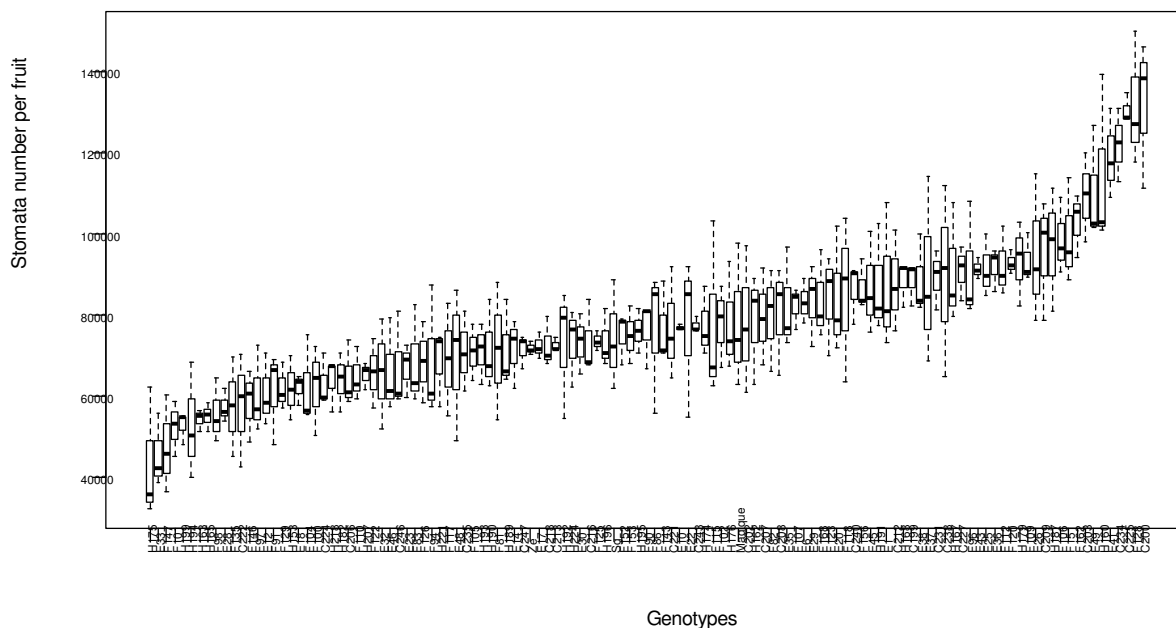


Figure 1: Stomata number distribution for 121 nectarine genotypes of the BC2 population ordered by increasing mean number. Within-genotype variability comes from 3 fruit.

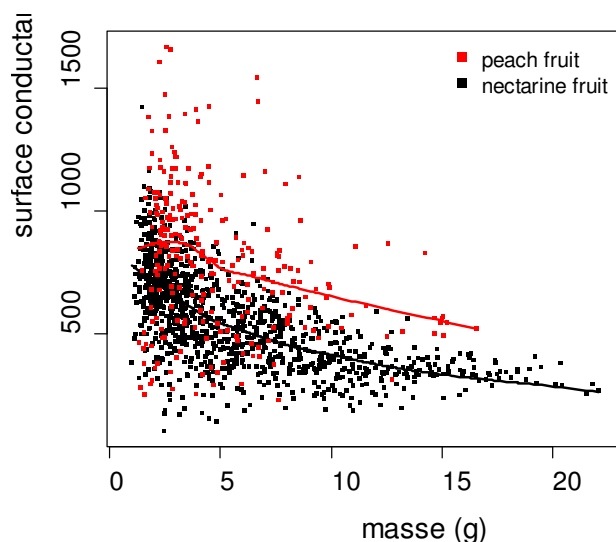


Figure 2: Surface conductance (cm/h) according to fruit mass (g) for both harvest series at stage I (black: nectarine fruit; red: peach fruit). The black and red lines were obtained by smoothing nectarine and peach data respectively.

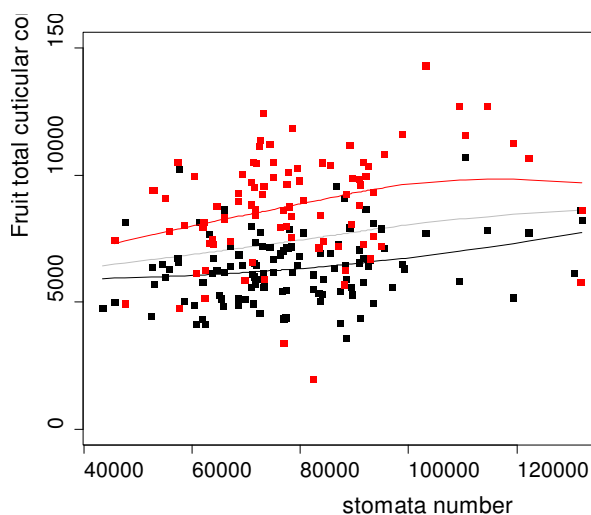


Figure 3: Fruit total surface conductance (cm<sup>3</sup>/h) according to fruit stomata number depending on the harvest series. In black data from 1<sup>st</sup> series and red from 2<sup>nd</sup> series. The black and red lines were obtained by smoothing 1<sup>st</sup> series and 2<sup>nd</sup> series data respectively; the grey line was obtained by considering both series together.



Figure 3: Young fruit at stage I after infection by drop. a) resistant fruit, b) 'classic' infection c) 'clear spot' reaction.

The stomata number has a near-normal distribution in the population with a right tail towards large values. It proved to be highly variable between genotypes ranging from 43 459 to 132 050 (Figure 1). Between-genotype variance represented 76% of the total variance and the p-value associated (Fisher test) was widely significant ( $<2 \cdot 10^{-16}$ ). Hence, between-fruit (within genotype) variation was small compared to the genotype effect. Thus, fruit stomata number appeared stable between fruit of a same genotype and highly variable between genotypes.

Considering the small within-genotype variability of stomata number, we further considered the number of stomata per genotype as a genotypic constant and kept the mean value of stomata number estimated for each genotype as specific of the genotype. Then using this genotypic stomata number, we could estimate stomatal density of every fruit harvested for a given genotype at any stage by dividing this genotypic stomata number by the surface of the fruit considered. This extrapolation was further used to explore links between variables and to perform QTL research (see below).

The fruit surface conductance is the second variable characterized on the young fruit of the population via monitoring transpiratory losses. It was assessed at two dates during stage I. The general trend of fruit surface conductance data plotted against fruit mass follows a decreasing curve (Figure 2). Smallest fruit displayed high values of surface conductance that sharply falls and then remain stable when fruit grow. Although evolutions with fruit growth are similar, a notable difference of levels was observed between peach and nectarine fruit, the former displaying higher values of surface conductance.

In order to decipher the role of stomata on surface conductance at stage I, we explored the relationship between the fruit total surface conductance ( $\text{cm}^3/\text{h}$ ) and the fruit stomata number (Figure 3). A positive relationship was observed which is reinforced when looking at both harvest date separately. The correlation between the two variables is significant whatever the dataset considered: for the 1<sup>st</sup> harvest series, the correlation is 0.26 (p-value = 0.004692), for the 2<sup>nd</sup> harvest series it is 0.29 (p-value = 0.005993) and when considering both series together it is 0.23 (p-value=0.0008914). This result suggests that stomata number may partially contribute to the level of fruit surface conductance at stage I: the more stomata the higher surface conductance.

To be able to predict the conductance of any fruit from the other experiments (fruit used to count stomata number and for infection tests) from its mass and genotype, we smoothed the relationship between mass and conductance for each genotype (*Annexe 1. Supplementary Fig S5*). Then for every fruit harvested for a given genotype at any stage, we extrapolated its fruit surface conductance from the corresponding fruit mass. This extrapolation was then used to further explore links between fruit variables and research of QTLs (see below).

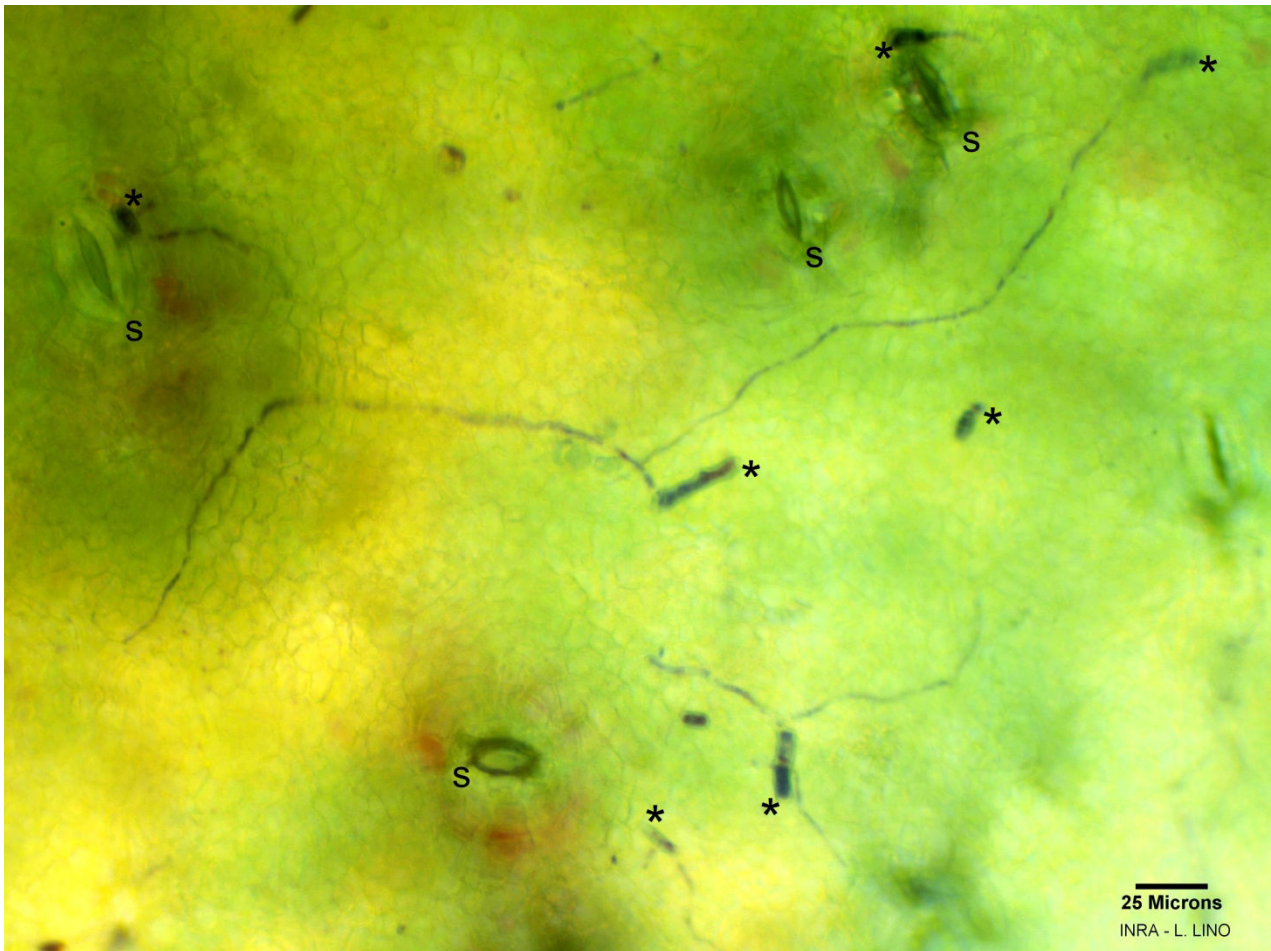


Figure 4: Light microscopy image of spore germination on 'clear spot' area at immature nectarine surface with Toluidine bleu, 0.1%. This image illustrates a germination of spores (asterisks) around stomata (s) and the colonization of surface by hyphae.

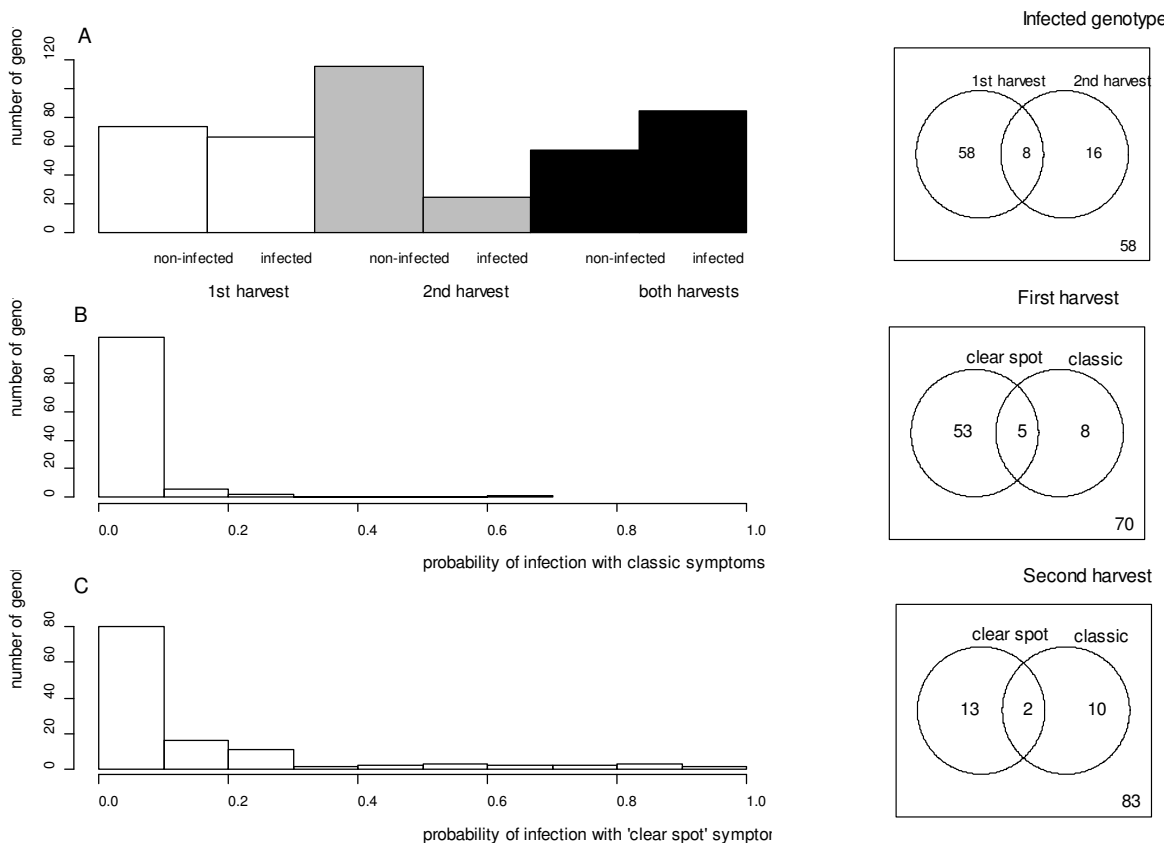


Figure 5 : Left panel: Number of genotypes with no infected fruit (non-infected) or infected fruit at both harvests during stage I (A). Maximal probability of infection between the 2 harvests with 'classic' symptoms (B) and 'clear spot' symptoms (C). Right panel: Number of infected genotypes at each harvest and both harvests. Number of genotypes with 'clear spot' and/or classic symptoms at first and second harvests. The numbers at the bottom right correspond to non-infected genotypes.

### 3.2 Infection probability

Infection probability of fruit at stage I was investigated by drop test in laboratory at two harvest dates within stage I. Besides fruit which did not develop infection, we observed two types of reaction: either fruit with an infection considered as "classic" displaying a development of brown rot up to cover the whole fruit, or fruit with a 'clear spot' (Figure 3) that reached a limited size of about 5 mm of diameter and then stopped to grow. Observation with a binocular microscope (x10) with solution of toluidine blue at 0.1%, showed hyphae, and signs of infection in the middle of the spots (Figure 4). This kind of symptoms has never been described before in the literature although it represents in our experiment a majority of cases of infection.

More genotypes were infected at the first harvest than at the second but 16 were infected only at the second harvest. For the 1<sup>st</sup> harvest, 50% of genotypes were susceptible and 88% of them displayed 'clear spots'. As for the 2<sup>nd</sup> harvest, only 23% of the genotypes were infected and 60% of them displayed 'clear spots' (Figure 5 right panel). Thus 'clear spots' symptoms were more frequent at the 1<sup>st</sup> harvest. Finally, 57 BC2 genotypes proved to be resistant at both harvests within stage I. Among the susceptible genotypes, the infection probability was generally low, especially for the ones displaying classic symptoms (Figure 5B). For the genotypes presenting 'clear spot' symptoms (Figure 5C) infection probability ranged from 0.1 to 1.

The huge variability of the phenology within the progeny (flowering date, time and growth rate) may have resulted in shifting the two harvest dates on either side of the peak of sensitivity expected at stage I. To take into account this probable phenology shift between genotypes, the maximum of infection probability between the 2 harvests for each genotype was further considered.

The evolution of the diameter of the lesion caused by the fungus was monitored (Figure 6). These profiles displayed different kinetics according to the type of symptoms. Lesions on fruits displaying classic symptoms (Figure 6A and B) progressed towards large diameters whereas lesions with 'clear spot' symptoms stopped growing around 5 mm of diameter (Figure 6 C and D), In addition, the maximum speed of progression of the lesion was lower for 'clear spot' lesions than for classic ones (Figure 7). However in case of the classic lesions a large inter-genotype variability was observed and certain genotypes showed a greater predisposition to the rapid development of the fungus once infected.

### 3.3 Links between fruits characteristics and fungal susceptibility

The effects of fruit mass, surface conductance (total or per area unit) and number of stomata on the sensitivity of fruit to *M. laxa* in the early stage of their growth were investigated (Figure 8). Among the fruit characteristics, only surface conductance (cm/h) was significantly related to fungal susceptibility (Table 1). Higher conductance levels were associated with susceptible genotypes and

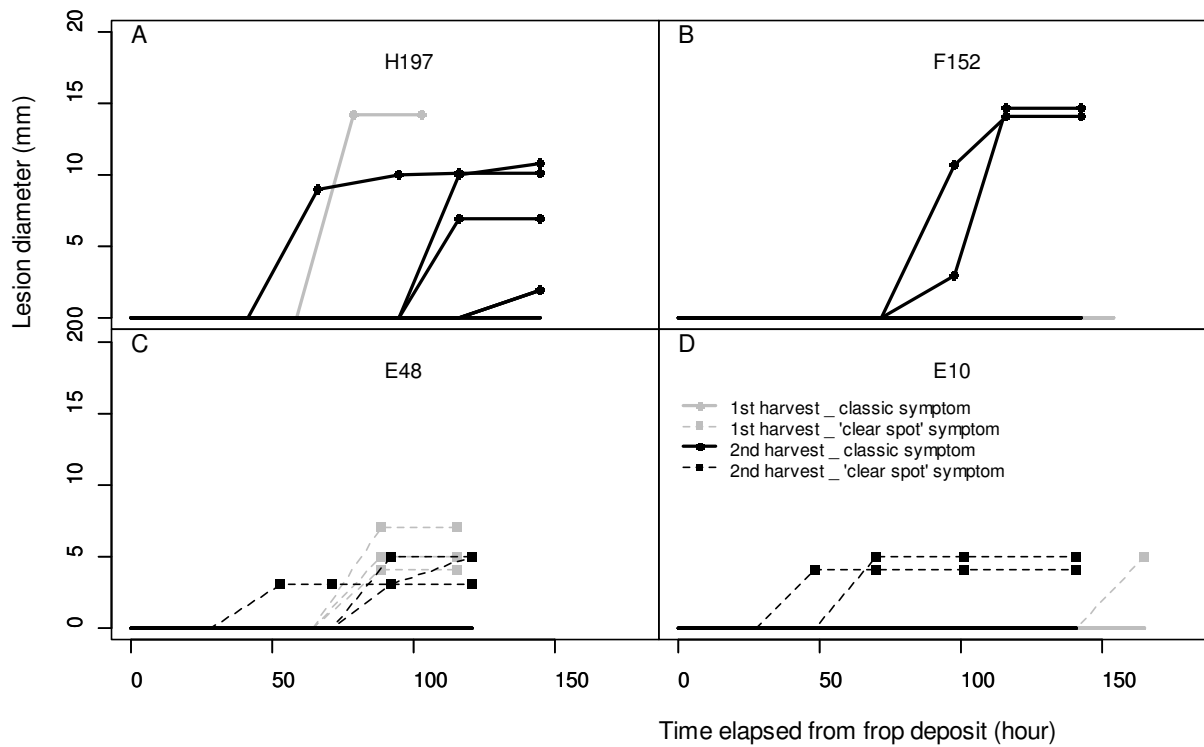


Figure 6: Evolution of the diameter of the lesion of fruits of 4 genotypes displaying 'classic' (A and B) or 'clear spot' (C and D) infection at both harvests during stage I.

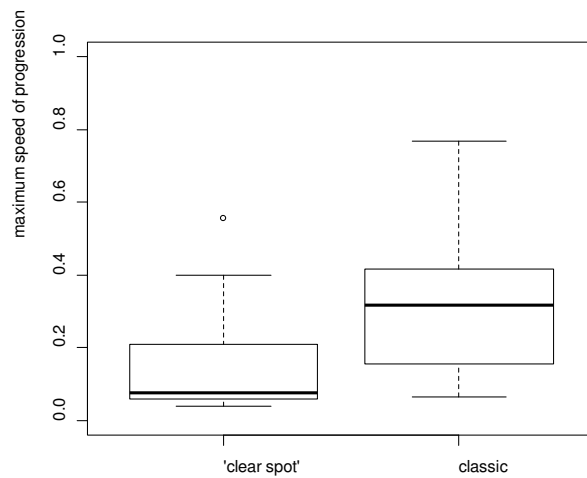


Figure 7: Variability between genotypes of the maximum speed of progression of the lesion according to the type of symptoms.

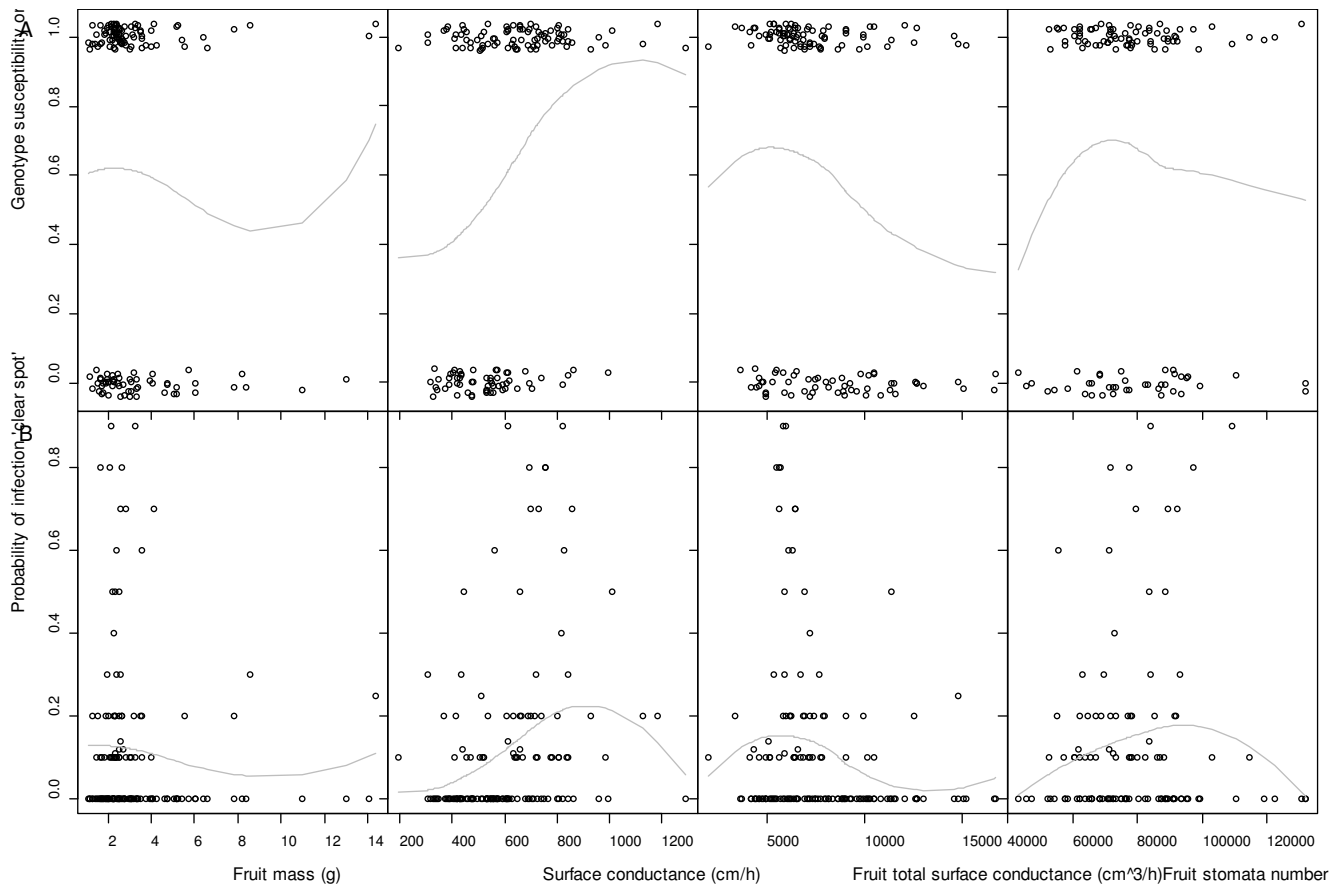


Figure 8: Relationships between 2 variables of infection and tentative explanatory variables, i.e. Fruit mass, Surface conductance, Fruit total surface conductance and Fruit stomata number (nectarines only). A. Genotype susceptibility or resistance (respectively 1 or 0, more or less a little variation added to view all points (R 'jitter' function)). B. Probability of infection with 'clear spot'. The grey lines are tendencies plotted with a local polynomial regression fitting.

Table 1: Summary of statistical analyses (p-value and significance) from anova on genotype dataset \_ (1 data per genotype corresponding to the harvest with maximal infection)

Explanatory variables	Variables of interest	
	Genotype susceptibility or resistance	Probability of infection ('clear spot')
Mean fruit mass	ns	ns
Surface conductance (cm/h)	Pval 1.43e-06 *** Var expl 18.95%	Pval 4.09e-05 *** Var exp 13.96%
Fruit total surface conductance (cm <sup>3</sup> /h)	ns	Pval 0.00074 ** Var exp 5.54%
Stomata number §	ns	ns

Signif. Codes : '\*\*\*\*' 0.0001 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 'ns'

§ only nectarines

higher infection probability. The surface conductance explained 20% and 14% of the variation of 'genotype susceptibility or resistance' and 'probability of infection with 'clear spot'', respectively. Even though stomata number was slightly linked to surface conductance, no significant effect of stomata number on fungal susceptibility was observed.

### 3.4 QTL location

The QTL detection was performed on the two maps of the population, monitoring the polymorphism between D and *P. persica* 'Summergrand' (S) genomes and the heterozygosity of Z, respectively.

For the number of stomata, available for nectarine only, a putative QTL was detected on the 'DvsS' map on linkage group 5 (Table 2). The *P. davidiana* allele reduced the number of stomata. No QTL for stomata density was detected.

For surface conductance, either total or per area unit, QTLs were detected on different linkage groups (1, 3 and 4) suggesting a polygenic determinism. In all cases, the wild allele reduced the conductance. In the preliminary analysis, QTL detected on LG5 and LG7 were strongly associated with the peach-nectarine locus and with a QTL for fruit mass, respectively and disappeared when integrating these later traits as covariates. On LG3, QTL for both surface conductance and fruit total surface conductance were detected on 'DvsS' map and a QTL for surface conductance was detected on 'SNP\_Z' map on LG3 as well, in the same position (Figure?).

For resistance to *M. laxa*, QTL were detected for the different variables computed, both on 'DvsS' and 'SNP\_Z' maps. On the 'SNP\_Z' map, three QTL were detected on LG3 and LG4 for the probability of infection of 'clear spot' type and one QTL on LG6 for the lesion diameter. On 'DvsS' map, QTL were detected on LG4, LG5 and LG7, for 'Genotype susceptibility or resistance' (LG4, LG5), speed of progression of the lesion (LG4, LG5), lesion diameter (LG5) and intra-genotype variation of the time elapsed between the drop deposit and the first observation of an infection spot (LG7). On LG6, QTL previously detected for the probability of infection disappeared when adding fruit mass as covariate. The effects of the D wild alleles were varying according to the QTL. They reduced the 'Clear Spot' susceptibility but tended to increase the lesion diameter. As for the polymorphism of Z, it controls the probability of 'Clear Spot' infection only.

## 4 Discussion

### 4.1 Marker density and population size – factors controlling QTL detection

Despite the complex structure of the studied population limiting the number of SNPs that were useful for the construction of the genetic maps, the 'DvsS' map used in this study is fairly accurate compared to the maps usually used for genetic mapping studies in *Prunus* (Salazar et al, 2013).



Table 2. Summary of the QTL detected on the ‘DvsS’ and ‘SNP\_Z’ maps

Map	Fruit characteristics	Trait	LG	QTL symbol	Marker	LOD peak position (cM)	LOD	pval	Genome-wide threshold (pval<0.05)	Wild allele effect (D)	R <sup>2</sup> (%)	Covariable	Method <sup>ε</sup>
DvsS	transpiration	Surface conductance	1	Cond	SNP_IGA_14438	8.7	3.27	0.00115	2.94	-	4.4	Peach versus Nectarine	mr
DvsS	transpiration	Surface conductance	3	Cond	CC8	41.6	3.96	2.64E-05	2.94	-	7.5	Peach versus Nectarine	mr
DvsS	transpiration	Fruit total surface conductance	3	TotalCond	CC8	41.6	4.55	0	2.61	-	3.8	_	np
DvsS	susceptibility or resistance	Genotype susceptibility or resistance	4	Inf	AC1	4.6	2.70	0.035	2.47	-	8.9	_	np
DvsS	infection propagation	speed of progression of the lesion ('classic')	4	LesionSpeed-class	SNP_IGA_382502	6.1	3.26	0.018	2.71	-	36.0	_	mr
DvsS	transpiration	Fruit total surface conductance	4	TotalCond	PMS40	19.8	3.49	0.015	2.89	-	8.3	_	mr
DvsS	infection propagation	speed of progression of the lesion	5	LesionSpeed	CPPCT040	6.2	3.05	0.005	2.32	+	16.9	_	np
DvsS	infection propagation	lesion diameter	5	LesionDiam	SNP_IGA_587450	24.5	3.08	0.001	2.13	+	35.0	_	np
DvsS	stomata	number of stomata	5	nbStom	SNP_IGA_589972	26.6	2.15	0.028	1.89*	-	6.2	_	mr
DvsS	susceptibility or resistance	Genotype susceptibility or resistance ('clear spot')	5	Inf-CS	pchgms4	32.8	2.96	0.00028	2.91	-	11.1	Fruit Mass	bin
DvsS	infection propagation	intra-genotype variation of the time elapsed between the drop deposit and the first observation of an infection spot	7	LesionDelay	SNP_IGA_784825	35.6	5.14	0.00063	4.72	+	22.1	Fruit Mass	mr
SNP_Z	susceptibility or resistance	Probability of infection ('clear spot')	3	InfProb-CS_Z	SNP_IGA_346651	28.0	2.48	0.029	2.21		10.8	_	np
SNP_Z	transpiration	Surface conductance	3	Cond_Z	SNP_IGA_346651	32.8	2.72	0.012	2.22		7.5	_	np
SNP_Z	susceptibility or resistance	Probability of infection ('clear spot')	4	InfProb-CS_Z	SNP_IGA_397391	18.5	4.25	0.019	3.35		14.4	_	2part
SNP_Z	infection propagation	lesion diameter	6	LesionDiam_Z	SNP_IGA_612236	28.0	2.41	0.026	2.11		23.3	_	np

\* linkage-group-wide significance level of 5%; <sup>ε</sup> 'mr': marker regression; 'np': non-parametric method; '2part': method for data L-shaped

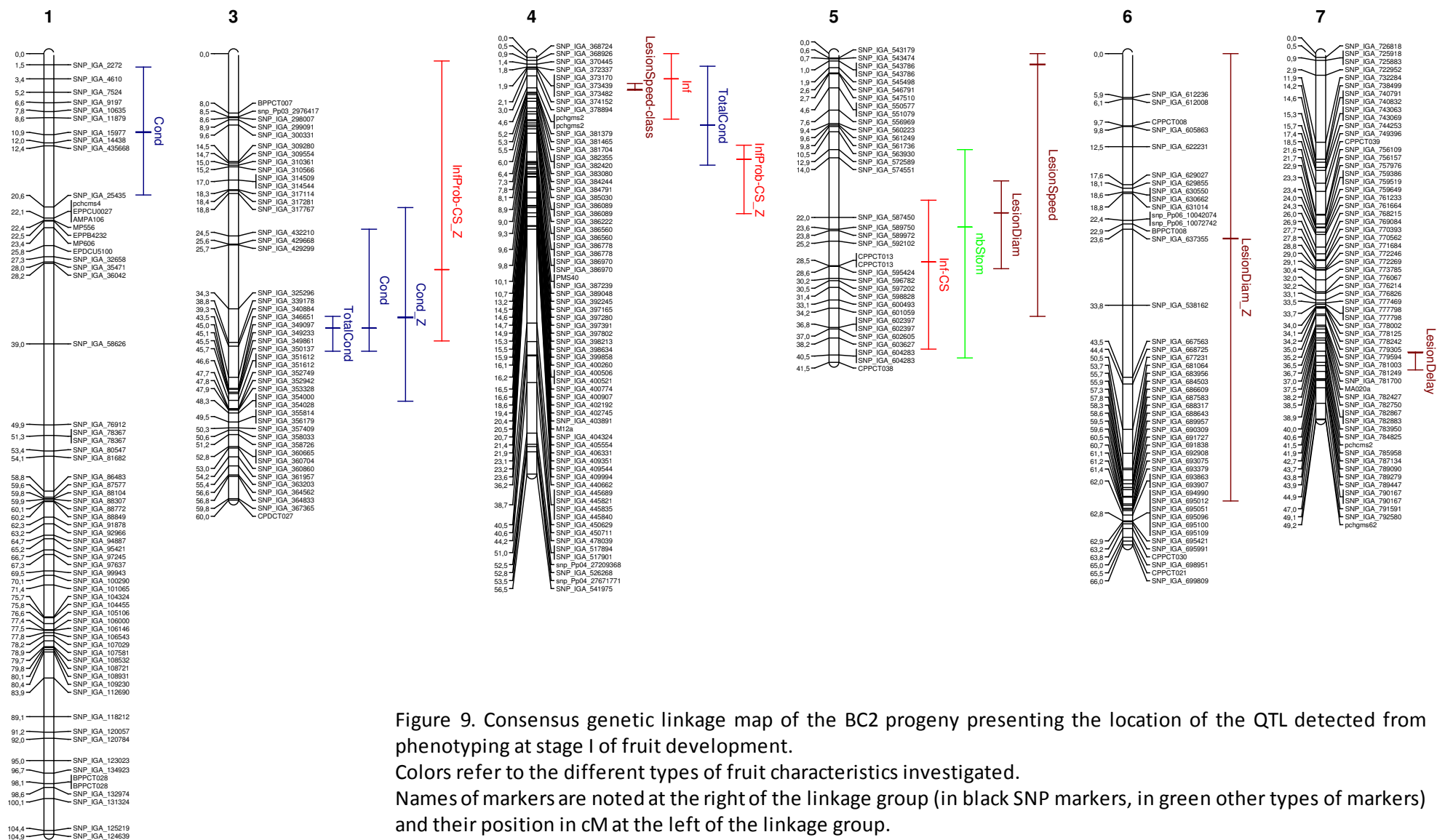


Figure 9. Consensus genetic linkage map of the BC2 progeny presenting the location of the QTL detected from phenotyping at stage I of fruit development.

Colors refer to the different types of fruit characteristics investigated.

Names of markers are noted at the right of the linkage group (in black SNP markers, in green other types of markers) and their position in cM at the left of the linkage group.

Abbreviations: Cond, Surface conductance (cm/h) ; Inf, Genotype susceptibility or resistance ; InfProb, Probability of infection ; LesionDelay, intra-genotype variation of the time elapsed between the drop deposit and the first observation of an infection spot (h); LesionDiam, lesion diameter (mm) ; LesionSpeed, speed of progression of the lesion; mass, fruit mass (g); nbStom, number of stomata ; PN, Peach versus nectarine ; TotalCond, Fruit total surface conductance (cm<sup>3</sup>/h); -class, 'classic' infection ; -CS, 'clear spot' reaction. Z at the end of name of the QTL indicates that the QTL was detected on the 'SNP\_Z' map.

Therefore, the limiting factor impairing QTL detection and resolution in this study might be the population size, although it falls in the range typically studied in *Prunus*, the number of hybrids produced by trees being limited by the single seed of a fruit. Even though the number of genotypes studied for the different traits was reasonable (the smallest dataset counted 121 genotypes for stomata number since only nectarines were analyzed), the number of genotypes bearing the D wild allele was always low (only  $\frac{1}{4}$  individuals in average) which impaired the power of QTL detection. In addition, many traits stemmed from infection tests were recorded for infected fruit only which reduced the number of genotypes considering the high proportion of resistant fruit observed in this study. Despite these limitations, the comparative analysis of different traits of stage I and their genetic control is innovative and brings interesting results to further explore. A majority of the traits studied proved to be highly variable in the population and under complex genetic control.

#### **4.2 Counting stomata number on a mapping population**

The number of stomata is determined during ontogenesis and remains constant during fruit development (Hieke et al, 2002). The density of stomata for young green fruit is very high and very variable depending on the genotype. With the expansion of the fruit, the stomata are diluted on the surface of the fruit, leading to the decrease in density (Blanke, 1992) (Knoche et al, 2001). In peach the stomatal density of the peduncle zone decreases throughout fruit growth from 65 stomata/mm<sup>2</sup> to 5 stomata/mm<sup>2</sup> (Yamaguchi et al, 2003). Very few studies were published yet on fruit stomata number avocado and apple: (Hieke et al, 2002); cherry: (Peschel et al, 2003); peach: (Yamaguchi et al, 2003). Hence no need to say that phenotyping fruit stomata number of an entire mapping population is pioneering. Although this phenotyping is time-consuming, the critical point is probably sampling. Indeed, stomatal density varies from the pistil to the peduncle zone (Peschel et al, 2003), (Yamaguchi et al, 2003), (Gibert et al, 2007) which would encourage to count all stomata from each fruit or to define a statistical model for the evolution of the stomata density as a function of the position on the fruit. The first solution is conceivable only in the case of small fruit and if the count can be automated. Unfortunately, in the case of nectarines, stomatal density of small fruit is high and the cuticle relief very rugged which makes counting and automation difficult. The second solution, applied to a population, would require that the model is the same regardless the genotype. But preliminary experiments to settle the protocol showed different profiles of stomata distribution on the fruit for 2 genotypes of the population.

Therefore, we chose to sample only on the equator. This choice did not allowed to grasp the within fruit variability of stomata density but it probably contributed in obtaining a low intra-genotype variability compared to the inter-genotype variability. Similarly, the limited number of fruit studied per genotype resulted in a low precision in the estimation of stomata number for each

genotype. Therefore in the light of these two elements, the reliability of the average number of stomata calculated for each genotype may be questionable. The number of stomata calculated for the BC2 population is between 43,459 and 132,050 stomata; the observed densities ranged from 21 to 156 stomata/mm<sup>2</sup> for stage I. For Zephir cultivar, 71846 ( $\pm$  1246) stomata were counted on average for 3 sampled fruit. The average value obtained for the same cultivar by Gibert (2007) with a different sampling methodology was 70592 ( $\pm$  5048). These remarkably similar results support the choice of the methodology used for our experiments. Furthermore, the detection of a QTL on the LG5 is also encouraging.

In *Arabidopsis*, several genes involved in the regulation of stomatal density have been identified: the EPF1 and EPF2 genes (epidermal patterning factor 1 and 2) control stomatal density (Hara et al, 2007); (Hunt & Gray, 2009); the STOMAGEN / EPFL9 gene is a regulator of stomatal development (Hunt et al, 2010); a mutation in *sdd1* (stomatal density and distribution) causes specific alterations in the density and distribution of stomata (Schlüter et al, 2003). We searched for homologues of these genes on the peach genome sequence (Verde et al, 2013). Homologues of EPF1, EPF2 and STOMAGEN were detected on LG3 and LG8. Only a homologue of *SDD1* gene (Berger & Altmann, 2000) was located on the LG5 in the QTL region we detected for stomata number. This makes *SDD1* a good functional and positional candidate for this QTL.

#### **4.3 Exploring the link between stomata number and conductance**

High surface conductance was observed in the 1<sup>st</sup> series of harvest, but also a strong decrease thereof over a small range of fruit mass. In the 2<sup>nd</sup> series of harvest, mass was more dispersed but surface conductance more stable. The high surface conductance for very small fruit may be explained either by the high density of stomata or/and by the low cuticle thickness. Indeed, surface conductance of the fruit can be decomposed into three components: cuticular, stomatal and crack conductance (Gibert et al, 2010). The latter is considered negligible at stage I of fruit development. We found a significant relationship between the fruit total surface conductance and the fruit stomata number. Between genotypes, the latter only explained 5.3% of the variation of fruit total surface conductance. As it was not possible to measure both traits on same fruit in our study, it is difficult to assess this relationship with accuracy at the fruit level, but our estimations (stomatal density of a fruit calculated from the stomata number of the corresponding genotype and the fruit surface) ended up to 36.1% of variation of surface conductance explained by stomatal density at the fruit level. Such trend has been observed on cherry fruit at later stages of fruit development (Knoche et al, 2000). At stage I, small green peach fruit are certainly photosynthetic and the stomata may be functional (Pavel & DeJong, 1993). In the experimental conditions of measuring transpiration, the chamber was ventilated which may have resulted in the closure of stomata. If it was the case, this

would explain the low impact of stomata number on total surface conductance. Besides stomata effect, the other hypothesis to explain the high surface conductance of young fruit is that their cuticle is still not well-formed, i.e. waxes not deposited, which would make fruit very permeable. On cherry fruit, (Knoche et al, 2001) obtained a negative linear relationship between cuticular membrane thickness and cuticular conductance. On peach fruit, the amounts of cutin and cuticular wax compounds are very low at stage I (data not shown), suggesting that cuticular membrane deposition has just begun, and resulting on high permeability to water.

#### **4.4 Identification of loci governing surface conductance**

To our knowledge, fruit surface conductance is rarely studied on early stage of fruit development and has never been studied so far on a peach mapping population. The magnitude of conductance we measured are consistent with the results obtained by (Gibert et al, 2005) on peach fruit at 35 days after full bloom, which corresponds approximately to the 2<sup>nd</sup> harvest of this study. For the search of QTL, data from each harvest and the average were used. The main difficulties in the QTL analyses lay on the one hand in the link of surface conductance with fruit size and on the other hand in the very rapid evolution of surface conductance for a very small range of fruit mass, in a short time lapse. In addition, the fruit type, either peach or nectarine, had an impact on surface conductance. Considering these features, the QTL detection for surface conductance was performed with fruit mass and peach/nectarine type as covariates and QTL were detected on linkage groups 1, 3 and 4, the one on the LG3 being detected on both maps and for both traits (total or per area unit surface conductance). No QTL was detected on LG5 where a QTL for stomata number was detected.

#### **4.5 Resistant fruit, 'classic' infection and 'clear spot' symptoms: a very mixed population**

The BC2 population showed a high variability in susceptibility to *M. laxa*, with probabilities of infection ranging from 0 to 100%. (Mari et al, 2003) and (Biggs & Northover, 1988) showed that fruit were very sensitive to *M. laxa* in stage I. Based on these results we expected to observe a high probability of infection for the majority of the genotypes. However, contrary to our expectations, 41 BC2 genotypes proved to be resistant at both harvests within stage I. So either resistance factors segregate in this population or the susceptible stage was very short and we missed it. For the probability of infection, the results were somehow consistent with what is known, since fruit stage I undergo a phase of increased susceptibility before pit-hardening. By contrast, the observation of 'clear spot' symptom in response to *M. laxa* was new. Fruit showing these symptoms have been kept nearly a month in the boxes and no 'classic' brown rot symptoms developed. The infection started as the fungi developed hyphae, and stopped. This fruit reaction prevented the spread of local infection. These elements suggest a hypersensitivity reaction of the fruit probably due to an active biochemical

response of the fruit, leading in some cases to stop fungus development. Unfortunately it was not possible to observe if the fungus has grown in micro-existing injury or if it has caused them himself.

In addition to the likelihood of infection, the diameter of infection was monitored for 6 days. This monitoring allowed calculating for each infected fruit an average speed of progression of the infection, the minimum time and the average time towards the apparition of a lesion. The variability of these parameters also suggested that additional mechanisms were involved once the infection was declared.

#### **4.6 Identification of loci governing infection probability and progression**

Four QTL for susceptibility or resistance and infection probability were detected of which 2 on the 'SNP\_Z' map and with a colocation between the 2 maps. In addition, 5 QTL were detected for traits related to infection progression. Several hypotheses may explain the low number of QTL with significant LOD score detected for infection variables. First, this trait may certainly be under control of many low effects QTL. The population size is likely too small to detect these QTLs, particularly because many genotypes displayed an infection probability of zero. In addition, resistance to *M. laxa* is also largely influenced by the environment.

The effects of the wild allele from *P. davidiana* were sometimes favorable to limit infection but other times they were promoting lesion propagation. Contrary to our expectations, infection associated QTL were detected on the map marking Zephir polymorphism, suggesting that this cultivar bears interesting alleles for resistance. On LG3 of 'SNP\_Z' map, the QTL for infection probability was collocated with a QTL for surface conductance. The same way, on LG5 of the 'DvsS' map, the QTL for lesion diameter collocated with the QTL for stomata number. Finally, on LG 4, there were also collocations of QTL for infection with a QTL for fruit total surface conductance.

#### **4.7 Hypotheses to explain the variations in susceptibility of young peach to *M. laxa***

Various hypotheses can be advanced to explain the variations in susceptibility of peach to *M. laxa* in the first stage of fruit growth. They are either mechanical or biochemical. In stage I, the mechanical barriers may have insufficient efficacy. On the skin of nectarines, (Lee & Bostock, 2006) observed the formation of appressoria by *M. fructicola* on the lips of stomatal guard cells, suggesting that stomata can be a gateway into the fruit for the fungus. This is all the more likely that at stage I stomatal density is important and that stomata are probably open. While we observed no relationship between the number of stomata of a genotype and susceptibility to *M. laxa* or between stomatal density of the fruit and the presence of infection, we detected a collocation between QTL for lesion diameter and QTL for stomata number. This collocation, although very hypothetical, casts doubt on the role of stomata on infection at stage I.

More evidence is in favor of an effect of very thin cuticle on susceptibility of peach fruit. At stage I, cuticle deposition on peach fruit has just started (see chapter II of this thesis) and thus the cuticular membrane does not already play its role of barrier to water transport resulting to the high surface conductance levels we observed. In addition, this immature cuticular membrane certainly does not play its role of defense barrier against pathogen. This would explain the significant relationships we found between surface conductance and infection traits and the collocation of QTL for those traits.

Our results, combined with those in the literature tend to confirm that a plurality of resistance factors intervene to limit the infection by *M. laxa*. Also, the wisest strategy in terms of varietal innovation would be to try to combine these factors to increase new varieties resistance level. We must continue to explore the characteristics of fruit at stage I to uncover the role of cuticular membrane in the infection and decipher whether it is passive (impermeable barrier) or active (inhibition of the fungus) so as to gain knowledge on resistance factors. The same way, further analyzes of the mechanisms of 'clear spot' onset are needed to better understand young fruit defense reactions. This information may be of utmost importance to solve the problem of brown rot at fruit maturity.

## 5 Conclusion

The work presented here focused on the early stage of fruit growth. It aimed at deciphering the relationship between physical characteristics of young green fruit and susceptibility to *M. laxa*. It was the occasion to develop a methodology of counting stomata for a population. The main conclusion from this study is that the number of stomata had probably little effect on the probability of infection in stage I. On the contrary, surface conductance was linked to susceptibility but did not explain the whole genotypic variation observed for infection probability. These results suggested a probable important role of cuticle depositing delay on young fruit behavior.

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## Chapter 4: Etude de la variabilité génétique et cartographie QTL de la résistance à la pourriture brune (*Monilinia laxa*) dans une descendance interspécifique issue d'un croisement entre *Prunus persica* et *P. davidiana*

A maturité, nous avons étudié le contrôle génétique de la résistance à la pourriture brune dans une descendance interspécifique issue d'un croisement entre de cultivars *Prunus persica* et un clone de *P. davidiana*, nommée BC2. Les fruits à maturité ont été infectés avec une suspension de spores de *M. laxa* selon deux modalités d'infection: par vaporisation sur l'ensemble du fruit dans le verger et par dépôt d'une goutte dans des conditions contrôlées en laboratoire. En plus de la probabilité d'infection, cette dernière modalité a permis d'estimer le début, la progression et la vitesse de l'infection. Ces expérimentations ont été répétées 3 années successives. En outre, les infections naturelles des rameaux au printemps et des fruits à maturité ont été observées en 2015. La descendance BC2 a montré une forte variabilité de résistance à la moniliose. Malgré une très faible stabilité entre les années, trois génotypes à haut niveau de résistance ont été identifiés. Pour la cartographie de QTL, deux cartes de la descendance ont été utilisées, l'une pour tracer la différence entre les allèles de *P. davidiana* et du cultivar Summergrand (*P. persica*) (carte 'DvsS') et l'autre pour l'hétérozygotie du cultivar Zephir (*P. persica*) ('SNP\_Z' carte). Sur la carte 'DvsS', des QTL contrôlant la probabilité d'infection ont été détectés sur les groupes de liaison (LG) 1 et 2. Un QTL fruit contrôlant l'infection naturelle a été détecté sur LG7 et un QTL contrôlant la brûlure des rameaux a été détecté sur LG5. Dans tous les cas l'allèle sauvage provenant de *P. davidiana* a augmenté la sensibilité à la moniliose, sauf pour l'infection naturelle. En revanche, les cultivars Summergrand et Zephir portent des allèles favorables à la résistance à la moniliose. Sur la carte 'SNP\_Z', trois QTL ont été détectés, deux au même endroit sur LG4 pour la sensibilité et la vitesse de progression des lésions notés 2 années différentes, et un sur LG6 pour le diamètre de la lésion. En complément, nous avons également détecté sur LG2 et 6 des QTL contrôlant le maximum de conductance cuticulaire des fruits à maturité, ce qui peut être considéré comme un proxy de la fissuration des fruits. Aucune colocalisation n'a été observée entre les positions des QTL contrôlant la conductance cuticulaire et la résistance. Cela suggère que les QTL détectés pour la résistance à la moniliose ne sont pas liés à la sensibilité des génotypes à la fissuration. Le QTL contrôlant la probabilité d'infection détecté sur LG1 était co-localisé avec un QTL contrôlant la teneur en fructose détecté par Desnoues, Baldazzi et al. (2016) sur la descendance BC2. Il était également à la même position que le QTL majeur détecté par Martinez-Garcia, Parfitt et al. (2013) pour la résistance du pêcher à *M. fructicola* pour lequel le meilleur gène candidat proposé était un transporteur de sucre de la famille SWEET. Ce gène était également le meilleur candidat pour le QTL de fructose. Les QTL détectés sur LG4 et 6 de la carte

'SNP\_Z' étaient dans les mêmes régions que des QTL détectés par Pacheco, Bassi et al. (2014) pour la résistance de l'épiderme et de la pulpe de la pêche à *M. fructicola*. Même si une grande instabilité entre les années et les tests est observée au sein d'une expérimentation donnée, la comparaison de différentes études peut permettre d'identifier des QTL stables.

# QTL mapping for brown rot (*Monilinia laxa*) resistance in an interspecific peach progeny derived from a cross between *Prunus persica* and *P. davidiana*

## Abstract

At maturity stage, we investigated the genetic control of brown rot (BR) resistance in an interspecific progeny derived from a cross between *Prunus persica* cultivars and a clone of *P. davidiana*. For three years, mature fruit were infected with a suspension of spores of *M. laxa* with two modalities of infection: spray on the whole fruit until runoff, in the orchard and drop in controlled conditions in the laboratory. In addition to infection probability, the latter modality enabled to estimate the beginning, progression and speed of infection. In addition, natural infections on stem at spring and on fruit at maturity were scored in 2015. The BC2 progeny displayed high variability for BR resistance. Despite very low stability between years, three genotypes with high level of resistance were identified. For the QTL cartography, two maps of the progeny were used, one tracing the difference between *P. davidiana* and *P. persica* cultivar Summergrand alleles ('DvsS' map) and the other for the heterozygosis of *P. persica* cultivar Zephir ('SNP\_Z' map). On the 'DvsS' map, QTL controlling infection probability were detected on linkage groups (LG) 1 and 2. One QTL controlling fruit natural infection was detected on LG7 and one QTL for blossom and twig blight was detected on LG5. Except for natural infection, in all cases *P. davidiana* wild allele increased the susceptibility to BR. In contrast, the cultivars Summergrand and Zephir carried favorable alleles for BR resistance. On the 'SNP\_Z map', three QTL were detected, two at same location on LG4 for susceptibility and speed of lesion progression for 2 different years, and one on LG6 for the lesion diameter. In complement we also detected on LG2 and 6 QTL controlling maximal surface conductance at maturity, which can be considered as a proxy of fruit cracking. No colocation was observed between the positions of QTL controlling surface conductance and BR resistance. This suggested that QTL detected for BR resistance were not linked to cracking susceptibility of the genotypes. The QTL detected on LG1 for infection probability was co-located with a QTL controlling fructose content detected by Desnoues, Baldazzi et al. (2016) on the BC2 progeny. It was also at the same position as the major QTL detected by Martinez-Garcia, Parfitt et al. (2013) for the peach resistance to *M. fructicola* for which the best candidate gene proposed was a sugar transporter from the SWEET family. This gene was also best candidate for the fructose QTL. The QTL detected on LG4 and 6 of 'SNP\_Z' map were in the same regions as QTL detected by Pacheco, Bassi et al. (2014) for skin and flesh resistances. Even though instability between years and tests was observed inside specific experiments, the comparison of different studies may allow identifying valuable QTL.

## 1 Introduction

Brown rot (BR) on *Prunus* fruit caused by *Monilinia spp.* is a common disease that can provoke large losses in the orchard and after harvest. In peach and all cultivated species of the genus *Prunus*, this aerial fungal disease is a serious problem. Indeed, the relatively long period of incidence, from flowering to post-harvest, the development of resistance to fungicides and the low resistance of the cultivars, are the main reasons to explain the extent of the problem (Fan, Guo et al. 2010). In organic orchards obviously, the problem is even worse and this disease is the major factor limiting the production due to difficult control (Larena, Torres et al. 2005). Studies conducted in apple organic orchards reported yield losses of the order of 42% in the pre-harvest stage, and 80% in the post-harvest period, during which an aggravating factor was the presence of damage on fruit surface (Holb 2004).

Researches have shown that prophylaxis measures could influence the health performance of the orchard and certain cultural practices can reduce the impact of brown rot. For example it is well known in the literature that strong tree thinning and abundant irrigation are very favorable conditions for the development of infection (Gibert, Chadœuf et al. 2007), (Mercier, Bussi et al. 2009). Recently another study conducted by (Bussi, Plenet et al. 2015), showed that tree form and size are also likely to modulate the incidence of brown rot in peach orchard. However these methods alone are not enough to eradicate the disease from the orchard.

Currently, no other alternative than chemical treatment is available hence fungicide applications are required until pre-harvest. Regular use of fungicides presents a significant risk for the environment. Residues could persist in the soil, migrate and eventually contaminate waterways (Gevao, Semple et al. 2000), (Komárek, Čadková et al. 2010), and pose a potential risk to the long-term fertility of the soil (Wightwick, Walters et al. 2010). Besides, chemical control can also have adverse consequences for human health. Inappropriate uses of fungicides can result in residues on fruit and impair the health of consumers. Moreover, the emergence of pathogens resistant to certain active ingredients has already been observed in case of brown rot fungi.

In this context and because the regulation of fungicide use strengthens, the discovery of genetic resistance is a major priority for bringing a durable solution to this problem. Presently there are no complete resistant varieties to brown rot available for peach and nectarine production. In the literature, the Brazilian cultivar 'Bolinha' is recognized as highly resistant to brown rot (Feliciano, Feliciano et al. 1987), (Gradziel, Thorpe et al. 1998), (Wagner Júnior, Raseira et al. 2005), (Lee and Bostock 2007). However, it is a clingstone cultivar with low fruit quality (dos Santos, Raseira et al. 2012). This cultivar was used in fruit breeding programs by Embrapa Pelotas (Brazil) and other institutions but these works never led to the creation of resistant commercial varieties. The

resistance of 'Bolinha' is believed to be related to the thickness of the cuticle, compression of the epidermal cells and production of phenolic compounds, giving a major role to the epidermis in the resistance process. In this way (Pascal, Levigneron et al. 1994) observed no variability in disease progression between different fruit species of *Prunus*, when injured fruit were infected. In contrast, they noticed a large variability of susceptibility between cultivars when infecting fruit with intact epidermis. Other studies focused on small collections of cultivars have shown same contrasting behaviors of cultivars of peach and apricot (Bassi, Rizzo et al. 1998), (Gradziel, Thorpe et al. 1998), (Walter, McLaren et al. 2004), (Wagner Júnior, Raseira et al. 2008), indicating a quantitative inheritance for BR susceptibility and the possibility for its genetic improvement in *Prunus*.

To generate new cultivars that require less fungicide, the identification of genes or loci associated with resistance to brown rot would allow the accumulation of favorable alleles in elite genitors to supply crossing breeding programs. The search for QTL (Quantitative Trait Locus) is an effective tool to identify loci involved in a quantitative trait such as polygenic resistance. Even though several research groups around the world work on brown rot matters, so far only two publications, recent, report QTL detection of resistance to brown rot in *Prunus*. The difficulty of scoring BR susceptibility at the level of a progeny may explain that very few studies have been undertaken. The first study published focused on the introgression of resistance to brown rot from almond to peach (Martinez-Garcia, Parfitt et al. 2013). The authors evaluated a progeny derived from an interspecific cross over three years by inoculating fruit, wounded or not, with a 10 $\mu$ L droplet containing conidia of *M. fructicola* at a concentration of  $2.5 \times 10^4$  spores per mL. No resistance of the flesh was detected on injured fruit. QTL detection on the test with non-injured fruit has in turn enabled the detection of QTL on linkage groups 1 and 4. The second work, conducted at the University of Milan, focused on a F1 progeny stemmed from Contender (fairly resistant) and Elegant Lady (susceptible) cultivars. Artificial infection was performed by two years on fruit, wounded or not, with a 10 $\mu$ L droplet containing conidia of *M. fructigena* at a higher concentration of  $5 \times 10^6$  spores per mL (Pacheco, Bassi et al. 2014). This study enabled the detection of different QTL for resistance of the flesh (wounded fruit) and epidermis (unwounded fruit), respectively on linkage groups 2 and 4 and on linkage groups 3 and 6. These studies ended up on different genomic regions, but this can be explained by differences in the vegetal material studied, the fungus species used and the infection test methods employed. Anyway, these two studies allowed the identification of different genitors that potentially carry different genes of resistance. The most striking and common results of these studies are firstly the low number of QTL detected and the generally low variation explained by the QTL, except the major one detected on LG1 ; and secondly the high instability of the QTL between years. This latter result highlights the importance of environmental factors on the infection probability, at the

infection test level, even though done in laboratory semi-controlled conditions, but perhaps above all because they affect fruit characteristics and therefore susceptibility levels.

The pre-breeding program of peach at INRA-Avignon focuses a major part of its work on resistance to pests, in particular using wild peach species such as *Prunus davidiana* as sources of resistance. For example, *P. davidiana* has already shown interest as mildew resistance source, and for resistance to green peach aphid (Sauge, Kervella et al. 1998) (Foulongne, Pascal et al. 2003) and plum pox virus (Rubio, Pascal et al. 2010). More recently, the team has started to work again on brown rot, developing both quantitative genetics studies and more integrative approaches to design peach ideotypes with reduced sensitivity to brown rot (Quilot-Turion, Ould-Sidi et al. 2012).

In this general context, the objective of this study is to identify new sources of resistance to BR and progress in the characterization of the genetic control of BR resistance by identifying genomic regions associated with this trait. We studied a progeny derived from *P. davidiana* over three years. We focused on the resistance factors linked to epidermis, by performing infection tests in laboratory on non-injured fruit only. This test in controlled conditions enabled the scoring of different parameters describing infection progression. In addition, we also phenotyped the progeny in the orchard using same method as the one used by Pacheco et al (2014) allowing the estimation of the infection probability per genotype. We also scored natural occurrence of BR in the orchard affecting the branches at spring and fruit at maturity. At last we looked at fruit surface conductance, a proxy of epidermis alteration (by microcracks for example). These datasets were then used to perform QTL analyses.

## 2 Materials and methods

### 2.1 Plant material

This study was carried out on the genotypes of a progeny called BC2. This progeny is derived from an interspecific cross between a clone of *P. davidiana* (D), a wild species closely related to *P. persica*, and *P. persica* cv 'Summergrand' (S) (Pascal, Kervella et al. 1998). One hybrid from the F1 progeny resistant to powdery mildew was back-crossed to (S) to produce a BC1 progeny. Finally BC1 individuals were used to pollinate *P. persica* cv 'Zéphir' (Z) to derive the breeding progeny BC2. S and Z are yellow and white nectarine cultivars, respectively, with large tasty fruits.

The study was conducted at the INRA Research Centre of Avignon (southern France). BC2 genotypes and the three parents were planted in 2001 in an orchard with one tree per genotype. The trees were 15 years old in 2013. All the genotypes were grafted onto GF305 seedling rootstocks and were grown under normal irrigation, fertilization and pest-control conditions. All trees were homogeneously pruned and thinned. Experimentations on the BC2 were performed at maturity in 2013, 2014 and 2015.



## 2.2 *Monilinia laxa* strain

The strain of *Monilinia laxa* used for infections was *MI3*. It was isolated from an apricot fruit mummy on March 25th 2011, and cultured in the laboratory INRA Gotheron (Drôme). A mono-spore strain was isolated and stored at -20°C. This fungus was bred on V8 agar medium at a temperature of 21°C ± 2°C (16 hour light and 8 hour darkness) and kept in a glycerol solution in aliquots of 45µl, stored at -20°C. It was multiplied by transplanting aliquots on petri dishes containing V8 agar, medium based on vegetable juice, two weeks before an experiment that required a spore suspension. The petri dishes closed with parafilm were incubated at 21°C ± 2°C and 16 hours of light

## 2.3 Infection tests

A spore suspension of *M. laxa* was prepared before each infection test from a culture dish of 15 days. 5mL of sterile distilled water was added on the culture medium, the agar surface was scraped with a sterile spatula which allowed taking off the spores and the mycelium. The resulting mixture was transferred into a vial containing small glass beads and wherein a drop of Tween was added. The vial was vortexed for approximately 30 seconds to separate the spores from the mycelium. Thereafter, the entire suspension was sampled using a syringe and passed through a filter of 30 microns of porosity to obtain a suspension with a minimum of hyphae and a maximum of spores. To estimate the spore concentration of the suspension, the number of spores of a 1/10th dilution of the mother solution was counted on a Malassez cell under a microscope.

For each suspension spore viability was verified. Five to seven separate drops of 5µL of the final suspension were deposited on a PDA culture medium which transparency facilitates microscopic observation. Seeded Petri dishes were closed and placed at room temperature. After 24h, the germination rate was calculated for each drop by microscopic counting of the spores germinated or not. A spore was considered germinated when it had a germ tube larger than the spore.

## 2.4 Phenotyping

Two modalities of test were used: a spray test in the orchard and a drop test in the laboratory with control of humidity and temperature conditions.

Under laboratory conditions, 20 fruit of similar size and maturity were measured (mass and between cheeks diameter). In order to record potential variation associated with maturity stage, the physiological stage was determined by the IAD index (Ziosi, Noferini et al. 2008), with one measure on the equatorial zone of each cheek. The fruits were disinfected in water bath at 55°C for 40 seconds in 2013 and 2014. Each fruit were numbered and placed in a crystal plastic box, finally the fruits were infected by a drop of 10µL of spore suspension (concentration of 100 spores/µl) deposited on the fruit intact surface (at the cheek), without wounding the fruit. In order to maintain sufficient humidity in the boxes, water cups were added to favor the development of the fungus. The

boxes were placed in climatic chambers with controlled light duration and temperature (8 hours of darkness at 18°C and 16 hours of light at 24°C). An observation was made every day and during five days to record fruit infection or not and to measure the diameter of the lesion if any. From this monitoring the maximum speed of progression of the lesion and the time elapsed between the drop deposit and the first observation of an infection spot were calculated for each genotype.

In 2015 the same procedure was adopted except that, no water bath was made and a spray until runoff was used for infecting 10 fruit per genotype.

In the orchard, 10 peach fruit per genotype, homogeneously distributed in the tree, were chosen and inoculated until runoff with a suspension of spores with the same concentration as for the laboratory test. Infected fruit were protected by paper bag for maintenance of humidity. Six days after inoculation, the paper bag was opened and the number of fruits infected counted (Pacheco, Perini et al. 2015).

In addition, we also scored natural infection at the orchard in 2015, both at the level of stem in spring and of fruit at maturity. As BR attack on flowers was heavy that year, tree branches were then highly affected. So we recorded the number of branches infected in the tree. At fruit maturity, we estimated natural infection probability.

## 2.5 Measurements acquired in the laboratory test

Each day after lab infection, fruits were inspected to verify the appearance of first symptoms of BR.

The **infection occurrence** ('inf01') was recorded as 0 or 1: a genotype with at least 1 fruit infected was recorded as infected (1).

The **infection probability** ('infprob') was estimated in percentage dividing the number of fruit showing BR symptoms by the total of fruit per genotype.

The suffixes 'lab' and 'orchard' were affixed to the names of these 2 traits to differentiate the tests done in the laboratory and orchard, respectively.

The **infection delay** ('infdelay') represent the time in hour between drop deposit and the observation of lesion for the first time, at naked eyes. Maximal delay observed per genotype was considered.

The **mean infection diameter at 72 hours after infection** ('lesiondiam') is the average of the lesion diameter (mm) recorded 3 days after infection for the different infected fruit of a genotype.

The infection **speed** ('lesionspeed') for a given fruit was calculated as the maximum increase of lesion diameter in mm/hour. Maximal speed observed per genotype was considered.

In 2015 in the beginning of spring the **number of stems infected** was counted. To correct this measure with the effect of tree size, it was centered and reduced ('infprob\_stem'). At fruit harvest

time, the **natural infection probability** ('infprob\_natural') was measured in the orchard counting the number of infected fruits, mummies and the number of uninfected for each three.

The year numbers were added to the trait names when necessary.

## 2.6 Fruit surface conductance assessment

Fruit conductance to water vapor was estimated for 87 genotypes from total fruit transpiration measurements during fruit development in four years (2012, 2013, 2014 and 2015). Data were obtained from 5, 10 or 20 fruits, depending on the genotype and of the available number of fruits in the orchard.

Freshly harvested fruits were measured (cheek, suture and height diameters) and placed in a ventilated chamber. Temperature and relative humidity of the chamber were continuously registered (Sefram Log 1520; St Etienne, France). Each fruit was weighed hourly for about 7h. The method to calculate hourly surface conductance from transpirational water loss and to deduce fruit surface conductance is detailed in (Gibert, Lescourret et al. 2005).

Fruit surface conductance results from the integration of three components: stomata, cuticular and cracks conductance. At maturity stage, surface conductance can increase due to the apparition of micro-cracks. From the temporal kinetics of each genotype, we computed the maximal value of surface conductance at the end of fruit growth ('SurfCond') as a proxy of epidermis alteration by cracking.

## 2.7 Genetic linkage map construction and QTL analysis

Genotyping of the BC2 progeny was performed using the International Peach SNP Consortium (IPSC) 9K peach SNP array v1 (Illumina Inc. San Diego, CA, USA), which was described in Verde et al. (2012). SNPs showing severe segregation distortion ( $\chi^2$  test,  $p < 10^{-6}$ ) and more than 1% of missing data were excluded.

Two different genetic linkage maps of the BC2 progeny were developed from the dataset of the 9k SNP array by filtering on the basis of parental alleles, one monitoring the polymorphism between D and *P. persica* 'Summergrand' (S) genomes and the second tracking the heterozygosity of Z. The SNP dataset was combined with the previous mapping dataset used in Illa et al. (2009). For the SNP map tracking the polymorphism between D and S genomes, we only kept markers with D nucleotide being different from both S and Z nucleotides ('DvsS'). The map built on 163 individuals is 420 cM long with 340 independent SNP markers and a density of 1.24 cM between markers. The second map, monitoring Zephyr heterozygosity, was based on the SNP having a specific nucleotide for one of the two alleles of Z ('SNP\_Z'). The map developed on 111 individuals included 117 independent SNP markers, covered 389 cM with a density of 3.3 cM between markers. Linkage group

Table 1. Number of genotypes evaluated as resistant or susceptible in the orchard and under laboratory conditions.

Test	Lab <sup>s</sup>			Orchard		
	2013	2014	2015	2013	2014	2015
susceptible	73	95	70	67	100	72
resistant	52	27	2	30	18	29
# genotypes tested	125	122	72	97	118	101
% susceptible	58.40	77.87	97.22	69.07	84.75	71.29
% resistant	41.60	22.13	2.78	30.93	15.25	28.71

<sup>s</sup> Lab test in 2015 was performed with spray not drop and without water bath

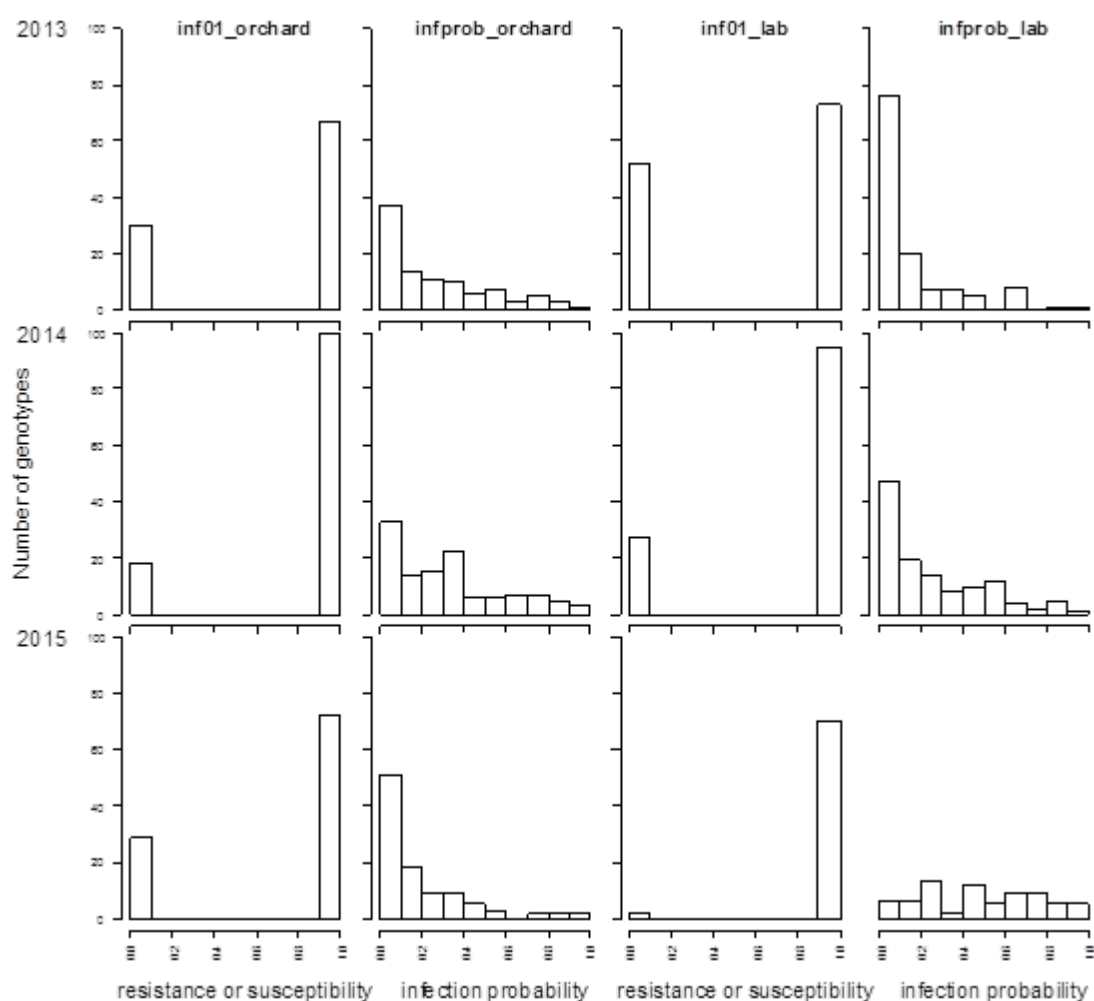


Figure 1. Distribution frequency of infection occurrence and probability in the orchard and the lab, among the BC2 progeny for the 3 years. Frequency is expressed in the y-axis and the number of individuals falling on each phenotypic interval (x-axis). Trait names are indicated at the top of each histogram.

2 was almost missing possibly due to very high homozygosity of Zephyr. Details on DNA extraction, SNP genotyping and map construction are available on Desnoues et al. (2016).

The QTL research was carried out by using R/qtl (Broman et al., 2003) implemented in R (R Development Core Team (2014)). For all traits, a nonparametric test (model 'np' of function 'scanone') was used based on the method described by Kruglyak and Lander (1995), to search phenotype-marker associations without assuming a normal distribution of phenotypic data. Regarding the traits that follow a normal distribution, or those that could be transformed to follow a normal distribution, QTL detection was performed by marker regression ('mr' method) (Soller et al. 1976). Considering the particular characteristics of the BC2 progeny, the analysis was performed only at the genetic markers (no interval mapping), and individuals with missing genotypes were discarded. The likelihood value for presence of a QTL was expressed as a LOD score. Permutation tests were used to estimate genome wide significance thresholds.

## 2.8 Statistical analysis

The effect of traits on infection probability was tested using the aov function of R. The Venn and Euler diagram was plotted with VennDiagram-package functions. The 'loess' function, allowing adjustment by local polynomial regression, was used to add trends of the relationships on graphs.

## 3 Results

### 3.1 Screening for brown rot resistance with infection tests

A set of 139 genotypes of the BC2 progeny were phenotyped for *M. laxa* resistance at least once during 3 years using infection tests in the orchard and in laboratory conditions. The three genitors of the progeny proved to be susceptible to brown rot disease (Figure 1). The progeny showed a high variability in susceptibility to *M. laxa*, with both susceptible and resistant genotypes (Table 1) and probabilities of infection ranging from 0 to 100% in both tests (Figure 1). The genotype effect on infection probability was highly significant for both tests (Table 2).

Depending on the years, the number of genotypes available for phenotyping was not the same. For example the total number of common genotypes phenotyped by the infection test in the orchard the 3 years was only 68. Among those genotypes, 15 proved to be 'low susceptible' (infection probability <0.2) the three years and 20 were 'low susceptible' in 2 out of the 3 years (Figure 2 A). In total, 4 genotypes (E14, F104, F153 and H197) appeared resistant since no infection was observed for them during the 3 years (Table 3). Twenty and 14 genotypes appeared resistant 2 years in orchard and lab respectively. As for susceptible ones, no genotype was scored 'highly susceptible' (infection probability  $\geq 0.7$ ) the three consecutive years in orchard but were classified 'highly susceptible' 2 years out of the 3. Regarding infection tests in the laboratory, performed with

Table 2. ANOVA test for infection probability in the orchard (A) in the lab (B)  
A.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
genotype	122	11.457	0.0939	2.218	6.61E-06	***
fruit mass	1	0.857	0.8573	20.246	1.55E-05	***
IAD	1	0.313	0.3135	7.403	0.00745	*
date of infection (jullian day)	1	0	0	0	0.98804	
year	1	0.078	0.0781	1.845	0.17685	
residuals	124	5.251	0.0423			

Signif. codes: <0.0001 '\*\*\*' 0.001 '\*\*' 0.01 '\*'

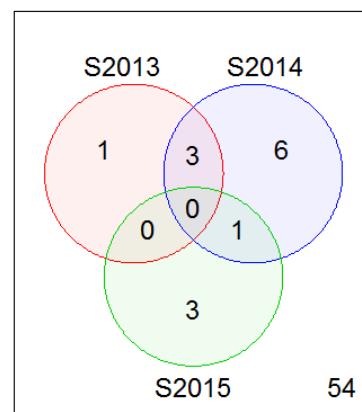
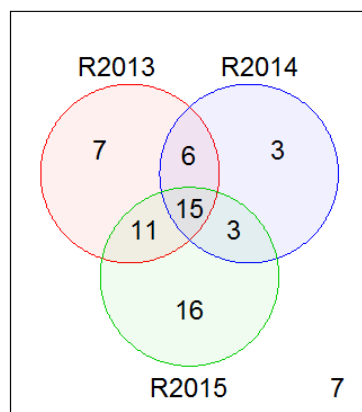
B.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
genotype	115	7.761	0.0675	2.338	0.000144	**
fruit mass	1	0.599	0.5986	20.739	2.42e-05	***
IAD	1	0.122	0.1223	4.238	0.043615	
date of infection (jullian day)	1	0.101	0.1005	3.483	0.066569	
year	1	0.061	0.0607	2.105	0.151719	
residuals	64	1.847	0.0289			

Signif. codes: <0.0001 '\*\*\*' 0.001 '\*\*' 0.01 '\*'

Resistant genotypes (Inf. probability<0.2)

Susceptible genotypes (Inf. probability>=0.7)



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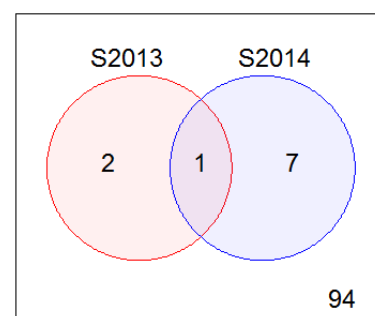
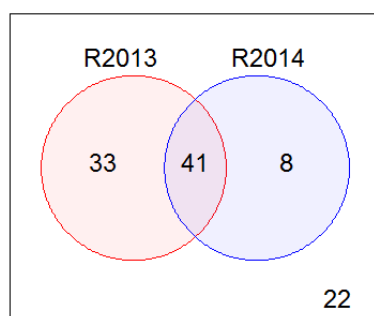


Figure 2. Venn and Euler diagrams presenting the classification of the BC2 genotypes according to the results of infection tests A. results from the 3 successive years (2013, 2014, 2015) with infection tests in the orchard (68 genotypes were common to the 3 years); B. results from the 2 successive years (2013, 2014) of drop tests in the laboratory (104 genotypes were common to both years). The left panel presents the low susceptible genotypes (infection probability <0.2) and the right panel the highly susceptible genotypes (infection probability >=0.7).

Table 6. Summary of the QTL detected on the 'DvsS' and 'SNP\_Z' maps.

Map	Fruit characteristics	Trait	LG	QTL symbol	Marker	LOD peak position (cM)	LOD	Pval	Genome wide threshold	Wild allele effect	R2 (%)
DvsS	transpiration	Surface conductance	2	SurfCond	SNP_IGA_284124	36.4	2.95	0.003	2.31	+	13.46
DvsS	transpiration	Surface conductance	6	SurfCond	SNP_IGA_687583	42	2.38	0.039	2.31	+	9.19
DvsS	susceptibility or resistance	Infection probability (artificial test in orchard)	1	infprob_orchard_2014	AMPPG021	30.3	2.69	0.02	2.39	+	9.46
DvsS	susceptibility or resistance	Infection probability (natural infection in orchard)	7	infprob_natural_2015	CFF10	38	3.56	0.003	2.36	-	9.04
DvsS	susceptibility or resistance	Blossom and twig blight (spring natural infection)	5	infprob_stem_2015	SNP_IGA_561798	16.2	3.90	0.002	2.48	+	13.00
DvsS	susceptibility or resistance	Infection probability (artificial test in orchard)	2	infprob_orchard_2015	SNP_IGA_279439	26.6	4.99	0.009	3.49	+	16.83
DvsS	susceptibility or resistance	Infection probability (artificial test in orchard)	1	infprob_orchard_2016	SNP_IGA_123089	79.6	3.85	0.022	3.33	+	14.56
SNP_Z	infection propagation	Speed of lesion progression (artificial test with drop in lab)	4	lesionspeed_lab_2013	SNP_IGA_445821	64.706	2.33	0.048	2.31		12.85
SNP_Z	susceptibility or resistance	Genotype susceptibility or resistance (artificial test with drop in lab)	4	inf01_lab_2014	snp_scaffold_4_27671771	76.186	2.53	0.028	2.27		8.52
SNP_Z	infection propagation	Lesion diameter (artificial test with drop in lab)	6	lesiondiam_lab_2014	SNP_IGA_612236	1.873	2.28	0.048	2.27		16.05

Abbreviations: linkage group (LG), LOD score (LOD). The (+) means contribution of wild allele for increase the trait and the (-) for decrease the trait.

suspension drop deposit (2013 and 2014), 41 genotypes had ‘low susceptibility’, and 10 genotypes were ‘highly susceptible’ at least one year (Figure 2 B.).

### **3.2 High variability of scoring between years**

The lab infection by drop deposit and the infection tests at orchard were repeated respectively over 2 and 3 years. The correlations between years of fruit mass were significant and quite high (ranging from 0.67 to 0.72), assessing the consistency of the datasets. Depending on the year, the genotypes were often not classified resistant or susceptible the same way, whatever the infection methodology. Considering probabilities of infection, low correlations between years were observed for a given test with the maximum of 31% of significant correlation between 2014 and 2015 for infection probability in the orchard, and 28% between 2013 and 2014 for infection probability in the lab (Table 4). However, no significant effect of year on infection probability was found in the orchard (Table 2A.), only mass and IAD had an influence on infection probability. The same way in lab conditions, a significant effect of mass was detected on infection probability scored with ‘drop’ tests. At last, the timing of infection during the season had no effect, whatever the infection methodology.

### **3.3 Natural infection of stems in spring**

In spring 2015, as we remarked a huge natural attack of blight, we recorded the number of stems presenting blossom and/or twig blight. In total, only 2 genotypes out of the 149 genotypes scored did not presented symptoms.

### **3.4 Natural infection of fruit in the orchard in 2015**

The number of mummies and currently infected fruit with tan-brown circular spots visible on the fruit were recorded at the time of fruit maturity of each genotype. Almost all the genotypes from the BC2 progeny were evaluated i.e. 144 genotypes. No significant effect of the number of fruit on the trees on the infection probability was identified (Table 6), ensuring that no bias was introduced in the scoring. A very significant effect of the date of observation on the natural infection probability was observed. At the end of the summer season, the probability of infection was sensibly higher than before (Figure 3).

### **3.5 Comparison between infection methodologies**

The infection procedures used in the orchard and in the lab in 2013 and 2014 were differing much, since same suspension with same spore concentration was used in both cases but infection was made either by spray until runoff or by drop deposit. Consequently the number of spores and the surface infected were highly different. This ended up in the fact that results of these two infection methodologies were not comparable and thus poor correlations were observed (Table 4).



Table 3. Number of resistant genotypes (null infection probability) across multiple years

Orchard tests	the 3 years	4
	2 years out of the 3	20
Drop tests (lab)	the 2 years	14
Common to infection tests (orchard and lab) a given year	2013	11
Common to orchard test and natural infection	2015	9

Table 4. Correlations between results of infection probability between years and between test modalities

	correlation	p-val
orchard 2013 vs orchard 2014	0.2861	0.0063 *
orchard 2013 vs orchard 2015	-0.0819	0.5004
orchard 2014 vs orchard 2015	0.3148	0.003 *
lab 2013 vs lab 2014	0.288	0.003 *
lab 2013 vs lab 2015	-0.0292	0.8162
lab 2014 vs lab 2015	0.2401	0.0522
orchard 2013 vs lab 2013	0.2947	0.0056 *
orchard 2013 vs lab 2014	-0.1151	0.3123
orchard 2013 vs lab 2015	0.049	0.7223
orchard 2014 vs lab 2014	0.1704	0.0884
orchard 2014 vs lab 2015	0.2301	0.1509
orchard 2015 vs lab 2015	0.4562	1e-04 ***
orchard 2015 vs Natural Inf 2015	0.3714	1e-04 ***

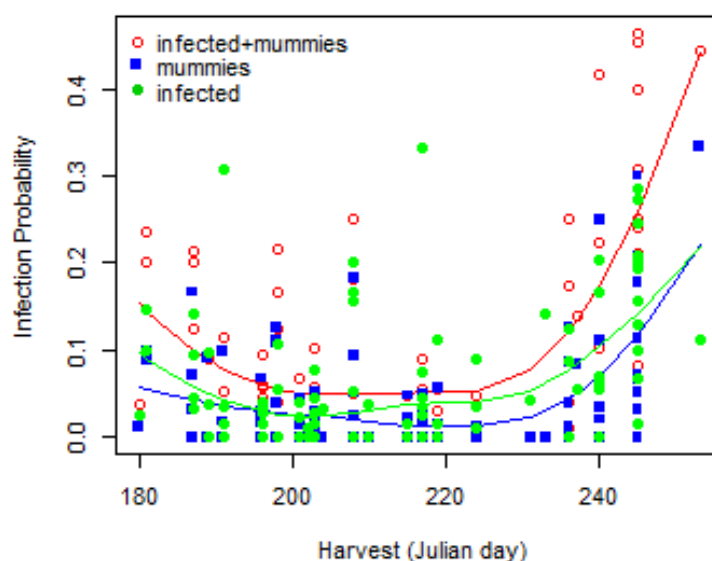


Figure 3. Natural infection probability scored in orchard in 2015. The blue squares represent only the mummies and the green ones the fruits infected at the time of the harvest. Red spots are the sum of both.

Table 5. ANOVA test for natural infection probability in orchard in 2015 at the moment of harvest

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
date of observation (jullian day)	1	0.2308	0.2308	22.74	5.68e-06	***
# fruit in the tree	1	0.0644	0.0643	6.34	0.0132	
residuals	111	1.1269	0.0101			

Signif. codes: <0.0001 '\*\*\*' 0.001 '\*\*' 0.01 '\*'

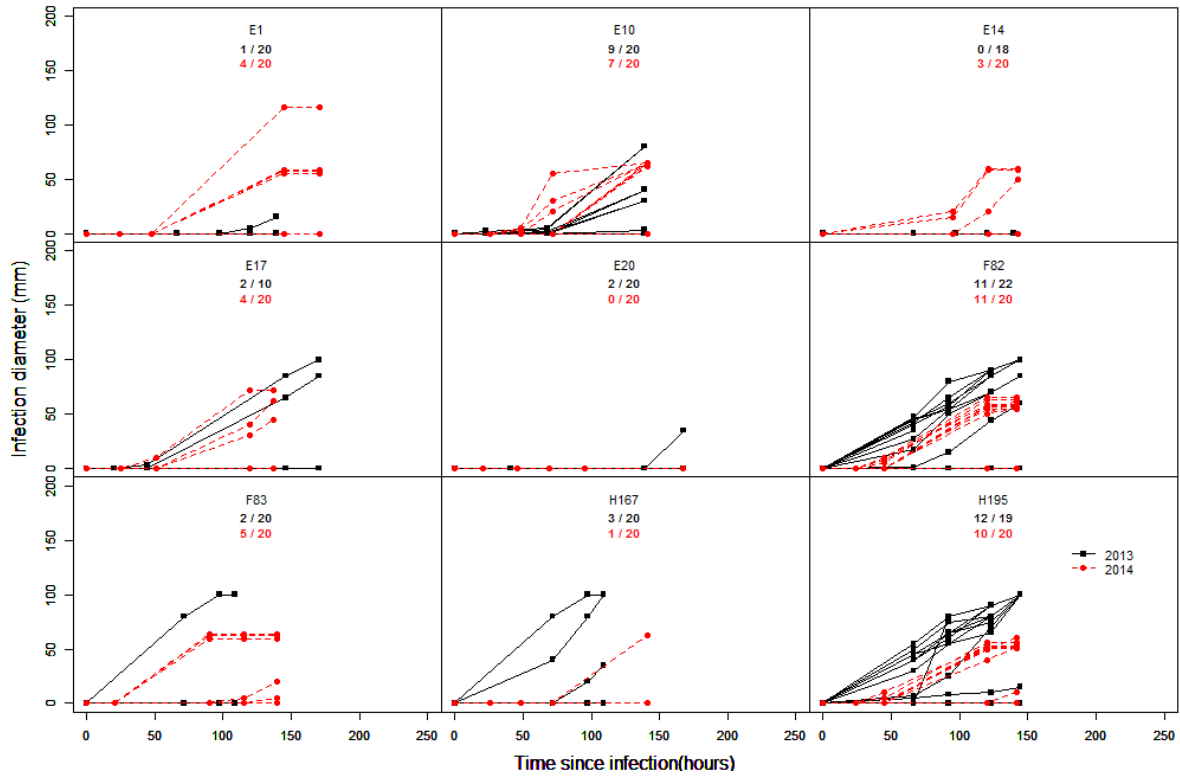


Figure 4. Evolution of infection diameter of infection causing by *M. laxa* in function of the time during two years of experimentation.

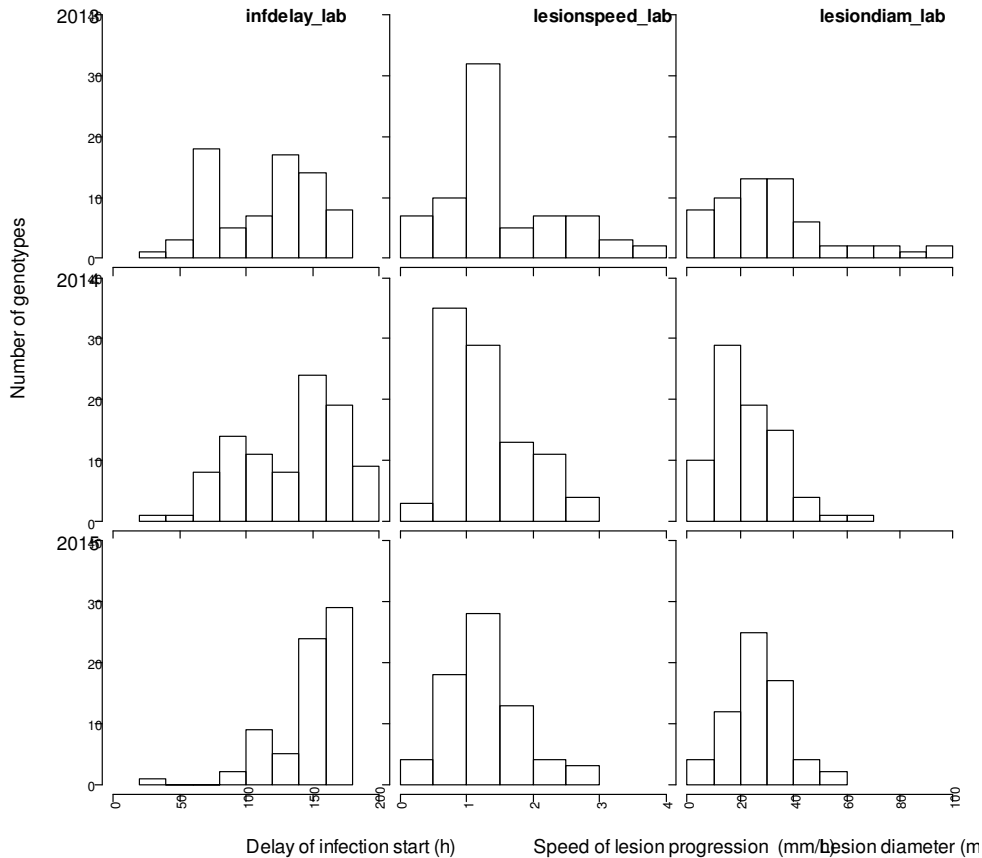


Figure 5. Distribution frequency of the traits linked to infection in the lab among the BC2 progeny for the 3 years. Frequency is expressed in the y-axis and the number of individuals falling on each phenotypic interval (x-axis). Trait names are indicated at the top of each histogram.

The lab test using drop deposit proved to be less stringent and so less distinctive between genotypes than the orchard test.

In the light of these results, the lab test was modified in 2015 and spray infection was used. The correlation between lab and orchard results in 2015 increased to a relatively high value 0.45 and turned out significant.

Finally, the scoring of natural infection in the orchard appeared well correlated to the spray infection in the orchard in 2015 (significant correlation of 0.37), even though the genotypes did not exceed 50% of probability of infection.

### **3.6 Survey of infection progression through lab tests**

The laboratory infection was designed mainly to investigate the progression of the infection along hours after infection (HAI). The BC2 genotypes showed a high diversity of response for these traits (Figure 7).

In general, it was possible to see the first spots of brown rot in fruit at 48 HAI. Some genotypes showed quickly the development of first signals, as F82, F83, H167 and H195. For others the infection developed very tardily in the end of experimentation (e.g. E14, E20). The lesion diameter also varied between the genotypes and for the same genotype between the years. For E1 as example, the lesion diameter was larger in 2014 than 2013 while the contrary occurred for F82, H167 and H195. No significant effect of year, mass, IAD and infection date were found on the mean lesion diameter.

The progression was characterized by the maximal delay of lesion appearance ('infdelay'), the mean diameter of the lesion at 72 HAI ('lesiondiam') and the maximal speed of progression ('lesionspeed'). The distribution frequency of these traits assess again of the large variation observed in the progeny (Figure 5).

### **3.7 Fruit surface conductance**

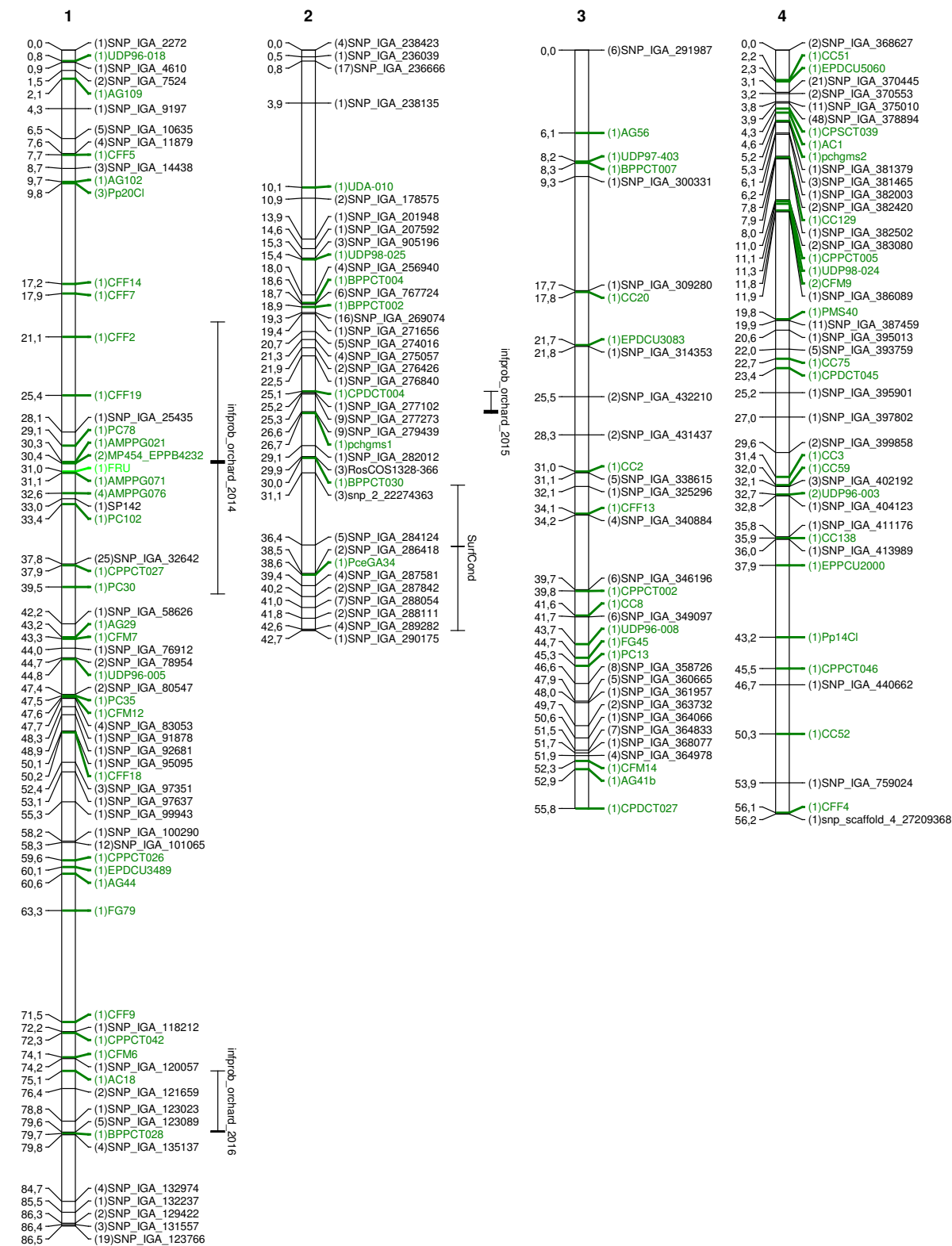
The maximal surface conductance scored at maturity stage showed a not normal distribution (Figure 5). In the progeny large variations were observed ranging from 71.42 to 685.48 g (cmh<sup>-1</sup>). The largest values observed correspond to fruit with large surface of cracks. The range of the values assessed of the diversity of susceptibility of cracking of the genotypes.

### **3.8 QTL location**

The QTL detection was performed on the two maps of the progeny monitoring the polymorphism between *Prunus davidiana* (D) and *P. persica* cultivar 'Summergrand' (S) genomes ('DvsS') and the heterozygosity of Zephir (Z) ('SNP\_Z'), respectively.

For the infection probability with the test spray in the orchard, a QTL was detected on the 'DvsS' map on linkage group 1 (Table 3) and QTL on linkage group 2 in 2015. For these 3 QTL, The *P.*

Figure 5. Genetic linkage map of the BC2 progeny monitoring the polymorphism between *P. davidiana* and *P. persica* ‘Summergrand’ genomes (‘DvsS’ map) and presenting the location of the QTL controlling brown rot disease occurrence and propagation from experiments done in 2015. Only the linkage groups carrying QTL are plotted. See Table 3 for details on QTL symbols.



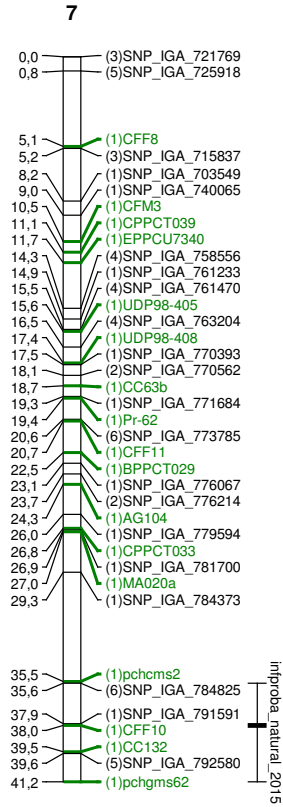
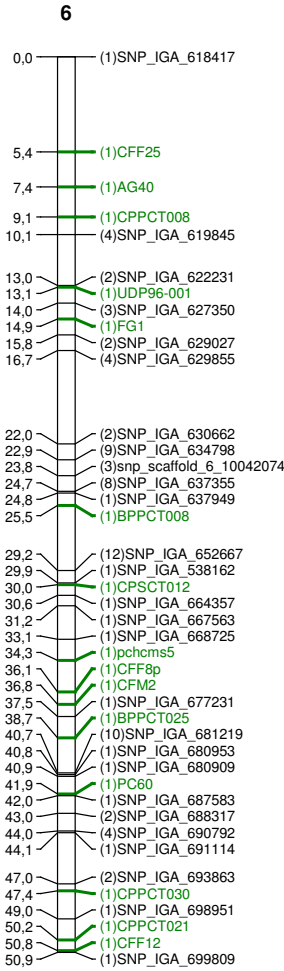
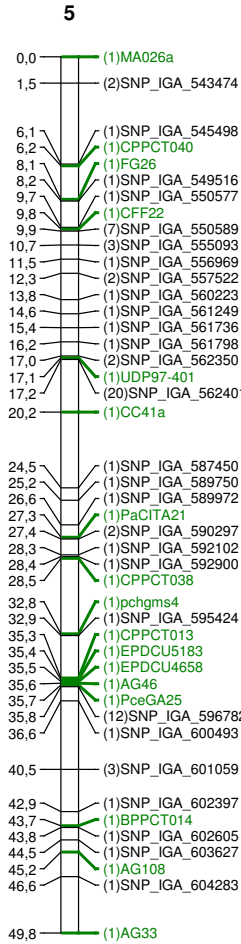
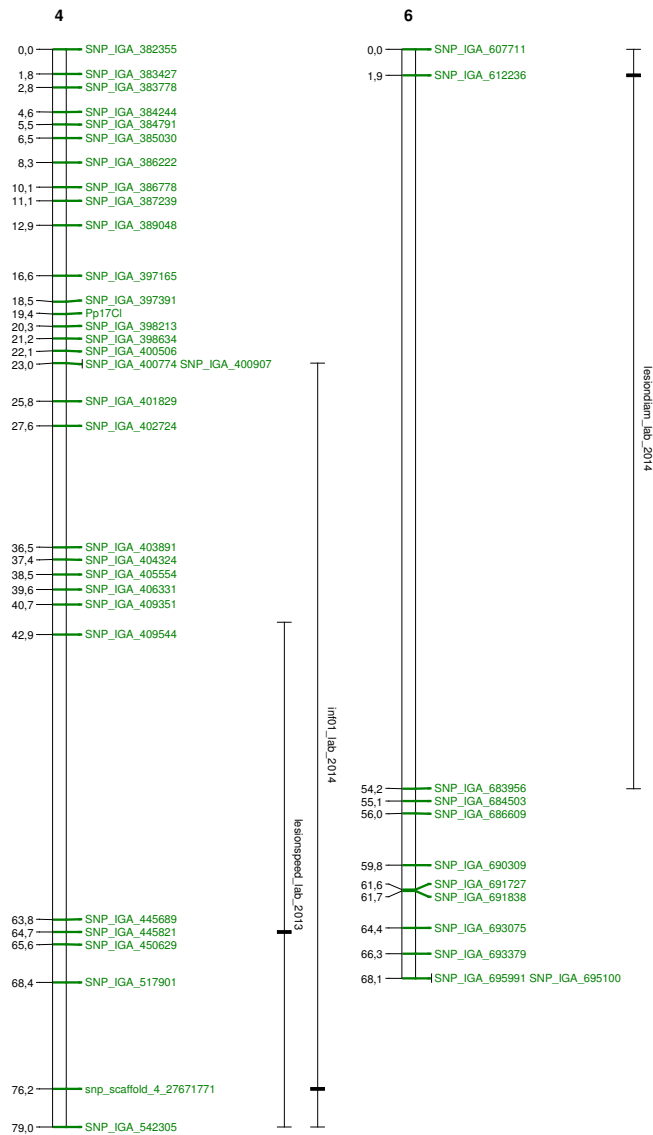


Figure 6. Genetic linkage map of the BC2 progeny tracking the heterozygosity of Zephyr cultivar ('SNP\_Z' map) and presenting the location of the QTL controlling brown rot disease occurrence and propagation from experiments done in 2015. Only the linkage groups carrying QTL are plotted. See Figure 8 for details.



*davidiana*  allele increased the infection probability. No QTL for the infection probability was detected for any year. One QTL was detected in the LG7 controlling fruit natural infection in 2015 and a QTL of blossom and twig blight was detected on the LG5. For surface conductance, QTLs were detected on different linkage groups (2 and 6). In all cases, the wild allele increases the conductance.

On the 'SNP\_Z map', one QTL for susceptibility was detected in the LG4. Other QTL were detected for traits linked to lesion progression. One QTL was detected for speed of progression lesion in LG4 for 2013, and one QTL in LG6 for the average lesion diameter in 2014.

These QTL and their confidence intervals were reported on the maps (Figure 5).

## 4 Discussion

### 4.1 The BC2 progeny: an interspecific back cross as a potential source of resistance to *M. laxa*

Currently, peach breeding programs lack of sources of resistance to BR. Results from the study of (Pacheco, Bassi et al. 2014) showed that 2 cultivars, Contender and Elegant Lady carry alleles with both negative and positive effects to the probability of infection of unwounded fruit by *M. fructigena*. The polygenic control of BR and the presence of favorable and unfavorable alleles in a cultivar could explain that transgressive seedlings were observed in their study. These results are providing a major boost to search of sources of resistance in the peach germplasm and to the creation of progenies from crosses of heterozygous genitors of moderate resistance to BR. In parallel, the recent study by (Martínez-García, Parfitt et al. 2013) have shown the potential improvement using *Prunus dulcis* as a source of resistance for BR. The two studies mentioned here did not detect QTL in same locations. Besides the fact the pathogen species used were not the same, this highlights that favorable alleles segregating in a progeny can be absent in progenies with different genetic background. In the case of the BR, it might be necessary to cumulate numerous favorable alleles coming from different sources of resistance to increase the level of BR resistance in new cultivars.

In this context, we studied the BC2 progeny, stemmed from a complex interspecific back cross between *P. davidiana* and *P. persica*. Fruits of the clone of *P. davidiana*, being small greenish even at maturity were scored highly susceptible in general. They presented sometimes a strange behavior against the fungi. Zephir and Summergrand cultivars proved to be moderately to highly susceptible, depending on the year and test modalities. Even though none of the 3 genitors of the progeny was resistant, the progeny displayed a large diversity of response of infection probability and infection propagation. In addition, some genotypes with a strong level of resistance were identified across the three years of experimentation. These observations of transgressive individuals are similar to those of (Pacheco, Bassi et al. 2014). The detection of QTL controlling different traits linked to BR infection

in the 2 maps indicates that the 3 genitors carry favorable and unfavorable alleles, the clone of *P. davidana* being the one carrying the less favorable alleles.

#### **4.2 Infection tests for scoring brown rot resistance: with or without wounding fruit?**

At the time we started this study, there was no established protocol for phenotyping BR resistance that made consensus in the literature. In particular, the question of wounding fruit or not was still unanswered. According to (Ogundiwin, Bostock et al. 2008), the brown rot resistance is associated with the pericarp (epidermal) or the mesocarp or both, depending on the genotype. Meanwhile studies focused on the effect of cuticle fracturing on infection probability reported that the incidence of fruit infection increased with cuticular cracks expansion (Gibert, Chadœuf et al. 2009). Indeed, *Monilinia* spp. have often been considered as opportunistic fungi that enter in the fruit tissue preferentially via naturally occurring entry points. So when the cuticle barrier is not intact, the defense of fruit decreases dramatically (Lee and Bostock 2007). In line with this, (Pascal, Levigneron et al. 1994) did not observed any sign of flesh resistance of peach or apricot cultivars, when wounding the fruit. In contrast, (Walter, McLaren et al. 2004) showed a significant correlation in BR infection area between wounded and not wounded apricots (47 % with  $p < 0.01$ ).

Since fruit once infected are lost, even though the lesion progression is slowed down by flesh resistance, we decided to perform tests on unwounded fruit only, hence focusing on skin resistance only. This choice was reinforced afterwards by the results of the two studies aiming at identifying QTL controlling BR. In these studies, both phenotyping methods, with and without wounding fruit, have been used. (Martínez-García, Parfitt et al. 2013) reported no resistance to BR in case of wounded fruit in their interspecific peach-almond progeny. As for (Pacheco, Bassi et al. 2014), they observed some variations of flesh infection and lesion diameter on wounded fruit in their progeny and could detect a stable QTL for lesion diameter recorded on wounded fruit. However, regarding their results and the fact that resistance to penetration is more interesting for a breeding program, these authors have strongly encouraged to concentrate phenotyping efforts on skin resistance scoring on non-wounded fruit.

#### **4.3 Infection tests for scoring brown rot resistance: drop or spray, orchard or lab, natural or artificial infections?**

Comparing the results of infection probability from the artificial infections in orchard and in laboratory, resulted in large inconsistencies. However, this was not totally surprising since the two methodologies differ in many points. Among them, the most important ones are the quantity of spores applied to one fruit and the surface of the fruit in contact with the pathogen. Both are larger in case of spray infection.



The 'drop' test is a localized test on a selected zone of the fruit. In this test we tried to overcome the factor 'microcracks' and depose the drop on an intact zone where the cuticle can play an entire role of barrier. This test was designed to explore the genetic variability of limiting active penetration of the fungi. In contrast, the 'spray' test is a comprehensive test that may reveal the total resistance of fruit, including its susceptibility to cracking. This test probably resembles more to natural infection that occurs in orchard. Thus part of the genotypes that showed high infection probability with this test may be susceptible to cracking. To further explore this likelihood, we looked for QTL of 'SurfCond' at fruit maturity which is a proxy of cuticle alteration by cracking. However, the two QTL controlling 'SurfCond' did not co-localize with any QTL of traits linked to susceptibility.

In addition to infection modalities, the two tests also differed by the environmental control. In case of the 'lab' test, the light and temperature were controlled and the humidity was always maximized in the boxes. On the contrary, with the 'orchard' test, the different genotypes were submitted to contrasted environmental conditions, depending on the day when the infections were done. We expected more stability of the results in case of the 'lab' test but the tentative of reducing year effect on BR by performing artificial lab infection was a failure.

Another objective of the 'lab' test was to monitor the infection propagation. This test was designed to investigate the pathogen development in post-harvest conditions. The different traits estimated from the survey of lesion diameter exhibited large variations in the progeny and enabled the detection of QTL controlling the fungi propagation. Two out of the three traits (i.e. 'lesiondiameter' and 'lesionspeed') may be considered as linked to flesh resistance as the third one ('infdelay') would be interesting to postpone onset of fruit infection. However no QTL was detected for the latter. For 'lesiondiameter' and 'lesionspeed', favorable alleles come from Zephir.

It's important to notice here that our tentative to perform a 'spray' test in the lab was not a real success since no QTL was detected. This modality was intended to perform a test i) comparable to the 'orchard' test but enlarging repeatability between genotypes and years thanks to controlled conditions, and ii) enabling to follow infection progression. Indeed the infection probabilities measured in orchard and lab in 2015 were significantly correlated with a reasonable  $R^2$ . However, the QTL detected for 'infprob\_orchard\_2015' was not confirmed with 'infprob\_lab\_2015'.

At last, we also scored natural infection in the orchard in 2015, both in spring on stem and in fruit at maturity. In both cases the progeny displayed variations, although these variations were reduced compared to the cases of artificial infections, one QTL was detected for each trait. The correlation between 'infprob\_orchard\_2015' and 'infprob\_natural\_2015' was significantly positive with a moderate  $R^2$  but the QTL detected for these 2 traits were in different LG. In conclusion, scoring natural infection of a progeny in case of high impact of BR in the orchard can be encouraged. Indeed

the incidence of blossom and twig blight can contribute to harvest losses (May-De Mio, Moreira et al. 2008).

#### **4.4 Trying to explain the high instability between years**

In general in our study, low correlations between years were found for the infection probability scored with a same test. This inconstancy of susceptibility is common in the literature as reported by (May-De Mio, Moreira et al. 2008) and (Wagner Júnior, Fabiane et al. 2011). Different factors may explain such instability, including climatic conditions in the orchard. Climatic factors as temperature and moisture have been reported as important for fungi development (Byrde and Willetts, 1977), (Gilbert et al., 2007). Having this in mind, one can expect large effect of years in case of natural infection, as observed by the producers. We observed an effect of maturity date on natural infection probability which is often mentioned in the literature. (Pacheco et al., 2014) also observed a positive correlation between natural infection probability and lateness of ripening. However, in our case it could also be explained by an increase of spores in the orchard due to the repetitions of sprays done for the 'spray' test along the season.

In case of artificial infection in the orchard, climatic factors may also play a role even though paper bags were used to maintain high humidity and favor the infection. We explored climatic data registered in the experimental orchard and checked the correlations between the infection probability in the orchard and different climatic factors as minimal and maximal temperature, moisture, wind velocity... but no correlation was found (data not shown).

More difficult to explain is the effect of year we observed in the case of 'lab' tests. However, such instabilities have also been reported by (Martinez-Garcia, et al., 2013) and (Pacheco et al., 2014), despite artificial infections performed in controlled conditions. For example, (Pacheco et al., 2014) noticed an inversion of the behavior of the two genitors "Contender" and "Elegant Lady" for rot diameter between 2009 and 2010.

Probably, indirect effects of environment on fruit growth and development before maturity may result in different susceptibility of the fruit. In this way (Pacheco et al., 2014) found a positive correlation between artificial infection probability and lateness of ripening. They suggested that late ripening cultivars may undergo higher decay of fruit barrier occurring during ripening process. In contrast, we found neither effect of year nor of maturity date on the artificial infection probability in the orchard but we observed that fruit mass and IAD had a significant influence. Fruit mass may encompass part of year effect which explains that no year effect was detected. As for the IAD index, it describes ripening level of the fruit. The survey of infection probability during fruit growth (Chapter 2 of this thesis) clearly stated that the more ripen a fruit is, the more it is susceptible. This is also well known in the literature (Gradziel and Wang 1993) Thus, precautions have been taken both at spring

when thinning trees to enable potential growth of fruit and at maturity to harvest fruit between commercial and physiological maturity stages which resulted in high to moderate correlations between years for fruit mass and IAD, respectively. Nevertheless, differences between years may be partly explained by differences in fruit mass and maturity stage of harvested fruit.

At last, in case of the 'drop' test, neither effect of mass nor IAD on infection probability was detected. On the other hand year displayed a strong effect. This is quite difficult to explain, as 'drop' test was performed in controlled conditions. Only environmental conditions in which the fruit has grown have been different between years.

#### **4.5 The QTL for brow rot resistance: where are we now?**

The genetic maps combined with multi-datasets (different years and tests) allowed a genetic analysis of the architecture of the genetic control of the resistance to *M. laxa* and of the propagation of BR disease in peach fruit. This is the first study reporting QTL of resistance to *M. laxa*.

Despite our efforts to minimize variations due to experimental discrepancies and environmental factors in the tests in laboratory, only one QTL was detected for resistance in these conditions. More QTL for resistance were detected in orchard conditions, artificial or natural. Still, 2 QTL for disease propagation were detected from lab experimentations. The comparison of the positions of QTL controlling surface conductance and QTL controlling BR resistance of progression resulted in no colocation. This suggested that QTL detected for BR resistance were not linked to cracking susceptibility of the genotypes.

The QTL for brown rot resistance detected on LG1 with the spray test in orchard had small likelihood and moderate effect. However we found again this QTL in our preliminary analyses of results of 2016 experiment with spray test in orchard. Above all, it was at the same position as the main QTL found by (Martinez-Garcia, Parfitt et al. 2013) for resistance to *M. fructicola*, under laboratory conditions. We could not inspect the candidate genes for this QTL, because the interval was quite large. However, it is important to notice that the peak of this QTL in our progeny is exactly associated with the major QTL for fructose content detected by (Desnoues, Baldazzi et al. 2016) in the BC2 progeny. One of the best candidate gene proposed was a sugar transporter from the SWEET family (Prupe.1G133300), the same as the candidate gene proposed by (Martinez-Garcia, Parfitt et al. 2013) for the resistance to *M. fructicola*. Indeed, different studies have reported that SWEET transporters can be involved in interactions with fungi (Chen, Hou et al. 2010), (Chong, Piron et al. 2014),

Another QTL for BR resistance was detected with the spray test on LG2 in 2015. The main QTL for skin resistance to *M. fructigena* detected by (Pacheco, Bassi et al. 2014) was at the same location. We also detected on LG4 of the 'SNP\_Z' map a QTL for BR resistance tested in lab in 2014 and a QTL

for speed of lesion propagation scored in lab in 2013. These 2 QTL were in the same region as QTL detected by (Pacheco, Bassi et al. 2014) for skin and flesh resistance.

We also detected a QTL on LG6 of the 'SNP\_Z' map controlling the lesion diameter scored in lab in 2014. This QTL was at the same position as the QTL for flesh resistance detected by (Pacheco, Bassi et al. 2014).

Finally, other 2 QTL were found in LG7 and LG5 for natural infection probability of, respectively, fruit and stems in the orchard. This is the first time that QTL for BR resistance were detected using scoring of natural occurrence of infections. Such phenotyping could represent a good method for screening progenies, but it requires both an important inoculum pressure in the orchard, and a total absence of chemical treatments during the fruit season. This can impair the future of the orchard.

## 5 Conclusion

The results of our study showed that the cultivars Summergrand and Zephir carry favorable alleles for brown rot resistance. Contrary to what was thought the alleles from the wild parent *P. davidiana* contributed for increasing the susceptibility to BR disease, except at the *infproba\_natural* QTL on LG7. The comparison of the present study with the two earlier QTL studies on peach-brown rot which resulted in the detection of common QTL, highlighted major findings. First, the genetic basis of resistance to BR disease is at least partially similar between the *Monilinia* species. Second, in the case of such a complex plant-pathogen system under high environmental control, even unstable or putative QTL found in one study may have their importance, since they proved to be detected in other studies. Finally, contrary to what one might think about the genetic control of BR resistance, the number of genes involved in BR resistance may not be so important since same QTL were detected in 3 progenies stemmed from highly different genetic backgrounds.

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## **Chapter 5: Etude de la variabilité des composés d'épiderme des fruits d'une descendance interspécifique issue d'un croisement entre *Prunus persica* et *P. davidiana* et liens avec la susceptibilité à la pourriture brune (*Monilinia laxa*)**

Ce chapitre présente succinctement les résultats, que l'on peut qualifier de préliminaires, d'une année d'expérimentation menée en 2015 sur la population BC2. L'objectif de cette étude est de rechercher un lien potentiel entre la composition biochimique des épidermes de fruits et leur sensibilité à la pourriture brune. Pour cela, nous avons caractérisé les composés secondaires des épidermes des fruits de 112 individus de la population et effectué en parallèle des tests d'infection avec *M. laxa*. Cette étude a été suivie en 2016 d'une seconde année de caractérisation identique. Les résultats des 2 années seront analysés ultérieurement dans le cadre d'un projet Agropolis Fondation-Embrapa qui vient de débiter.

Cependant cette première année de phénotypage que j'ai réalisé est présentée ici dans la mesure où elle constitue le point d'orgue de ma thèse. En effet, il s'agit d'étudier la relation entre la résistance à la pourriture brune, étudiée au Chapitre IV, et la présence et/ou les teneurs de certains composés de l'épiderme à maturité. Ce travail se situe dans le prolongement du Chapitre II qui, par l'étude de la cinétique des composés de surface des fruits, a permis d'identifier des composés potentiellement inhibiteurs ou activateurs de l'infection par *M. laxa*. La suite logique était donc de caractériser la variabilité génétique de ces composés dans la population BC2 à maturité, d'étudier le contrôle génétique de ces composés par une recherche de QTL et d'explorer les liens entre les teneurs de l'épiderme et la susceptibilité des fruits à la pourriture brune.

La méthode d'analyse des composés de surface par trempage, utilisée dans le Chapitre II, étant trop longue pour être utilisée dans le cas d'un grand nombre d'individus, les fruits ont été épluchés et l'épiderme analysé. Cette nouvelle méthode a permis de caractériser 43 composés. Parmi eux 8 composés, terpénoïdes et leurs dérivés, sont communs à ceux étudiés au Chapitre II et 11 sont des nouveaux composés, dérivés de terpénoïdes, acides hydroxycinnamiques et flavonol.

Le chapitre proposé ici n'est pas rédigé complètement sous forme d'un article, il ressemble plutôt à un relevé de conclusions tirées à l'issue d'une 1<sup>ère</sup> année d'expérimentation. Nous ne proposons pas ici d'introduction qui serait une redite des Chapitres II et IV. Les résultats obtenus ne sont pas présentés en totalité ni discutés de façon approfondie. L'accent a porté sur l'analyse du lien de la composition de l'épiderme avec la susceptibilité à l'infection, cœur de la problématique de cette thèse. Le phénotypage présenté dans ce chapitre ont été réalisées sur la population BC2. En conséquence le matériel végétal ainsi que les cartes génétiques sont identiques à ceux décrits dans les chapitres précédents (Chapitres III et IV) et les analyses QTL ont été réalisées en suivant la même

méthodologie. De même, les tests d'infection ont été réalisés avec la même souche MI3 en suivant les 2 protocoles d'infection par spray en verger et en laboratoire décrits dans le chapitre précédent (Chapitre IV). Aussi, dans un souci de concision, seuls les éléments nouveaux, spécifiques à ce chapitre, sont présentés ci-dessous dans la section 'Materials and Methods'. Il s'agit du protocole d'échantillonnage des fruits, de la préparation des échantillons d'épiderme et des analyses HPLC qui ont suivi.

## **1 Materials and Methods**

### **1.1 Fruit sampling**

Fruit were harvested in summer of 2015, at maturity stage. For each genotype, 15 fruit were harvested: 10 for infection tests with spray under laboratory conditions and 5 fruit for epidermis analyses. The fruit were weighed; the IAD index was taken with the  $\Delta$ A meter instrument (Syntelesia S.R.L., Italy) on the equatorial cheeks, and the 3 dimensions (fruit height, between cheeks width and the distance from the suture to the opposite side) were measured using a caliper.

Fruit of 112 genotypes of the BC2 progeny were sampled for epidermis biochemical analyses.

### **1.2 Epidermis preparation**

Sets of 5 fruits per genotype were entirely peeled with a ceramic knife. Care was given to peel in a very thin manner not to incorporate flesh. The epidermis was immediately frozen in liquid nitrogen. Then the samples were ground into a fine powder with the mill IKA A11B and liquid nitrogen keeping the sample frozen. Finally the samples were lyophilized in a Cryonext lyophilizer to ensure the conservation of secondary metabolites. In this work, the duration of lyophilization ranged from 4 to 6 days depending on the quantity of matter. Fresh and dry masses were weighted to calculate dry matter content.

### **1.3 Extraction**

An aliquot of 50mg of epidermis dry powder was weighted for each genotype and put in a Falcon tube of 15 ml. Eight ml of ethanol at 95% v/v at -20°C and 25  $\mu$ l of 6-Methoxy-Flavone at 0.4mg/ml of concentration as internal standard were added. The mix was homogenized with the disperser IKA Ultra-Turrax during 1 minute and the disperser element was rinsed with 2ml of ethanol 95%. For extraction, the tube was placed on the rotary shaker in cold room (4°C) at least 4 hours. After extraction, the tube was centrifuged at 5000 rpm during 5 min at 4°C. Then, the supernatant was collected and concentrated under vacuum in a Speed Vac<sup>TM</sup> concentrator (SC210A; Thermo Electron Corporation), without warming for about 15 hours or overnight until complete evaporation of liquid. The dry residue was dissolved in 1ml of Methanol, vortexed until complete dissolution and



then filtered with a syringe filter (membrane PTFE 0.45 µm). The filtered extract was collected into a 1.5ml autosampler vial for HPLC analysis and kept at -20°C until analyses.

#### 1.4 HPLC analyses

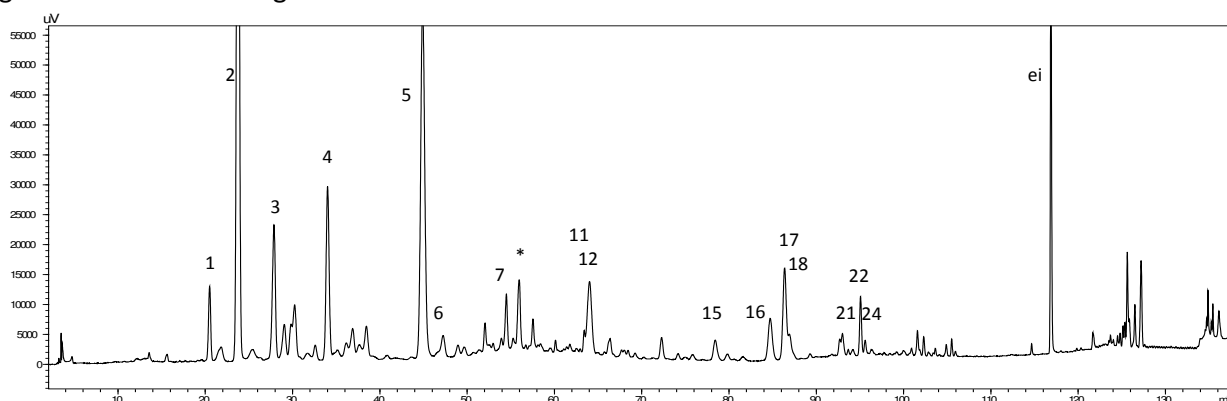
The quantitative analyses were performed with a High Performance Liquid Chromatography system -SHIMADZU- Prominence equipped with a reversed phase C18 column (MERCK Superspher RP18 endcapped) coupled with a photodiode array detector.

The mobile phase was a mixture of solvent A; ultrapure water (Millipore Synergy-UV) acidified at pH 2.6 with orthophosphoric acid at 85% (Prolabo Normapur) and filtered with membrane Millipore Durapore HVLP 04700 0.45µm and B; Methanol (Prolabo Hypersolv Chromanorm). A linear gradient elution from 3 to 12% B in 5 min was employed, followed by linear gradient to 14% B in 6 min, 6 min isocratic elution with 14% B, linear gradient to 16% B in 4 min, 5 min isocratic elution with 16% B, linear gradient to 17% B in 2 min, 10 min isocratic elution with 17% B, linear gradient to 35% B in 18 min, 20 min isocratic elution with 35% B, linear gradient to 55% B in 19 min, linear gradient to 90% B in 21 min, 10 min isocratic elution with 90% B, linear gradient to 100% B in 1 min, 5 min isocratic elution with 100% B, and column equilibration for 22 min with initial conditions. The column temperature was set to 30°C and the flow rate was 0.7ml/min throughout the analysis. The chromatograms were simultaneously analyzed for triterpenoids (wavelength 210 nm), flavan-3-ols (280 nm), hydroxycinnamic acids and terpenoids derivatives (315 nm), flavonols (350 nm) and anthocyanidins (520 nm).

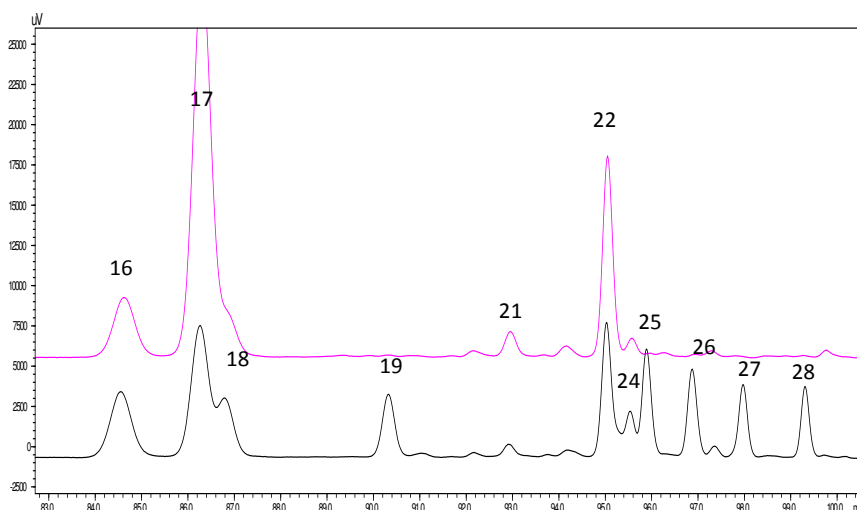
The compounds were characterized according to their UV and mass spectra (cf Chapter 2 of this thesis for details), and retention time; co-chromatographies were performed with known standards when available. For quantitative analyses, a calibration curve was obtained by injection of known concentrations of standard compounds (Extrasynthèse, Genay, France; Sigma-Aldrich, Saint-Quentin Fallavier, France): betulinic acid, oleanolic acid, ursolic acid, procyanidin B1, catechin, neochlorogenic acid (3-O-caffeoylquinic), chlorogenic acid (5-O-caffeoylquinic), 3,5-O-dicaffeoylquinic acid, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, kaempferol-3-O-galactoside, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, isorhamnetin-3-glucoside, isorhamnetin-3-rutinoside, cyanidin-3-glucoside and cyanidin-3-rutinoside.

For Terpenoid 1 and Terpenoid 2, oleanolic and ursolic acids were used as standards respectively. For ac 3 cafeoylquinique (CIS), NeoCHLORO was used as standard, for ac cryptochlorogenic (ac 4 cafeoylquinique) and ac 5 cafeoylquinique (CIS), acid CHLORO was used as standard and for ac 3,4 cafeoylquinique, DHC4 and ac 4,5 cafeoylquinique, ac 3,5 cafeoylquinique was used as standard.

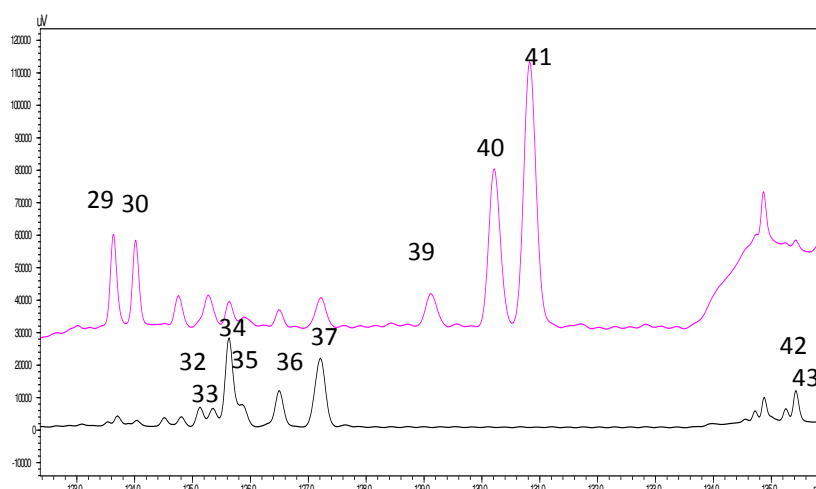
Figure 1. HPLC chromatograms



**Chromatogram at 280nm for separation of phenolic compounds of nectarine (genotype F147) epidermis:** (1) 3 cafeoylquinic acid (cis), (2) neochlorogenic acid, (3) procyanidin B1, (4) catechin, (5) chlorogenic acid, (6) cryptochlorogenic acid, (7) 5 cafeoylquinic acid (cis), (\*) cinchonain + flavan-3-ol (no quantified), (11) Cyanidin-3-glucoside, (12) Cyanidin-3-rutinoside, (15) 3,5 dicafeoylquinic acid, (16) Quercetin-3-galactoside, (17) Quercetin-3-glucoside, (18) Quercetin-3-rutinoside, (21) Kaempferol-3-galactoside, (22) Kaempferol-3-glucoside, (24) Kaempferol-3-rutinoside.



**Chromatogram at 350nm for separation of Flavonol compounds from peach (genotype C233) (in black) and nectarine (genotype C207) (in pink) epidermis:** (16) Quercetin-3-galactoside, (17) Quercetin-3-glucoside, (18) Quercetin-3-rutinoside, (19) flavonol unknown, (21) Kaempferol-3-galactoside, (22) Kaempferol-3-glucoside, (24) Kaempferol-3-rutinoside, (25) Isorhamnetin-3-glucoside, (26) Isorhamnetin-3-rutinoside, (27) (28) unknown flavonols.



**Chromatogram at 210nm (in pink) and 315 nm (in black) for separation of terpenoids and derivatives of nectarine (genotype F147):** (29) (30) di-hydroxy-urs-12-en-28-oic acid, (32) (33) (34) (35) (36) (37) p-coumaroyl-dihydroxy-urs-12-en-28-oic acid, (39) betulinic acid, (40) oleanolic acid, (41) ursolic acid, (42) (43) 3β-p-coumaroyloxy-urs-12-en-28-oic acid.

Table 1. Characteristics of the 43 compounds analyzed in peach epidermis

Numbers on chromatograms	Family	Compound	Abbreviation	Retention time (min)	$\lambda_{max}^{\xi}$	Unit
1	Hydroxycinnamic acid	3 cafeoylquinic ac (CIS)	3CQcis	20.5	318	mg/Kg DW
2	Hydroxycinnamic acid	neochlorogenic ac (3 cafeoylquinic ac) (TRANS)	NeoCHLORO	23.8	325	mg/Kg DW
3	Flavan-3-ol	procyanidin B1	ProB1	27.7	278	mg/Kg DW
4	Flavan-3-ol	catechin	Cat	34	278	mg/Kg DW
5	Hydroxycinnamic acid	chlorogenic ac (5 cafeoylquinic ac) (TRANS)	CHLORO	44.7	326	mg/Kg DW
6	Hydroxycinnamic acid	cryptochlorogenic ac (4 cafeoylquinic ac)	Crypto	47.4	325	mg/Kg DW
7	Hydroxycinnamic acid	5 cafeoylquinic ac (CIS)	cisCHLORO	54.7	318	mg/Kg DW
8	Hydroxycinnamic acid	DHC1	DHC1	57.2	311	aire/KgDW
9	Hydroxycinnamic acid	DHC2	DHC2	58.2	309	aire/KgDW
10	Hydroxycinnamic acid	DHC3	DHC3	62.9	312	aire/KgDW
11	Anthocyanin	cyanidin-3-glucoside	CY1	63.8	279/519	mg/Kg DW
12	Anthocyanin	cyanidin-3-rutinoside	CY2	66.0	279/519	mg/Kg DW
13	Hydroxycinnamic acid	3,4 cafeoylquinic ac	3_4diCQ	76.0	325	mg/Kg DW
14	Hydroxycinnamic acid	DHC4	DHC4	78.6	325	mg/Kg DW
15	Hydroxycinnamic acid	3,5 cafeoylquinic ac	3_5diCQ	80.0	328	mg/Kg DW
16	Flavonol	quercetin-3-galactoside	Q3Gala	84.8	256/354	mg/Kg DW
17	Flavonol	quercetin-3-glucoside	Q3Glu	86.6	256/354	mg/Kg DW
18	Flavonol	quercetin-3-rutinoside	Q3Rut	87	256/354	mg/Kg DW
19	Flavonol	flavonol 1	FI1	90.5	256/354	aire/KgDW
20	Hydroxycinnamic acid	4,5 cafeoylquinic ac	4_5diCQ	92.5	325	mg/Kg DW
21	Flavonol	kaempferol-3-galactoside	K3Gala	93.2	266/347	mg/Kg DW
22	Flavonol	kaempferol-3-glucoside	K3Glu	95.3	266/347	mg/Kg DW
23	Flavonol	pic 95,4	pic95_4	95.4	255/352	aire/KgDW
24	Flavonol	kaempferol-3-rutinoside	K3Rut	95.8	266/347	mg/Kg DW
25	Flavonol	isorhamnetin-3-glucoside	IsoGlu	96	254/347	mg/Kg DW
26	Flavonol	isorhamnetin-3-rutinoside	IsoRut	97	254/347	mg/Kg DW
27	Flavonol	flavonol 2	FI2	98	265/344	aire/KgDW
28	Flavonol	flavonol 3	FI3	99.3	255/355	aire/KgDW
29	Terpenoid	terpenoid 1	Ter1	123.7	198	mg/Kg DW
30	Terpenoid	terpenoid 2	Ter2	124.1	198	mg/Kg DW
31	Terpenoid derivative	p-coumaroyl-dihydroxy-urs-12-en-28-oic acid	der7	124.8	198/307	mg/Kg DW
32	Terpenoid derivative	p-coumaroyl-dihydroxy-urs-12-en-28-oic acid	der11	125.1	198/312	mg/Kg DW
33	Terpenoid derivative	p-coumaroyl-dihydroxy-urs-12-en-28-oic acid	der8	125.4	198/308	mg/Kg DW
34	Terpenoid derivative	p-coumaroyl-dihydroxy-urs-12-en-28-oic acid	der1	125.6	198/311	mg/Kg DW
35	Terpenoid derivative	p-coumaroyl-dihydroxy-urs-12-en-28-oic acid	der9	125.9	198/311	mg/Kg DW
36	Terpenoid derivative	p-coumaroyl-dihydroxy-urs-12-en-28-oic acid	der2	126.5	198/311	mg/Kg DW
37	Terpenoid derivative	p-coumaroyl-dihydroxy-urs-12-en-28-oic acid	der3	127.3	198/310	mg/Kg DW
38	Terpenoid derivative	p-coumaroyl-dihydroxy-urs-12-en-28-oic acid	der10	127.7	198/322	mg/Kg DW
39	Terpenoid	betulinic acid	a_b	129.0	198	mg/Kg DW
40	Terpenoid	oleanolic acid	a_o	130.6	198	mg/Kg DW
41	Terpenoid	ursolic acid	a_u	131.2	198	mg/Kg DW
42	Terpenoid derivative	3 $\beta$ -p-coumaroyloxy-urs-12-en-28-oic acid	der12	135.2	199/313	mg/Kg DW
43	Terpenoid derivative	3 $\beta$ -p-coumaroyloxy-urs-12-en-28-oic acid	der5	135.4	199/313	mg/Kg DW

$\xi$   $\lambda_{max}$  is the wavelength for the maximum absorption

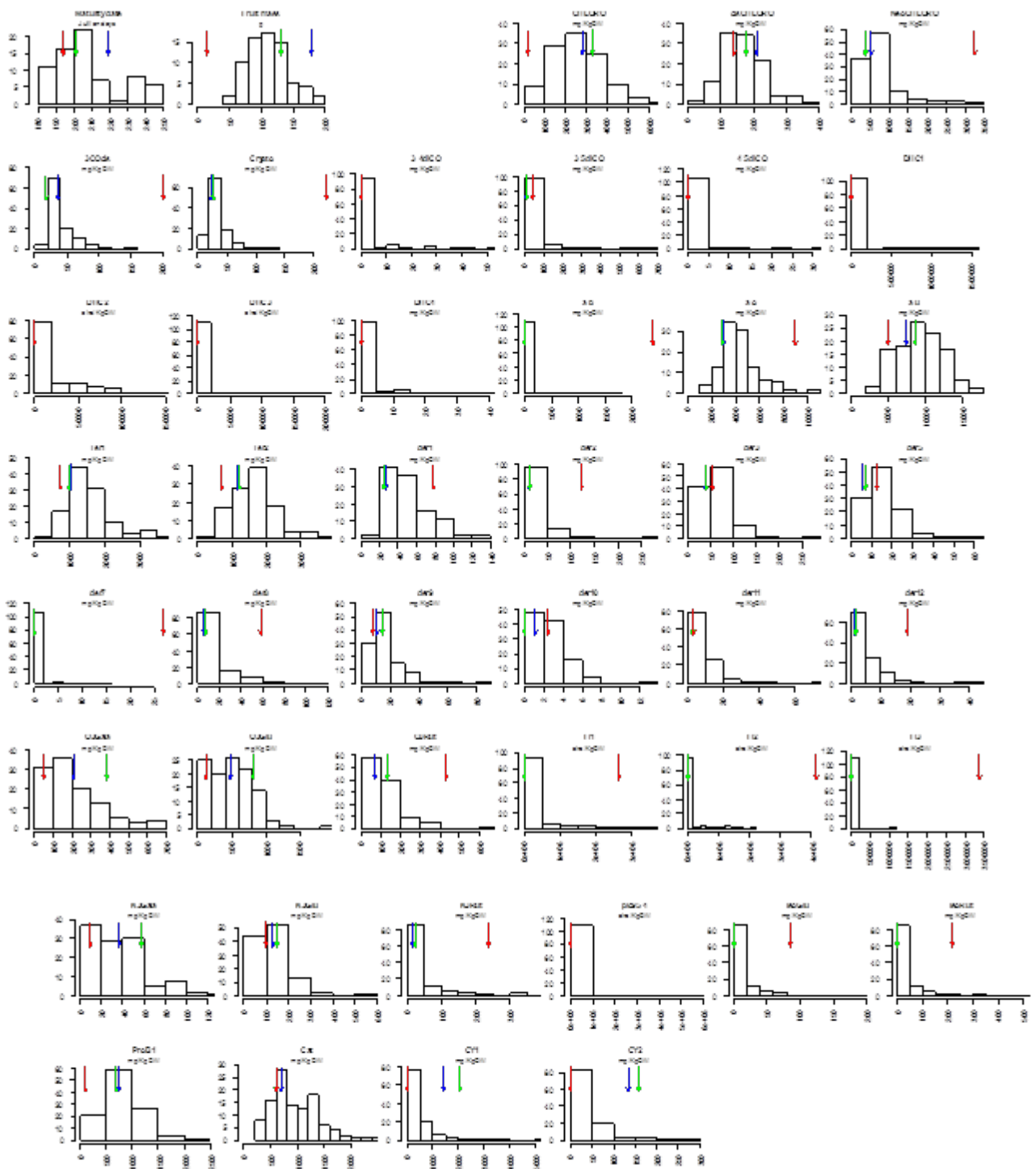


Figure 2. Distribution of frequency of BC2 progeny for maturity date, fruit mass and 43 epiderm compounds. Traits and units are indicated at the top of each histogram. Meanings of trait abbreviations are indicated in Table 1. Frequency is expressed in the y-axis as the number of individuals falling on each trait interval (x-axis). Arrows indicate the values for *P. davidiana* (red), Summergrand (green) and Zephir (blue).

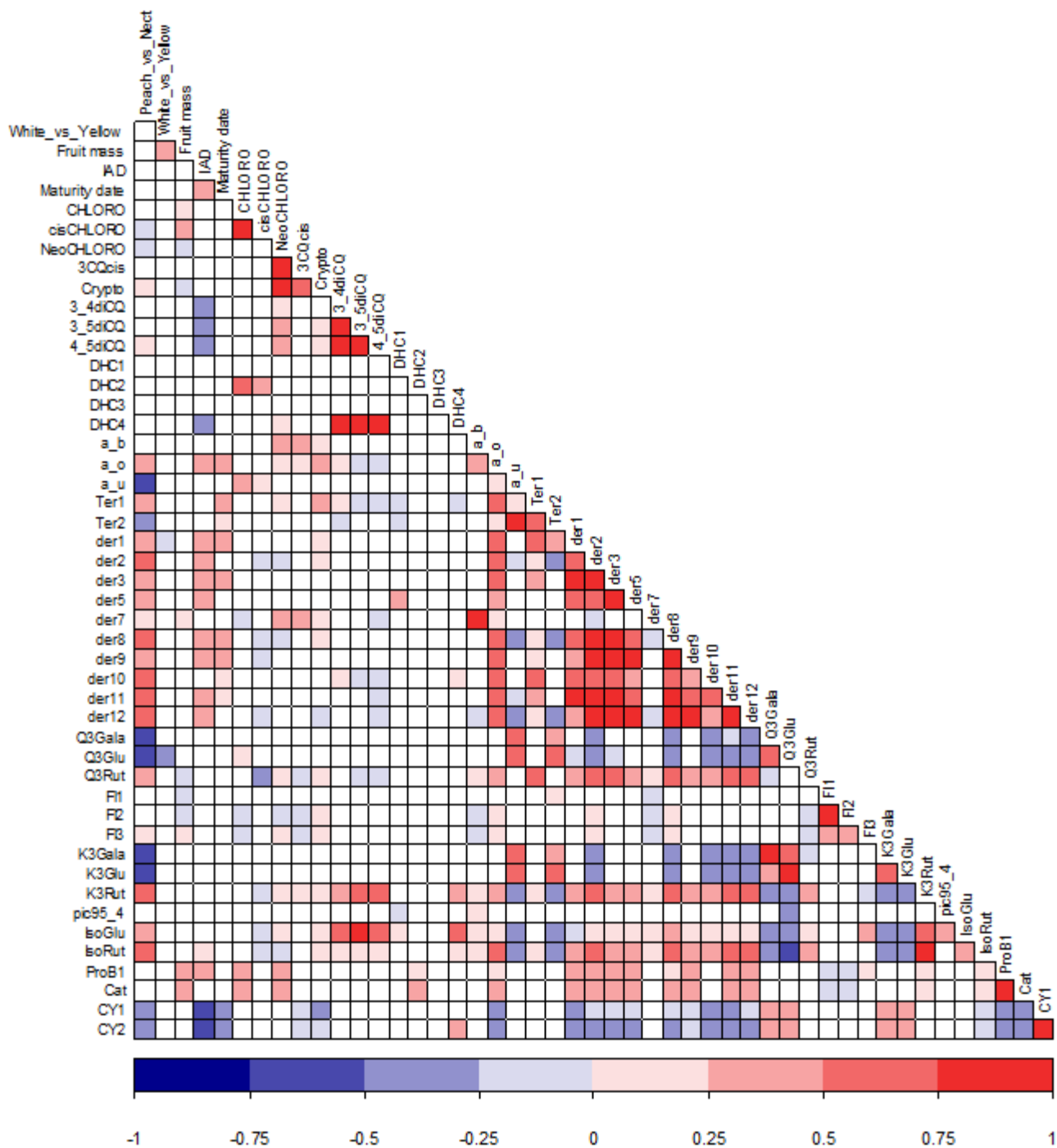


Figure 3. Visualization of Spearman's correlations between the compounds levels and the fruit traits (Peach versus nectarine, white and yellow flesh fruit, fruit mass, IAD and maturity date). The square color corresponds to the correlation value as shown in the legend: blue represents a negative correlation, and red represents a positive correlation. The white squares correspond to non-significant correlations (P value >0.01). Abbreviations: see Table 1.

In the case of the isomers der1, der2, der3, der5, der7, der8, der 9, der10, der11, der12 (p-Coumaroyl-dihydroxy-urs-12-en-28-oic acid), the p-coumaric acid was used as standard (Figure1).

The results were expressed in mg/Kg DW or in peak surface area (DHC1, DHC2, DHC3 and Flavonol1, Flavonol2, Flavonol3) in absence of identification.

## 2 Results and discussion

### 2.1 Epidermis compounds showed great variations within the BC2 progeny

The HPLC analyses ended up in the identification of 43 compounds (Table 1) of different families: 12 hydroxycinnamic acids, 5 terpenoids, 10 terpenoid derivatives, 12 flavonols, 2 flavan-3-ols and 2 anthocyanins. Among them, 8 are common to the compounds detected in fruit surface extracts (see Chapter 2).

The three genitors of the progeny exhibited much contrasted levels of the compounds, the clone of *P. davidiana* being very different from the 2 *P. persica* cultivars. The BC2 progeny exhibited considerable genotypic variations in all compounds (Figure 2). Two general trends were observed: compounds with a normal distribution or close to, and compounds with a L-shaped distribution. In the latter case, it happened that only very few genotypes contained the compounds, the majority of genotypes having not at all. It is the case of DHC3 compound present in epidermis of only 2 genotypes. This compound, together with 6 others (3,4diCQ, 4,5diCQ, DHC1, DHC2, DHC4 and pic95\_4) had null values for the 3 genitors. However for 6 other compounds, epidermis from *P. davidiana* showed the highest contents. On the contrary, the two cultivars never displayed the greatest levels. To conclude, transgressive segregants for high values were observed for a majority of compounds (37 out 43 compounds).

### 2.2 Some compounds were highly correlated between each other

The correlation coefficients between the fruit traits (fruit types, fruit mass and IAD), maturity date and between compounds are shown in Figure 3. A large number of compounds appeared to be correlated to the fruit type (peach versus nectarine).

Inside families, many correlations were observed. In this way, the two flavan-3-ols were highly correlated. The same applied to two out of the 3 unknown flavonols and to the two anthocyanins. The latter were also significantly linked to IAD index. Hence, more ripen the fruit is, more anthocyanins are accumulated in the epidermis. We also observed very high correlations between the two flavonols (quercetin and kaempferol) with galactoside group and with glucoside group, respectively. These two flavonols with rutinoside group were much less correlated. Quercetins with galactoside and glucoside groups were positively correlated whereas negative correlations were observed between them and the quercetin with rutinoside group. The same was observed inside the

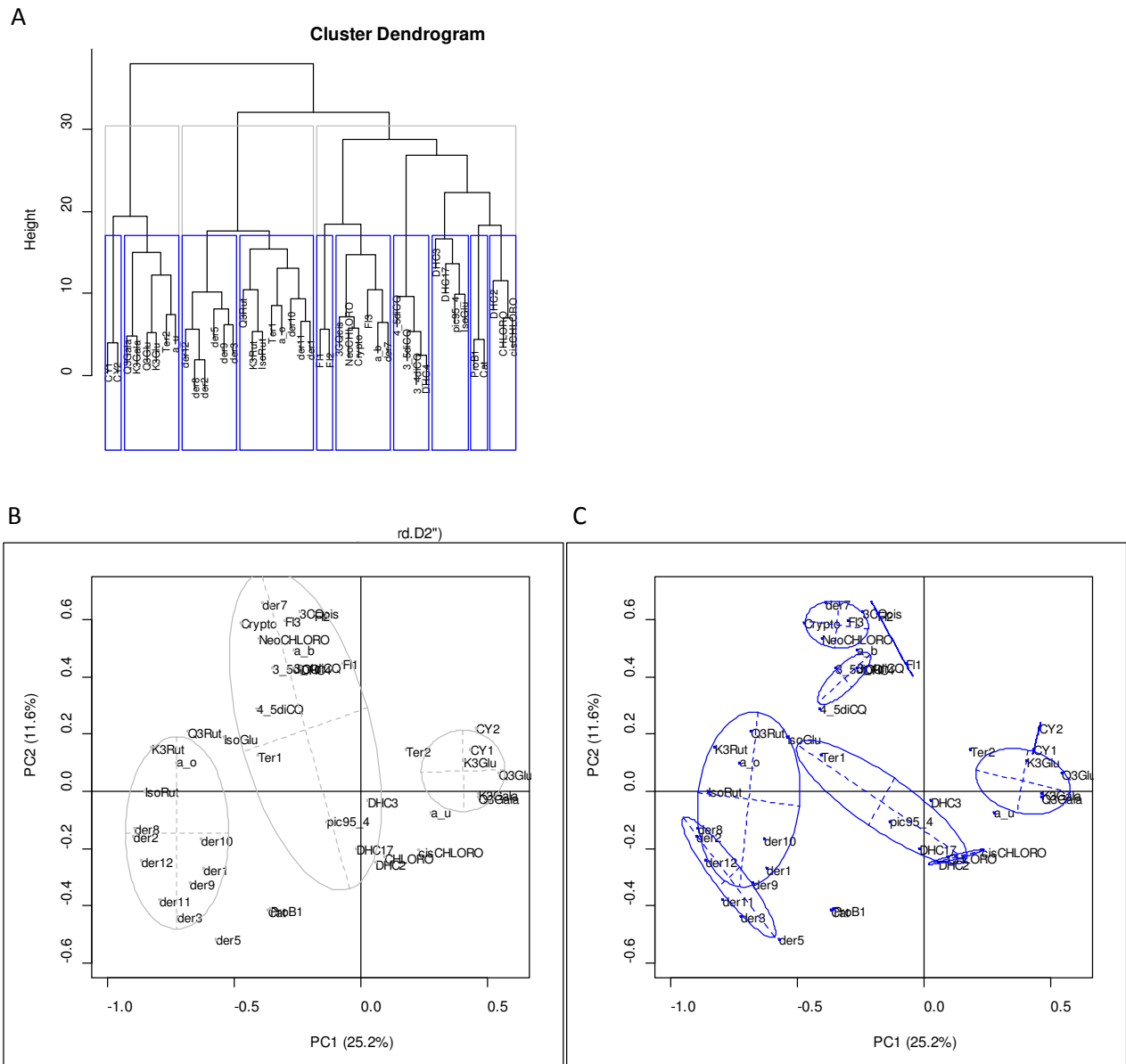


Figure 4. Exploration of the dataset of 43 compounds from the epidermis of fruit of the BC2 progeny. A. Dendrogram presenting variables clustered into 3 (grey) and 10 (blue) groups. B, C. Location of the epidermis compounds on the plan of the 2 first principal components of a Principal Component Analysis performed on the scaled dataset. The 3 and 10 clusters presented in A are reported respectively in B and C.

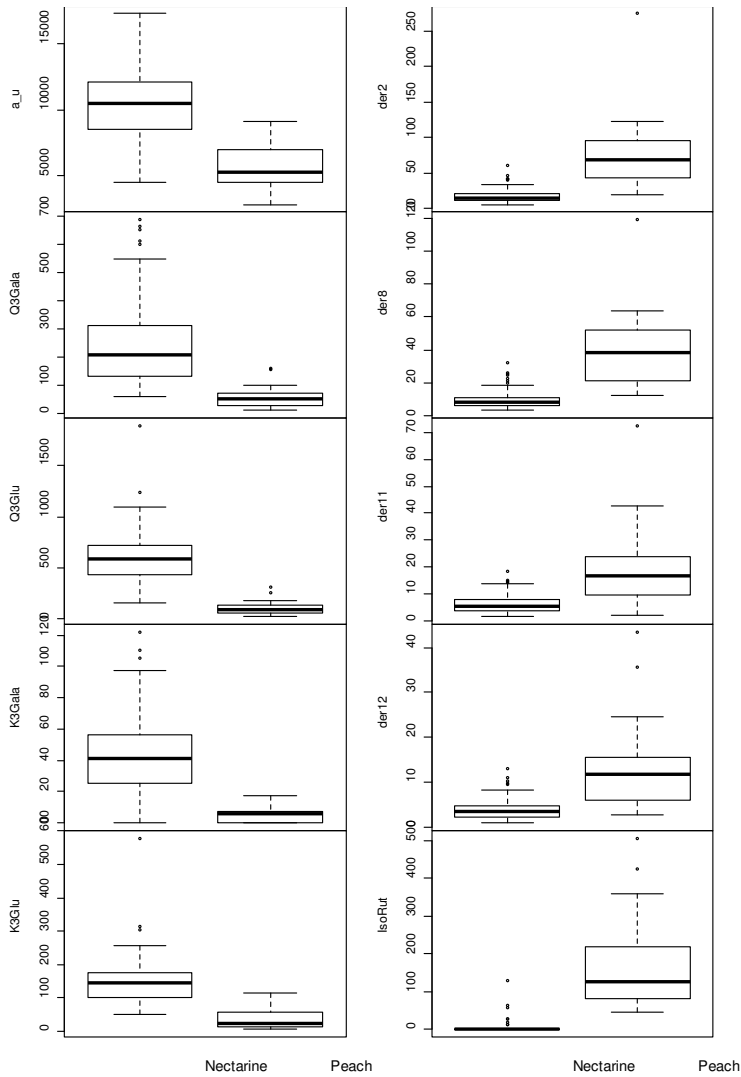


Figure 5. Boxplot representing significant differences of compounds levels (mg/KgDW) between peach and nectarine fruit.

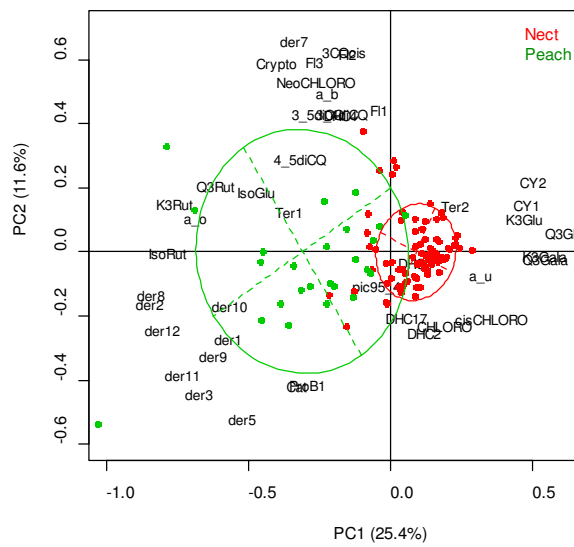


Figure 6. Genotype values were added to the PCA plan with different colors according to fruit type. Genotypes with peach fruit are in green and with nectarine fruit in red.



Table 2. Summary of the QTL detected on the 'DvsS' map for 41 compounds, fruit mass and index. Suffixes were added to QTL symbols when detected with the fruit skin pubescence trait as covariate (covPN) or from the 2 subsets of fruit type (P for the peach subset and N for the nectarine subset). a\_o/a\_u represents the ration between the two compounds.

Trait family	QTL symbol	Closest marker at the peak	LG	Peak position	LOD	pval	LOD threshold	R <sup>2</sup>	Confidence interval
Hydroxycinnamic acid	Crypto_P	CFF14	1	17.2	2.19	0.0180	1.87	56.50	12 53.1
Flavonol	K3Glu_P	CFF14	1	17.2	1.94	0.0490	1.94	37.36	6.5 28
fruit trait	mass	AMPPG021	1	30.3	2.71	0.0180	2.28	16.49	0 32.6
Hydroxycinnamic acid	4_5diCQ	SNP_IGA_32642	1	37.8	4.28	0.0190	3.70	6.10	28 50
Flavan-3-ol	Cat_N	PC30	1	39.5	3.58	0.0020	2.43	5.25	38 47.4
Flavan-3-ol	ProB1_N	PC30	1	39.5	3.66	0.0000	2.33	8.13	38 47.4
Hydroxycinnamic acid	3_5diCQ	SNP_IGA_76912	1	44	2.49	0.0440	2.41	31.50	26 52
Hydroxycinnamic acid	DHC4_pic78_5	lg1.loc46	1	46	4.06	0.0180	3.21	2.56	36 50.2
Terpenoid	Ter1	SNP_IGA_92681	1	48.9	3.50	0.0100	2.97	2.90	47.7 52.4
Terpenoid	Ter2_covPN	SNP_IGA_92681	1	48.9	3.08	0.0320	2.84	13.69	47.7 50.2
Hydroxycinnamic acid	3_4diCQ	SNP_IGA_92681	1	48.9	5.10	0.0030	3.06	4.96	47.7 50.2
Terpenoid	a_o/a_u	lg1.loc50	1	50	2.58	0.0360	2.41	7.14	0 58
Anthocyanin	CY1_N	SNP_IGA_123766	1	86.5	2.60	0.0270	2.28	3.93	38 86.5
Flavonol	IsoRut_N	SNP_IGA_256940	2	18	18.84	0.0000	3.96	33.78	16 18.6
Flavonol	IsoGlu	SNP_IGA_256940	2	18	6.24	0.0000	2.57	27.71	16 20
Flavonol	pic95_4_N	SNP_IGA_256940	2	18	8.99	0.0010	4.38	23.38	16 18.6
Flavonol	K3Rut_N	SNP_IGA_275057	2	21.3	2.92	0.0050	2.30	16.24	6 24
Hydroxycinnamic acid	3_5diCQ	SNP_IGA_276840	2	22.5	2.78	0.0230	2.41	4.63	20.7 28
Flavonol	Q3Gala_N	SNP_IGA_277102	2	25.2	3.35	0.0030	2.44	11.34	22.5 29.1
Terpenoid	Ter2	SNP_IGA_279439	2	26.6	5.31	0.0000	2.84	14.88	25.1 36.4
Terpenoid derivative	der5	pchgms1	2	26.7	4.50	0.0010	2.85	11.18	21.9 29.1
Hydroxycinnamic acid	DHC1_pic57_N	pchgms1	2	26.7	9.72	0.0000	3.99	17.84	24 31.1
Terpenoid derivative	der12_N	lg2.loc28	2	28	3.14	0.0010	2.23	21.00	18 32
Terpenoid	Ter1	BPPCT030	2	30	3.29	0.0190	2.97	6.21	19.4 36.4
Terpenoid	Ter2	BPPCT030	2	30	4.26	0.0000	2.48	16.04	26 34
Flavonol	Q3Glu_N	lg2.loc36	2	36	4.66	0.0000	2.34	19.09	28 42.7
Flavonol	K3Glu_N	SNP_IGA_287581	2	39.4	3.35	0.0020	2.33	6.82	28 42.7
Terpenoid derivative	der11_N	SNP_IGA_289282	2	42.6	4.20	0.0000	2.38	11.44	26.7 42.7
Anthocyanin	CY2_N	SNP_IGA_290175	2	42.7	3.12	0.0040	2.28	4.45	26.7 42.7
Terpenoid	a_b	lg3.loc6	3	6	20.57	0.0000	3.80	81.43	2 8
Terpenoid derivative	der7	lg3.loc6	3	6	20.57	0.0000	3.78	65.02	2 8
Hydroxycinnamic acid	NeoCHLORO	EPDCU3083	3	21.7	3.18	0.0070	2.50	19.26	18 28.3
Hydroxycinnamic acid	3CQcis	EPDCU3083	3	21.7	3.78	0.0010	2.47	24.54	18 28
Hydroxycinnamic acid	CHLORO_N	SNP_IGA_431437	3	28.3	2.95	0.0080	2.29	11.38	26 38
Hydroxycinnamic acid	cisCHLORO	SNP_IGA_431437	3	28.3	4.59	0.0000	2.50	13.08	26 31
Hydroxycinnamic acid	Crypto_P	SNP_IGA_431437	3	28.3	1.98	0.0340	1.87	56.56	18 31
Flavonol	Fl1	SNP_IGA_361957	3	48	15.78	0.0000	2.79	67.17	46.6 49.7
Flavonol	Fl2	SNP_IGA_361957	3	48	23.15	0.0000	3.15	76.05	46.6 49.7
Flavan-3-ol	Cat	SNP_IGA_364833	3	51.5	3.70	0.0040	2.49	7.85	42 55.8
Flavan-3-ol	ProB1	SNP_IGA_364833	3	51.5	2.52	0.0400	2.46	8.25	40 55.8
Flavonol	Fl3_P	SNP_IGA_368077	3	51.7	4.99	0.0040	3.54	99.00	46.6 55.8
Terpenoid	a_o/a_u	SNP_IGA_368627	4	0	4.80	0.0000	2.41	19.10	0 7.8
Flavan-3-ol	Cat_N	SNP_IGA_368627	4	0	2.52	0.0420	2.43	2.59	0 5.2
Terpenoid derivative	der8	SNP_IGA_368627	4	0	2.63	0.0200	2.37	7.63	0 26
Flavonol	K3Rut	SNP_IGA_368627	4	0	3.52	0.0020	2.41	4.83	0 25.2
Flavonol	Q3Rut	SNP_IGA_368627	4	0	3.65	0.0120	2.87	3.41	0 29.6
Terpenoid	Ter1	SNP_IGA_368627	4	0	3.69	0.0020	2.45	25.06	0 7.8
Terpenoid	Ter2_P	cch4.loc2	4	2	2.62	0.0050	1.97	58.19	0 16
Hydroxycinnamic acid	cisCHLORO_P	CPST039	4	4.3	2.60	0.0040	1.95	39.67	0 25.2
Hydroxycinnamic acid	3_5diCQ	pchgms2	4	5.2	9.07	0.0000	2.41	35.60	4.3 10
Hydroxycinnamic acid	DHC4_pic78_5	CC129	4	7.9	20.81	0.0000	3.21	56.25	6.2 10
Hydroxycinnamic acid	3_4diCQ	CC129	4	7.9	19.36	0.0000	3.06	50.72	6.2 10
Hydroxycinnamic acid	4_5diCQ	SNP_IGA_382502	4	8	8.21	0.0010	3.70	17.45	4.6 11.8
Terpenoid	a_u	SNP_IGA_393759	4	22	3.22	0.0190	2.80	35.70	20.6 27
Flavonol	IsoRut	SNP_IGA_393759	4	22	3.94	0.0020	2.54	13.39	0 25.2
Flavonol	IsoGlu	SNP_IGA_393759	4	22	4.53	0.0010	2.57	6.23	0 25.2
Hydroxycinnamic acid	Crypto	CPDCT045	4	23.4	2.52	0.0430	2.45	8.28	20.6 27
Terpenoid derivative	der10	SNP_IGA_440662	4	46.7	2.88	0.0250	2.54	4.93	0 56.2
Hydroxycinnamic acid	CHLORO	CC52	4	50.3	3.01	0.0150	2.58	11.08	46.7 53.9
fruit trait	mass_P	cch5.loc6	5	6	1.64	0.0450	1.60	56.51	0 49.8

Terpenoid	a_o/a_u_N	lg5.loc20	5	20	2.49	0.0490	2.48	10.52	12.3	27.3
Flavan-3-ol	Cat_P	CC41a	5	20.2	2.05	0.0390	1.98	3.46	4	24.5
Flavan-3-ol	ProB1_P	CC41a	5	20.2	2.29	0.0180	1.91	4.28	8.2	30
Flavonol	IsoRut_P	cch5.loc22	5	22	2.08	0.0300	1.97	3.39	4	32
Terpenoid derivative	der9_covPN	SNP_IGA_587450	5	24.5	3.05	0.0400	2.91	9.60	6.2	27.3
Terpenoid	a_o_N	SNP_IGA_589750	5	25.2	2.71	0.0230	2.41	10.51	17.2	27.3
Terpenoid derivative	der8_N	SNP_IGA_589750	5	25.2	2.70	0.0250	2.42	7.69	15.4	27.3
Flavonol	IsoGlu_N	CPPCT008	6	9.1	6.61	0.0020	3.90	21.33	7.4	12
Flavonol	pic95_4	FG1	6	14.9	4.33	0.0110	3.14	3.49	8	16.7
Anthocyanin	CY1	SNP_IGA_629027	6	15.8	2.89	0.0150	2.36	1.81	13.1	33.1
Flavonol	K3Glu_N	lg6.loc24	6	24	2.54	0.0250	2.33	3.99	16.7	36
Terpenoid derivative	der11_P	CPST012	6	30	2.90	0.0010	1.91	24.75	26	47.4
Terpenoid derivative	der1	SNP_IGA_664357	6	30.6	4.92	0.0000	2.87	18.96	25.5	47.4
Terpenoid derivative	der3	SNP_IGA_667563	6	31.2	3.38	0.0170	2.89	9.05	25.5	44.1
Flavonol	IsoRut_P	CFF8p	6	36.1	2.65	0.0020	1.97	27.53	26	50.9
Flavonol	K3Rut_P	CFF8p	6	36.1	3.23	0.0000	1.89	43.22	30.6	50.9
Flavonol	Q3Rut	CFF8p	6	36.1	4.31	0.0020	2.87	10.53	30.6	41.9
Flavan-3-ol	ProB1_P	CFM2	6	36.8	3.34	0.0000	1.91	17.92	31.2	49
Flavan-3-ol	Cat	SNP_IGA_690792	6	44	5.12	0.0000	2.49	23.72	38	47.4
Terpenoid	a_o/a_u_P	SNP_IGA_693863	6	47	2.83	0.0000	1.98	24.96	36.8	50.9
Hydroxycinnamic acid	3CQcis	lg6.loc50	6	50	3.08	0.0070	2.47	11.20	44.1	50.9
Hydroxycinnamic acid	Crypto	CPPCT021	6	50.2	2.89	0.0190	2.45	15.31	34.3	50.9
Hydroxycinnamic acid	NeoCHLORO_N	CPPCT021	6	50.2	3.10	0.0040	2.36	9.78	38	50.9
Terpenoid derivative	der9_N	lg7.loc14	7	14	2.78	0.0150	2.34	6.11	4	32
Terpenoid derivative	der2_P	UDP98-408	7	17.4	2.32	0.0100	1.98	23.13	6	22
Terpenoid derivative	der8_P	UDP98-408	7	17.4	2.15	0.0200	1.93	26.41	6	22
Terpenoid derivative	der3_P	Pr-62	7	19.4	2.06	0.0420	1.98	5.26	11.1	29.3
Flavan-3-ol	ProB1	CFF11	7	20.7	4.31	0.0010	2.46	11.89	16.5	32
Flavan-3-ol	Cat	lg7.loc22	7	22	4.08	0.0010	2.49	8.90	17.5	35.5
Terpenoid	a_o/a_u_P	SNP_IGA_779594	7	26	2.38	0.0100	1.98	4.95	11.1	29.3
Terpenoid	Ter2_N	CPPCT033	7	26.8	2.38	0.0470	2.35	9.40	16	32
Terpenoid	a_o_P	SNP_IGA_781700	7	26.9	2.12	0.0230	1.93	37.70	11.1	41.2
Terpenoid derivative	der12_P	cch7.loc28	7	28	3.33	0.0000	1.90	33.58	24.3	37.9
Terpenoid derivative	der5	lg7.loc28	7	28	5.23	0.0000	2.43	6.93	24	35.5
Hydroxycinnamic acid	NeoCHLORO_N	lg7.loc32	7	32	2.82	0.0100	2.36	23.38	24.3	37.9
Hydroxycinnamic acid	3_5diCQ	lg7.loc32	7	32	3.95	0.0020	2.41	2.57	24.3	39.5
Hydroxycinnamic acid	3CQcis	lg7.loc32	7	32	2.94	0.0120	2.47	17.78	27	37.9
Flavonol	IsoRut_covPN	pchms2	7	35.5	4.37	0.0260	3.71	4.92	29.3	39.5
Hydroxycinnamic acid	Crypto	SNP_IGA_784825	7	35.6	3.32	0.0080	2.45	11.68	29.3	37.9
Flavonol	K3Rut_P	cch7.loc36	7	36	2.43	0.0050	1.89	3.95	20.7	41.2
Terpenoid derivative	der11_P	SNP_IGA_791591	7	37.9	3.31	0.0000	1.91	6.29	26	41.2
Terpenoid derivative	der1_P	CFF10	7	38	3.02	0.0000	1.97	11.79	35.6	41.2
fruit trait	DA	SNP_IGA_800377	8	8.9	2.38	0.0340	2.29	12.96	4	36.9
Hydroxycinnamic acid	DHC1_pic57	lg8.loc12	8	12	7.47	0.0000	3.43	7.14	6	13.8

Table 3. Summary of the QTL detected on the ‘SNP\_Z’ map.

Trait family	QTL symbol	Closest marker at the peak	LG	Peak position	LOD	pval	LOD	R <sup>2</sup>	Confidence interval
Hydroxycinnamic	sumdiCQ_Z	c1.loc12	1	12	2.36	0.038	2.24	1.40	0 36
fruit trait	mass_Z	c1.loc16	1	16	2.40	0.037	2.16	17.7	0 42
Flavonol	Q3Rut_Z	c1.loc18	1	18	2.78	0.016	2.39	8.89	0 44
Flavonol	K3Gala_Z	snp_scaffold_3_2976417	3	0	3.06	0.010	2.21	14.1	0 10
Hydroxycinnamic	3_5diCQ_Z	c3.loc8	3	8	3.85	0.001	2.28	6.24	0 26
Hydroxycinnamic	CHLORO_Z	SNP_IGA_317114	3	13.586	2.91	0.007	2.20	15.5	4 32
Flavan-3-ol	ProB1_Z	c3.loc48	3	48	2.59	0.031	2.25	14.0	37.5 51.9
Anthocyanin	CY2_Z	c4.loc44	4	44	4.61	0.000	2.23	14.0	38 58
Anthocyanin	CY1_Z	c4.loc46	4	46	4.51	0.000	2.22	13.3	38 60
Hydroxycinnamic	Crypto_Z	c4.loc54	4	54	3.59	0.001	2.29	16.1	39.6 65.6
Terpenoid derivative	der9_Z	c4.loc74	4	74	3.98	0.000	2.30	19.3	64.7 78.9
Terpenoid derivative	der8_Z	c4.loc76	4	76	3.71	0.002	2.21	14.8	40.6 78.9
Terpenoid derivative	der2_Z	snp_scaffold_4_27671771	4	76.186	3.24	0.004	2.28	12.3	40 78.9
Flavonol	K3Rut_Z	snp_scaffold_4_27671771	4	76.186	3.12	0.005	2.26	11.6	42 78.9
Terpenoid derivative	der9_Z	snp_scaffold_4_27671771	4	76.186	4.74	0.000	2.35	22.8	65.6 78.9
Flavonol	IsoRut_Z	SNP_IGA_542305	4	78.964	2.57	0.020	2.20	11.1	42 78.9
fruit trait	mass_Z	SNP_IGA_776826	7	22.301	2.40	0.037	2.16	20.0	0 65.5
Terpenoid derivative	der12_Z	SNP_IGA_781249	7	41.589	2.19	0.041	2.12	6.68	0 48

kaempferol compounds. In contrast, isorhamnetins with glucoside and rutinoside groups were positively correlated.

Considering the hydroxycinnamic acids, very high positive correlations were observed among 3,4diCQ, 3,5diCQ, 4,5diCQ and DHC4 in one hand, and in the other hand between 3CQcis and NeoCHOLO.

At last, most of the terpenoid derivatives were positively correlated with each other, apart from the der7 compound correlated to none of the other terpenoid derivatives but which was highly correlated to betulinic acid.

The cluster and PCA analyses performed on this dataset gave an overview of the correlations that take place between the compounds (Figure 4). Three and ten clusters were proposed to group the compounds.

### **2.3 Peaches and nectarines displayed huge differences**

We explored more in details the impact of the fruit type on the composition of epidermis. Numerous compounds displayed contrasted levels according to fruit type. In general, flavonols with galactoside and glucoside groups showed higher levels for nectarine fruit whereas terpenoid derivatives had higher values for peach fruit (Figure 5). The addition of the genotypes with peach and nectarine fruit on the PCA plan performed on the dataset of compounds clearly highlighted that PC1 component separated peach and nectarine fruit (Figure 6). We can also notice that peach genotypes were more dispersed on the plan which may assess higher variability of peach than nectarine genotypes.

### **2.4 A hundred QTL were detected for epidermis compounds**

The QTL analyses performed on the 2 genetic maps of the progeny enabled the detection of QTL controlling 41 out of 43 compounds that were quite well distributed on the different linkage groups (apart on LG8, generally poor in QTL) (Tables 2 and 3). QTL was neither detected for DHC2 nor for DHC3 which both had a L-shaped distribution and few values different from null. For each of the other compounds we detected from a single QTL (for Q3Gala) to 6 QTL in different linkage groups (for cat). Only 2 QTL have been found in similar location between the 2 maps: a QTL controlling ProB1 on LG3 and a QTL controlling der12 on LG7.

Considering that fruit type had a very strong effect on epidermis composition, we performed the QTL analyses in different ways: i) on the whole dataset with no covariate, ii) on the whole dataset adding the peach versus nectarine trait as covariate, iii) on the peach and nectarine datasets separately. The first analysis resulted in numerous QTL located in the region of the G locus controlling fruit skin pubescence on LG5. Adding this trait as covariate enabled to detect other QTL for the compounds affected by the fruit type. We also detected significant QTL using the 2 subsets.

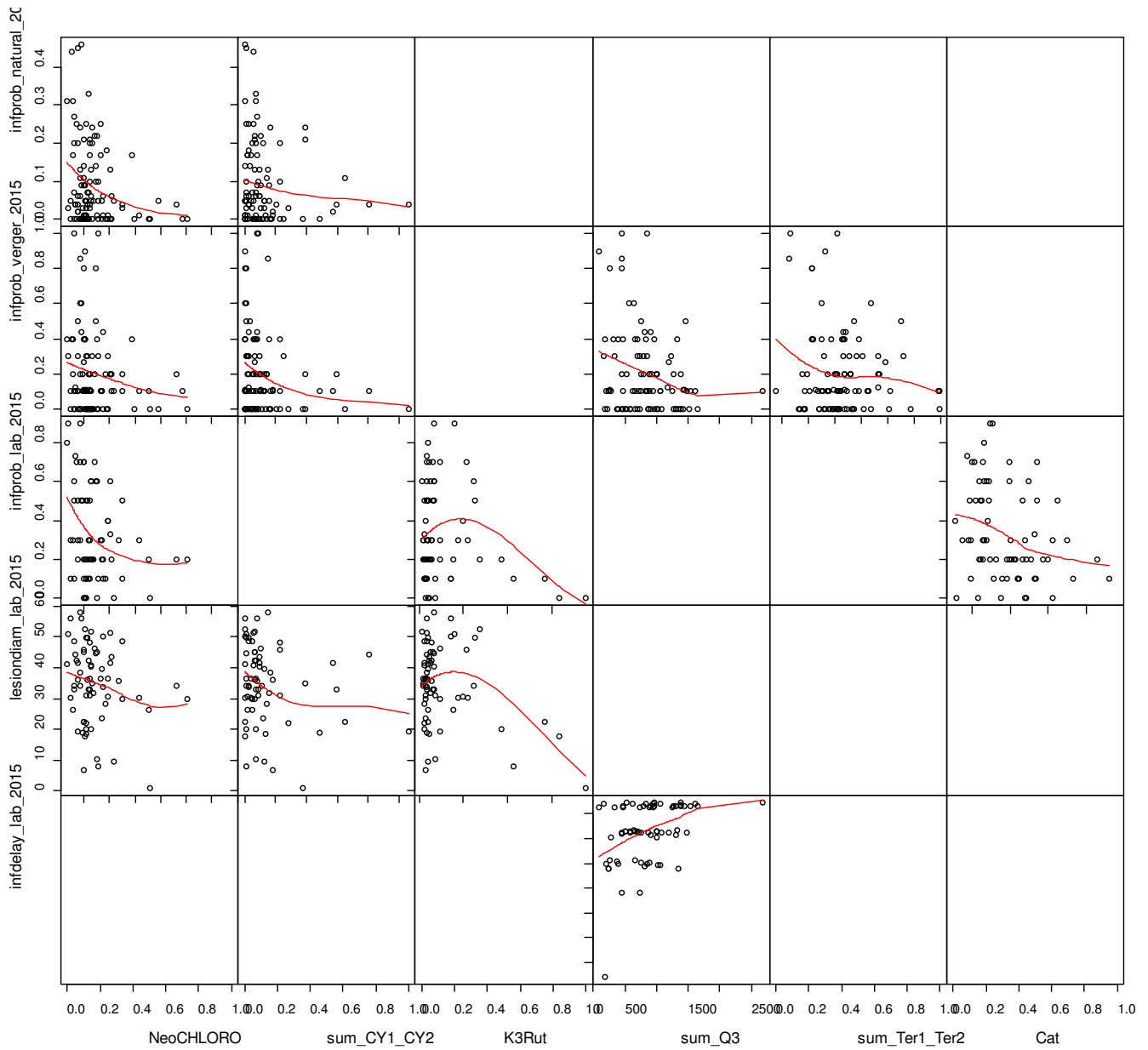


Figure 7. Relationships between epidermis compounds and traits linked to brown rot disease occurrence and propagation. Red lines are tendency lines obtained using 'loess' function in R. See Chapter 4 of this thesis for details on infection tests. Abbreviations: infprob\_natural\_2015: infection probability scored from natural infection in orchard in 2015; infprob\_orchard\_2015: infection probability scored by artificial test in orchard in 2015; infprob\_lab\_2015: infection probability scored by artificial test in orchard in 2015; lesiondiam\_lab\_2015: lesion diameter from artificial test with spray in lab; infdelay\_lab\_2015: infection delay from artificial test with spray in lab.

In general, QTL controlling compounds that were highly correlated were collocated forming clusters as on the middle of LG1, the middle and the end of LG2 and LG3, the top of LG4, the middle of LG5 and the second part of LG6 and LG7 (Figure 8). Whether same genes have pleiotropic effects on different compounds or different genes were arranged in clusters should be discussed case by case.

## **2.5 Some compounds exhibited significant relationships with infection traits**

We examined the relationships between compound variations in the BC2 progeny and infection level of the corresponding genotypes (Figure 7). On difficulty of such analysis is that, due to experimental constraints, fruit for infection tests and for biochemical analyses were not the same. In consequence only mean values per genotype can be used. Second difficulty is that few genotypes exhibited large values of the compounds. Accordingly, the relationships in the region of high values of the x-axes lay on a low number of data. The extreme case was for the 3,5diCQ for which a unique genotype had high-level and displayed very low susceptibility to *M. laxa*. However in different cases, data suggest that above thresholds some compounds may have inhibitory effects on the fungi. This is particularly the case for neoChlorogenic acid (NeoCHOLORO) and the sum of the two anthocyanins (sum\_CY1\_CY2) which displayed negative relationships respectively with 3 and 2 scores of the probability of infection (natural and artificial, in the orchard or lab.) The terpenoids Ter1 and Ter2 (sum\_Ter1\_Ter2) and the sum of the 3 quercetins (sum\_Q3) showed same negative links with probability of artificial infection in orchard, and the K3Rut and catechin compounds were linked to probability of artificial infection in lab only. Some compounds exhibited relationships with traits linked to infection progression as the sum of the two anthocyanins (sum\_CY1\_CY2) and K3Rut with lesion diameter: high-level of compounds were associated with low rot diameter. The same way, the sum of the 3 quercetins was associated with infection delay recorded in lab: genotypes with high-levels displayed delayed infection.

The study presented in Chapter 2 of this thesis enabled the identification of surface compounds linked to infection probability. Among them, 4 terpenoids (a\_u, a\_o, Ter1 and Ter2) and 4 terpenoid derivatives (der1, der2, der3 and der5) were also quantified in the present study. On these 8 compounds, only the terpenoids Ter1 and Ter2 were identified again in this chapter as compounds potentially linked to infection.

## **2.6 Colocation of QTL for epidermis compounds and *M. laxa* susceptibility were observed**

The two QTL controlling brown rot infection probabilities in orchard detected from the dataset acquired simultaneously as this epidermis composition dataset were located on LG2 and LG7.

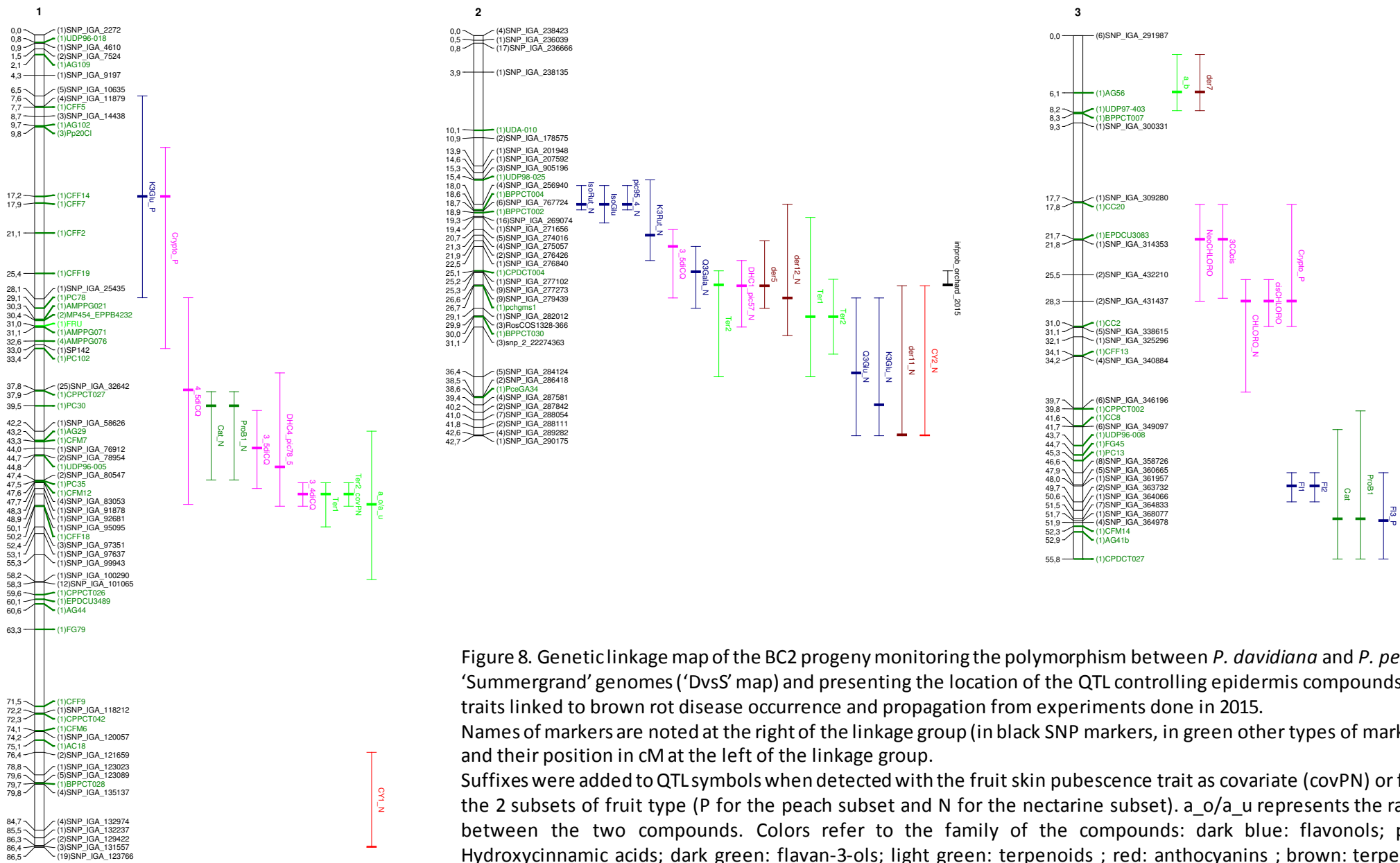
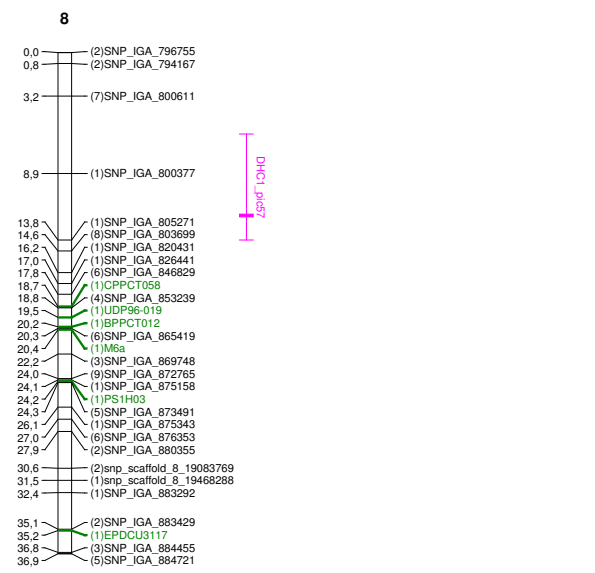
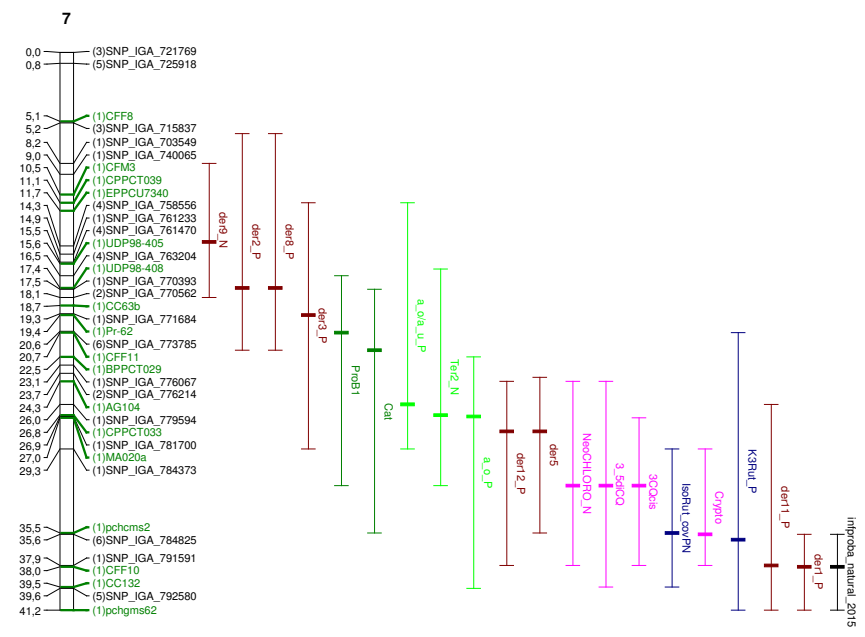
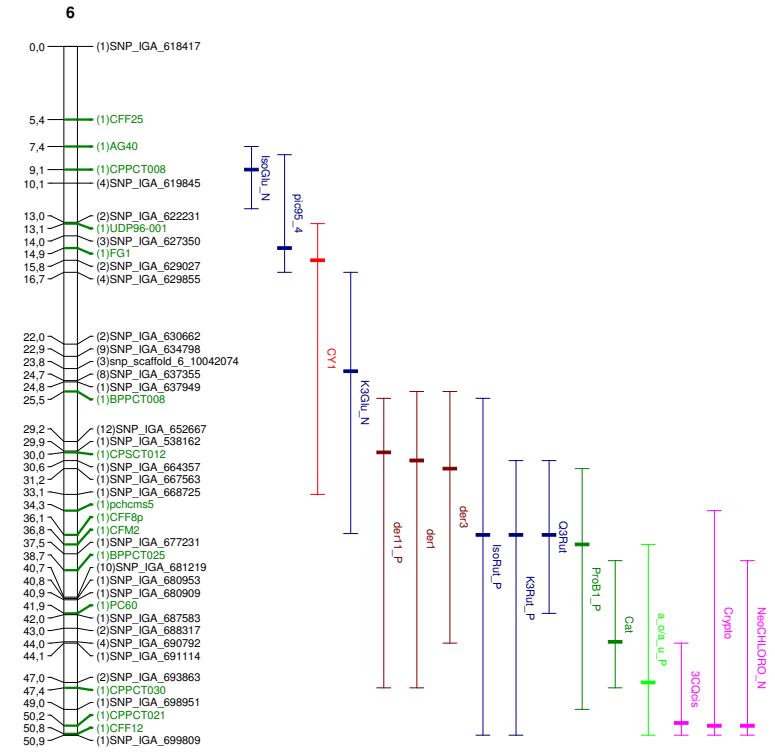
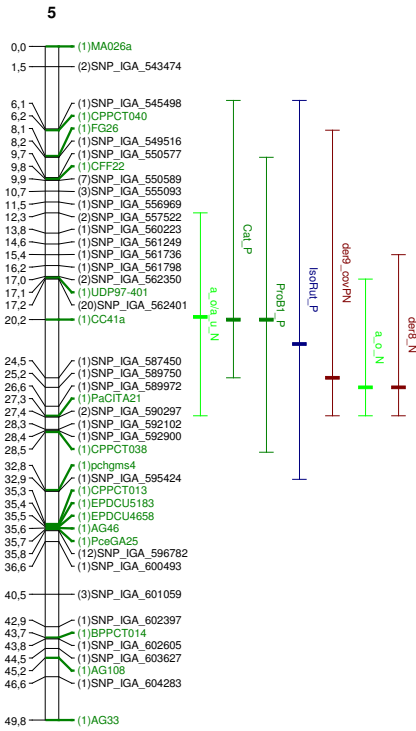
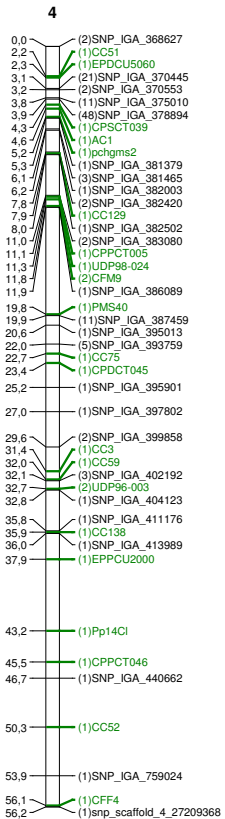


Figure 8. Genetic linkage map of the BC2 progeny monitoring the polymorphism between *P. davidiana* and *P. persica* ‘Summergrand’ genomes (‘DvsS’ map) and presenting the location of the QTL controlling epidermis compounds and traits linked to brown rot disease occurrence and propagation from experiments done in 2015. Names of markers are noted at the right of the linkage group (in black SNP markers, in green other types of markers) and their position in cM at the left of the linkage group. Suffixes were added to QTL symbols when detected with the fruit skin pubescence trait as covariate (covPN) or from the 2 subsets of fruit type (P for the peach subset and N for the nectarine subset). a\_o/a\_u represents the ration between the two compounds. Colors refer to the family of the compounds: dark blue: flavonols; pink: Hydroxycinnamic acids; dark green: flavan-3-ols; light green: terpenoids ; red: anthocyanins ; brown: terpenoid derivatives. In Black are QTL controlling traits linked to brown rot disease occurrence and propagation. See Chapter 4 of this thesis for details on infection tests. Abbreviations: infprob\_natural\_2015: infection probability scored from natural infection in orchard in 2015; infprob\_orchard\_2015: infection probability scored by artificial test in orchard in 2015; infprob\_lab\_2015: infection probability scored by artificial test in orchard in 2015; lesiondiam\_lab\_2015: lesion



Informa natural 2015

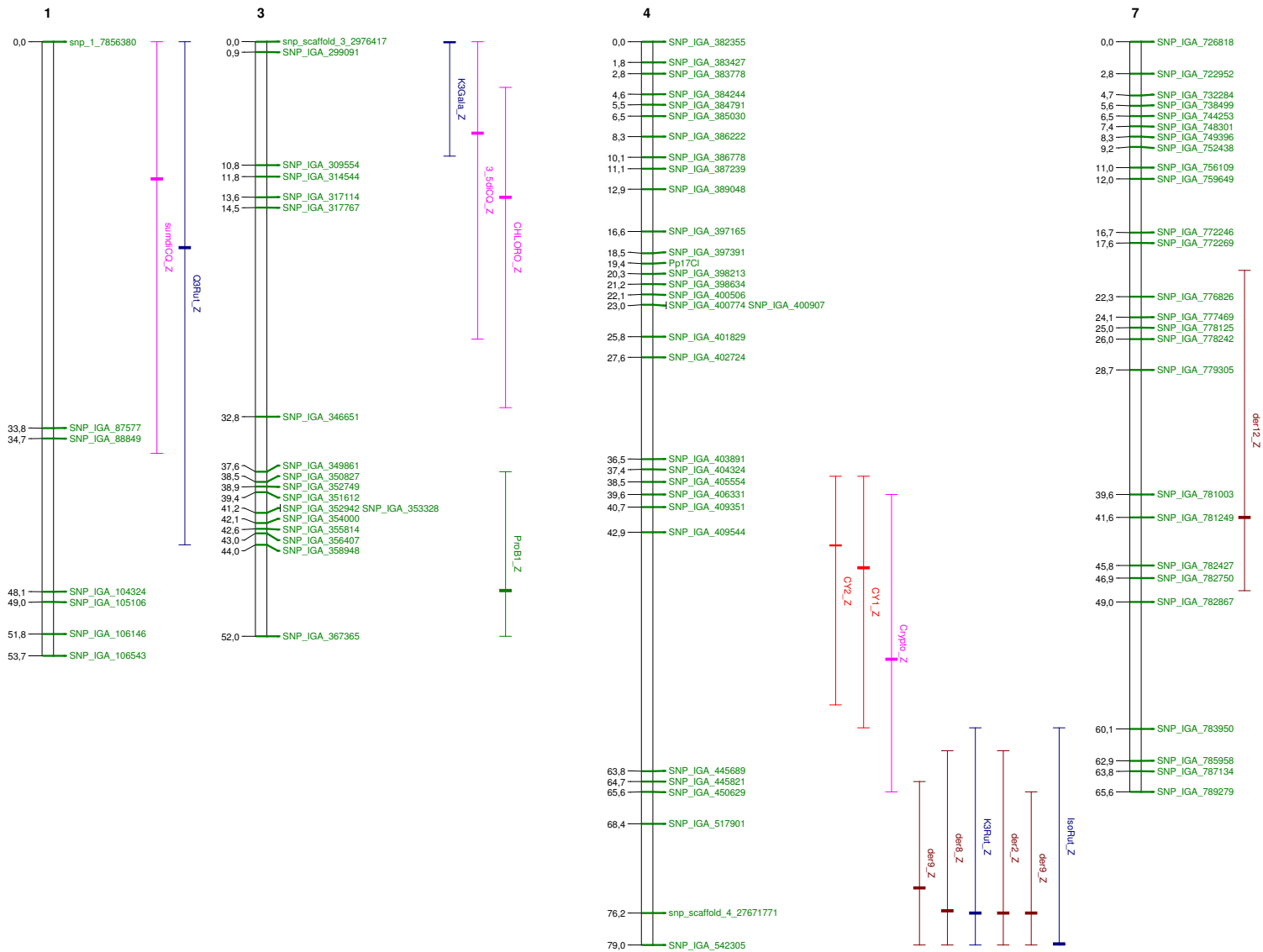


Figure 9. Genetic linkage map of the BC2 progeny tracking the heterozygosity of Zephir cultivar ('SNP\_Z' map) and presenting the location of the QTL controlling epidermis compounds and traits linked to brown rot disease occurrence and propagation from experiments done in 2015. Only the linkage groups carrying QTL are plotted. See Figure 8 for details.



In the region of the QTL controlling *infproba\_orchard\_2015* on LG2 we also detected QTL controlling terpenoids, terpenoid derivatives, flavonols and an hydroxycinnamic acid. Looking more specifically on the tiny region of the peak of the QTL for *infproba\_orchard\_2015*, peaks of QTL controlling Ter2, der5, DHC1 and Q3Gala were co-located. Among these 4 compounds, Ter2, der5 and Q3Gala were identified in Chapter 2 and in this Chapter as compounds potentially linked to infection.

In the region of the QTL controlling *infproba\_natural\_2015* on LG7 we detected QTL controlling terpenoids, terpenoid derivatives, hydroxycinnamic acids and a flavonol. The concordance of the peaks of the QTL enabled to select in priority the QTL controlling K3Rut, der1 and der11. Among these 3 compounds, der1 and K3Rut were identified in Chapter 2 and in this Chapter as compounds potentially linked to infection.

### **3 Conclusions**

This study is a first at different point of views. Primarily, we detected new compounds of fruit epidermis. Secondly, such large profiling of peach epidermis compounds has never been published before. Then, we do not know of any study on the variations of peach epidermis composition on a large collection of genotypes. Finally, the genetic control of such epidermis compounds from any fruit was not studied before.

The preliminary results presented here are very promising. Large variations were observed in the progeny and numerous correlations between compounds were detected, opening the door for further analyses of metabolic pathways. In case QTL positions are confirmed with results from 2016 experiments, efforts will be pointed to decipher the genetic control of metabolic pathways and the identification of candidate genes and their validation.

The same way, more year repetitions are needed to explore the relationships between the epidermis compounds and the resistance to brown rot disease. Few relationships were observed and the ones confirmed will be then checked in specific tests of fungicide activity.

## CONCLUSION GENERALE ET PERSPECTIVES

De nombreux travaux ont été entrepris pour comprendre la résistance aux monilioses. Certains éléments importants du processus d'infection et de résistance sont déjà connus (Oliveira Lino et al, 2016). Néanmoins, la communauté scientifique n'a pas rassemblé ces éléments de façon à générer un schéma complet des processus et facteurs de résistance aux monilioses qui expliquerait la diversité phénotypique observée au sein des espèces de *Prunus* et entre variétés. Sans doute nos connaissances sont-elles encore trop incomplètes et les lacunes ne permettent pas de construire un tel schéma.

L'influence notable de l'environnement dans le processus d'infection est une contrainte majeure persistante. Elle peut décourager toute velléité de criblage de matériel végétal tant les résultats sont instables entre les années. Quoiqu'il en soit, la persévérance semble appropriée, car les résultats récents suggèrent qu'un contrôle génétique intéressant pourrait être utilisé en création variétale dans l'avenir.

Les objectifs de travail étaient i) d'identifier les facteurs de résistance du fruit à *M. laxa* à différents stades de croissance des fruits et ii) d'étudier leur contrôle génétique, par l'étude d'une descendance interspécifique de cartographie. Nous avons utilisé une souche de *M. laxa*, qui était considérée en 2013 comme l'espèce de *Monilinia* la plus répandue dans le Sud de la France.

Pour répondre au premier objectif, nous avons étudié la probabilité d'infection à *M. laxa* au cours du développement du fruit en lien avec les caractéristiques structurales et biochimiques des fruits, chez 3 cultivars. Nous avons focalisé d'une part sur les stomates et la conductance cuticulaire des fruits et d'autre part sur les composés de surface des fruits.

Nous avons porté une attention plus particulière aux barrières physiques au stade I du développement des fruits. Notamment nous avons exploré l'importance des stomates, à un stade où leur densité est très forte, et de la conductance cuticulaire, dans la résistance à la pénétration du champignon dans les fruits.

Pour répondre au second objectif, nous avons examiné la résistance à *M. laxa* des géotypes de la descendance interspécifique à maturité. Différentes modalités de tests en laboratoire et en verger ont été essayées. Nous avons également quantifié les teneurs en composés d'épiderme au sein de la descendance interspécifique à maturité. Enfin, nous avons recherché des relations entre probabilité d'infection et différentes caractéristiques des fruits ; identifié des QTL de résistance aux monilioses à différents stades et analysé leur co-localisation avec des QTL contrôlant des caractéristiques physiques et biochimiques des fruits.

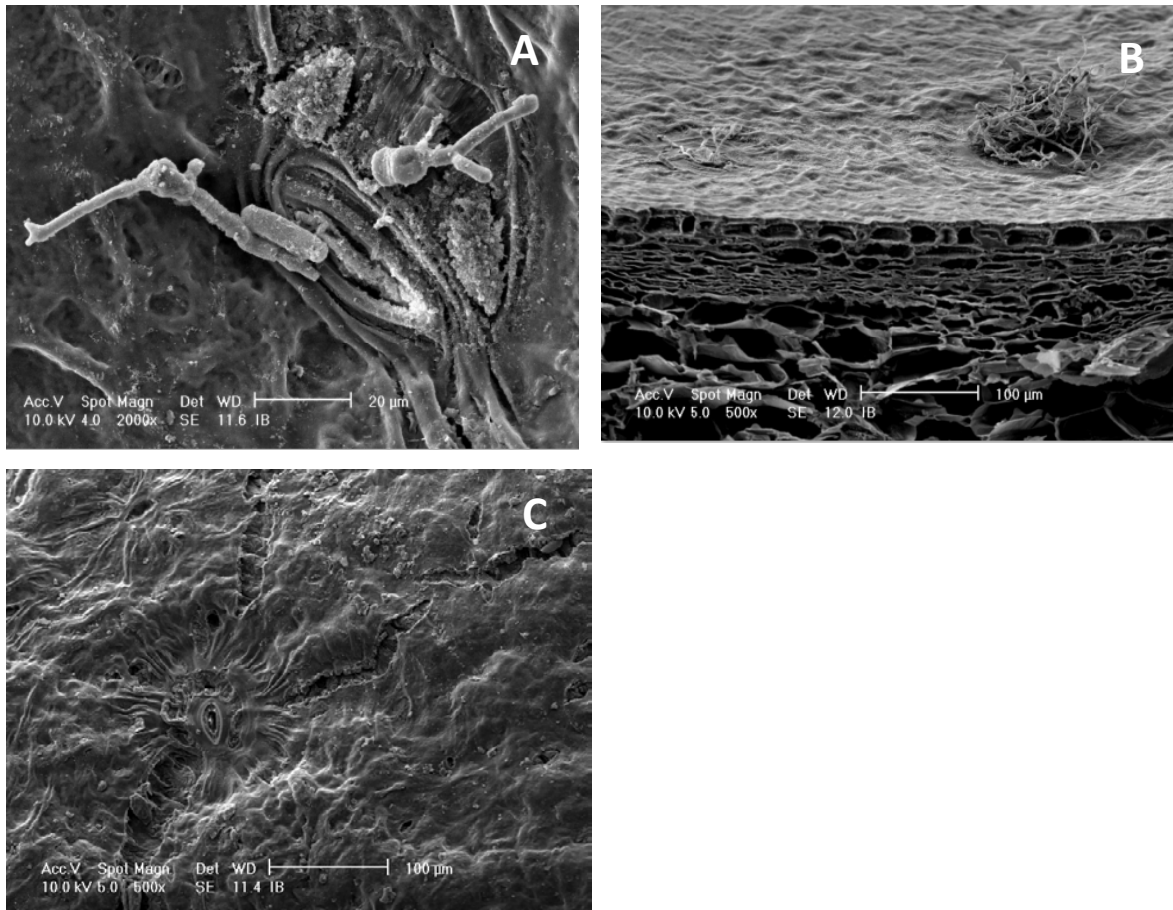


Figure 1. A et B Développement du champignon *M. laxa* autour des stomates de nectarines à maturité. C – Formation de microfissures autour des stomates à la surface de Summergrand à maturité.

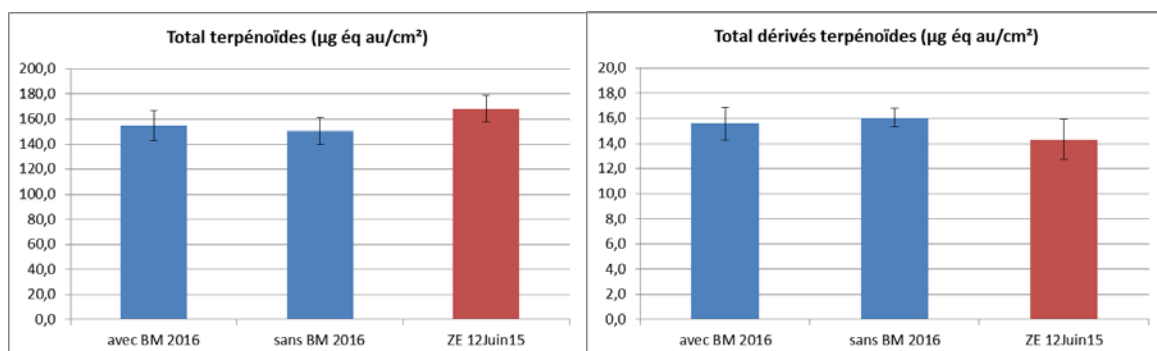


Figure 2. Total des tritérpnoides et dérivés de tritérpnoides en surface des fruits de Zéphir, pour différentes modalités de désinfection, avec bain-marie (BM) et sans bain-marie en 2016, et avec bain-marie en 2015.

Dans la littérature il est souvent mentionné que les stomates constituent une porte d'entrée pour les champignons et bactéries. Nos observations au microscope ont souvent montré des spores en germination autour et dans les stomates (Figure 1). Ceci nous a encouragé à étudier le lien entre le nombre de stomates et la probabilité d'infection, ce qui n'avait jamais été entrepris.

La première chose à signaler ici est peut-être notre surprise d'observer dans cette étude des symptômes d'infection inattendus. Nous pensions observer au stade I une très forte probabilité d'infection, ce qui n'a pas été le cas. Par contre nous avons observé des symptômes dits 'tache claire', jamais mentionné dans la littérature liée aux monilioses. Ces symptômes méritent sans aucun doute plus d'investigations. Nous pensons que ces fruits sur lesquels le champignon germe, mais où l'infection ne se développe pas, sont un excellent modèle d'étude qui est peut-être la clé pour comprendre comment le fruit détecte l'infection et quels moyens il met en œuvre pour se défendre. Si ces moyens de défense pouvaient être activés chez des fruits à maturité, ils pourraient participer à augmenter la résistance des fruits à ce stade.

Les résultats du Chapitre 3 n'ont révélé aucun lien entre la probabilité d'infection au stade fruit immature et le nombre de stomates, malgré la grande variabilité du nombre de stomates observé dans la descendance. Nous étions donc tentés de barrer les stomates de notre liste de facteurs potentiels liés à la résistance. Cependant nous avons détecté un QTL contrôlant le nombre de stomates dans la région de QTL d'infection... Pour aller plus loin, il nous manque sans doute des informations concernant le fonctionnement des stomates des fruits au cours de leur développement : à quel moment du développement du fruit deviennent-ils non fonctionnels ? ; se ferment-ils à ce moment-là ?

Dans cette étude au stade I, nous avons par contre mis en évidence un lien entre la conductance et la probabilité d'infection. La faible part de variation de la conductance cuticulaire expliquée par le nombre de stomates nous pousse à penser que le principal facteur de variation de la conductance est l'état de la cuticule elle-même. En pleine formation à ce stade, elle passe sans doute rapidement d'un état très mince et très perméable à l'eau à un état de cuticule formée jouant son rôle de barrière. En effet l'étude en cinétique des composés de surface et cires réalisée au chapitre 2 a montré des teneurs très faibles en composés et cires au 1<sup>er</sup> point de la cinétique. Or certains composés des cires ont été identifiés chez l'avocat comme inhibiteurs de champignons (Kolattukudy et al, 1995) et nous avons également observé des relations entre composés des cires et probabilité d'infection qui suggèrent un rôle des cires dans l'infection chez la pêche également.

Compte-tenu de ces résultats qui suggèrent un rôle pour la composition de la cuticule au stade I dans la forte sensibilité des fruits observée à ce stade, nous avons décidé d'explorer la diversité des composés de l'épiderme au sein de la population BC2 à maturité comme potentiel facteur expliquant les variations de sensibilité à la moniliose à ce stade.

# **1 Comment phénotyper la résistance à la pourriture brune n'est pas une question triviale**

Après 3 ans de travail, à essayer de concevoir des protocoles d'infection, à tester différentes modalités d'infection et différentes méthodologies, à effectuer un laborieux criblage des génotypes, nous avons acquis un peu d'expertise sur le phénotypage de la résistance à la pourriture brune. Certains résultats nous permettent de valider ou exclure certaines méthodologiques.

## **1.1 Résistance de l'épiderme et de la chair**

Nous avons déjà largement discuté de ce point-là dans le Chapitre 4. Nous concluons sans hésiter que nos meilleures chances d'identifier des facteurs de résistance utilisables pour la création variétale résident dans l'épiderme des fruits. Outre les résultats de la littérature (Martínez-García et al, 2013; Pacheco et al, 2014; Pascal et al, 1994) et ceux présentés dans ce travail, nous avons réalisé des expérimentations sur fruits blessés (non présentés dans ce mémoire) qui confirment une probabilité d'infection de 100% des fruits blessés et une très faible variabilité de propagation de l'infection dans notre matériel.

## **1.2 Effet du bain-marie sur la probabilité d'infection**

L'usage du bain-marie préconisé comme technique pour réduire les infections en post-récolte mérite attention. En effet, (Spadoni et al, 2015) ont montré un effet stimulant sur le tube germinatif des conidies de *M. fructicola* placées sur la surface du fruit immédiatement après un traitement thermique à 60°C pendant 60s. Ils suggèrent que les composés organiques volatils émis par les pêches traitées à la chaleur pourraient stimuler la germination des conidies de *M. fructicola*, et augmenter l'incidence de la pourriture brune lorsque l'inoculation se produit immédiatement après le bain.

Nos premiers tests en laboratoire (2013 et 2014) faisaient usage d'un bain-marie à 55°C pendant 45s de façon à réduire l'inoculum naturel se trouvant sur les fruits récoltés (*Monilinia spp.*, rhizopus,...). Nous avons comparé la probabilité d'infection avec et sans bain-marie pour les cultivars Zephir et Summergrand et en effet conclu que le bain-marie augmentait la sensibilité des fruits à *M. laxa*. Ceci peut expliquer en partie les différences obtenues entre les tests réalisés en verger (donc sans bain-marie) et ceux réalisés en laboratoire (années 2013 et 2014). Le bain-marie est très largement utilisé par les chercheurs pour réduire les pertes de fruits dues à l'inoculum naturel mais compte-tenu de son effet sur l'augmentation de la sensibilité des fruits nous avons décidé de ne plus l'utiliser.

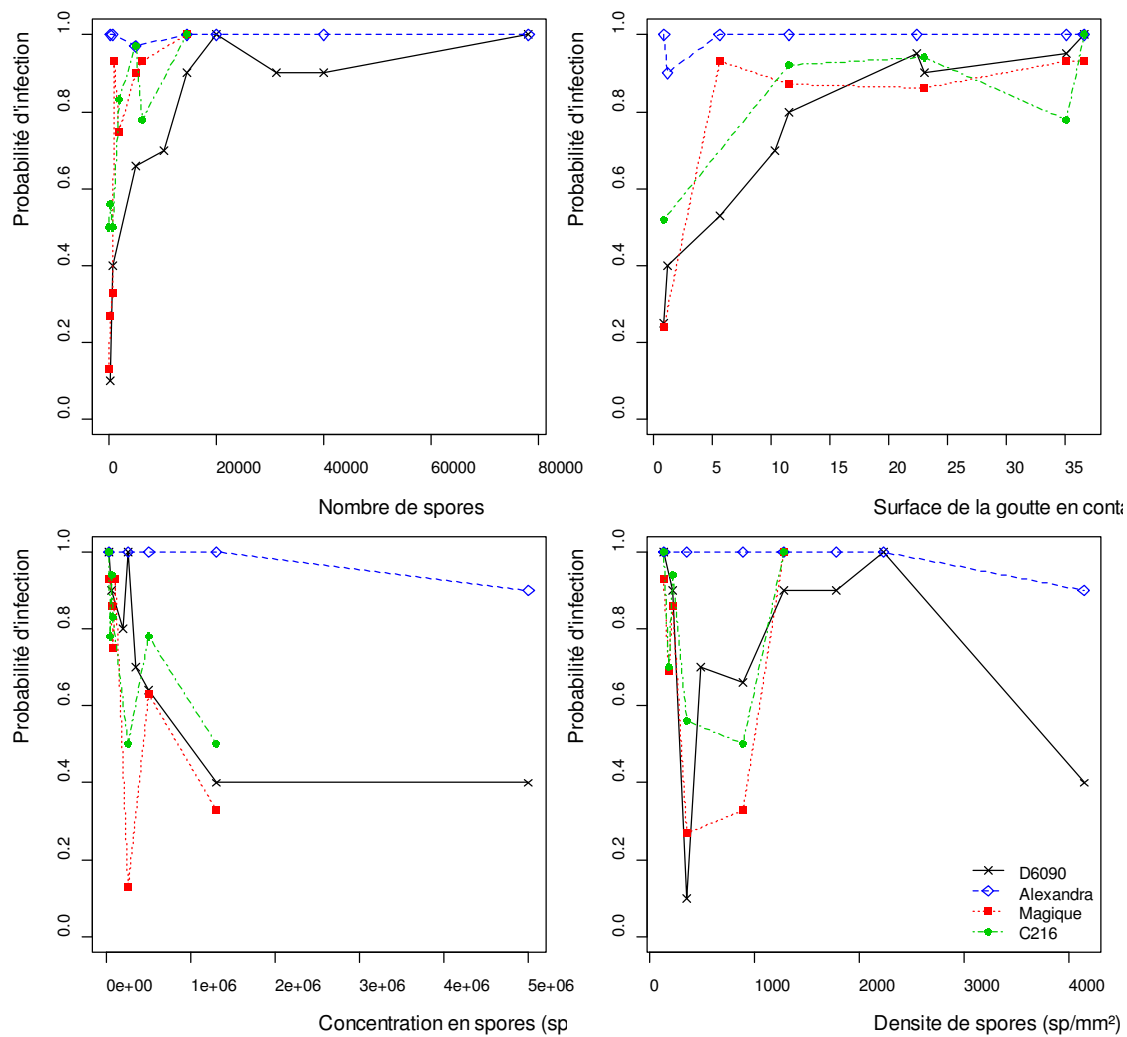


Figure 3. Evolution de la probabilité d'infection en fonction de 4 facteurs de la goutte de suspension de spores (nombre de spores, surface de la goutte en contact avec l'épiderme, concentration en spores et densité de spores dans la goutte). Cette étude a été réalisée sur 4 génotypes différents : D6090, Alexandra, Magique et C216.

Tableau 1. Résultats des Anova réalisées pour tester l'effet de 5 facteurs sur la probabilité d'infection (génotype, surface, nombre de spores, concentration, densité de spores). Seuls 3 facteurs montrent un effet significatif, les données des autres facteurs n'ont donc pas été précisées.

Facteurs	F-value	p-value	significativité
Génotype	4,357	0,017013	*
Surface	19,04	0,000334	***
nombre de spores	10,476	0,00365	**

Nous avons vérifié l'effet du bain-marie sur les composés de surface pour le cultivar Zephir. Nous n'avons pas trouvé de différences significatives entre les modalités avec et sans bain-marie pour les teneurs en triterpénoïdes ni en dérivés des terpénoïdes (Figure 2).

Outre sur les composés organiques volatils, le bain-marie a peut-être une influence sur d'autres composés comme les protéines et composés solubles dans l'eau participant au dialogue entre le fruit et le champignon et à la défense du fruit.

### **1.3 Concentration en spores de la suspension d'infection et volumes déposés**

Il n'y a pas de consensus dans la littérature sur la meilleure concentration en spores des suspensions pour les infections. Plusieurs concentrations et des volumes de gouttes différents sont utilisés dans les principales références mentionnées dans ce mémoire :  $1.5 \times 10^4$  spores/ml et 30  $\mu$ L (Walter et al, 2004),  $2.5 \times 10^4$  spores/ml et 10  $\mu$ L (Martinez-Garcia et al., 2013),  $5 \times 10^4$  spores/ml et 5  $\mu$ L (Lee & Bostock, 2006),  $10^6$  spores/ml et 20  $\mu$ L (Pascal et al., 1994),  $5 \times 10^6$  spores/ml et 10  $\mu$ L (Pacheco 2014). Cela aboutit à un nombre de spores déposées allant de 250 à 50000.

Suite à des tests préliminaires visant à trouver un bon compromis pour un test discriminant entre génotypes, nous avons utilisé 105 spores/ml et une goutte de 10  $\mu$ L ce qui représente 1000 spores déposées dans une goutte et une surface de goutte de 5,6mm<sup>2</sup>. Ces paramètres correspondent à un niveau moyen d'infection, ce qui permet d'observer une variabilité dans la sensibilité au pathogène (Figure 3 ; Tableau 1). En conditions trop virulentes, l'infection serait maximale pour tous les génotypes et aucune variabilité ne pourrait être observée. Bien sûr cet optimum doit certainement varier d'une souche à l'autre et d'une espèce de *Monilinia* à l'autre.

### **1.4 Goutte ou 'spray' : quel test utiliser ?**

Les tests réalisés avec une infection par dépôt de goutte (classique dans la littérature) ou avec une vaporisation sur l'ensemble du fruit (test mis au point en collaboration avec Igor Pacheco et l'Université de Milan dans le cadre du projet FruitBreedomics) sont bien différents. Nous avons discuté de cet aspect au Chapitre 4 de cette thèse. Le test 'spray' permet d'appréhender la globalité du fruit et l'hétérogénéité de l'apparition et de la densité des micro-fissures à sa surface (Gibert et al, 2007). Il permet d'évaluer la résistance globale du fruit à l'infection, y compris sa sensibilité à la formation de microfissures. Au contraire le test 'drop' doit être utilisé pour s'affranchir de la composante 'cracks', dans les études plus ponctuelles visant à caractériser la résistance de l'épiderme intact.

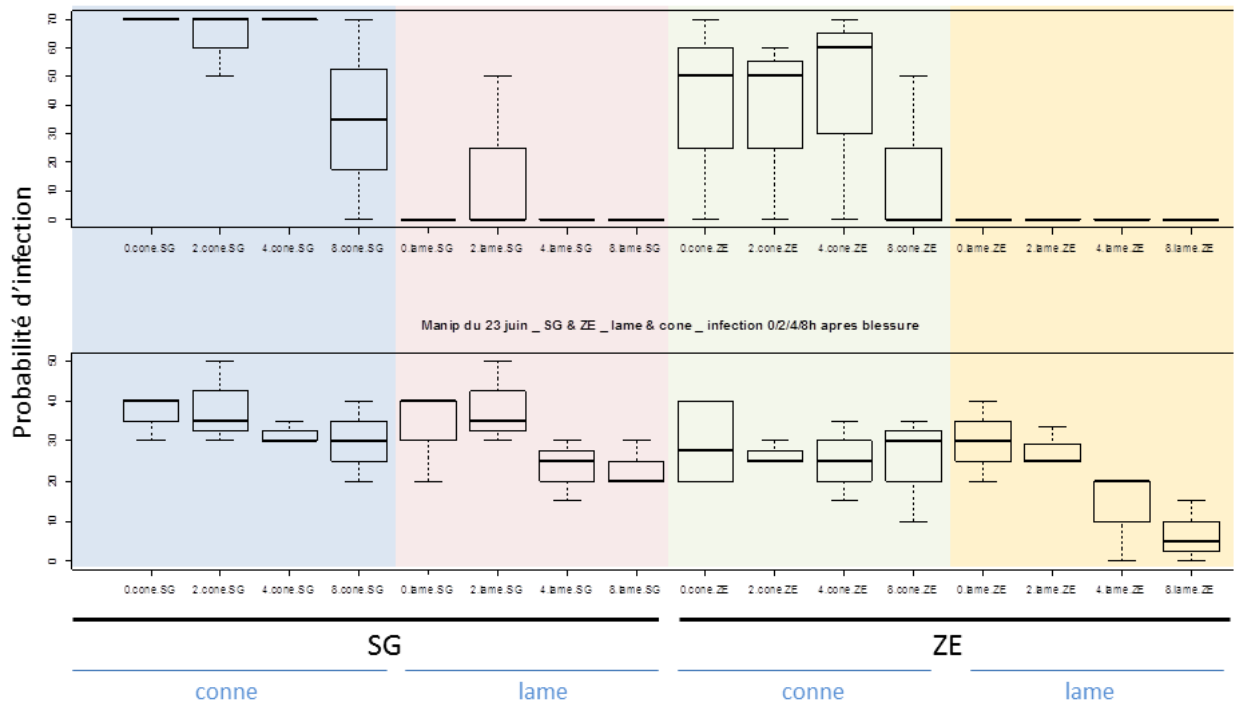


Figure 3. Probabilité d'infection des fruits de Summergrand (SG) et Zephir (ZE) en fonction du nombre d'heures écoulées entre le moment de la blessure (par cône ou par lame) et le moment du dépôt de la suspension sur la blessure (0, 2, 4 et 8 heures).

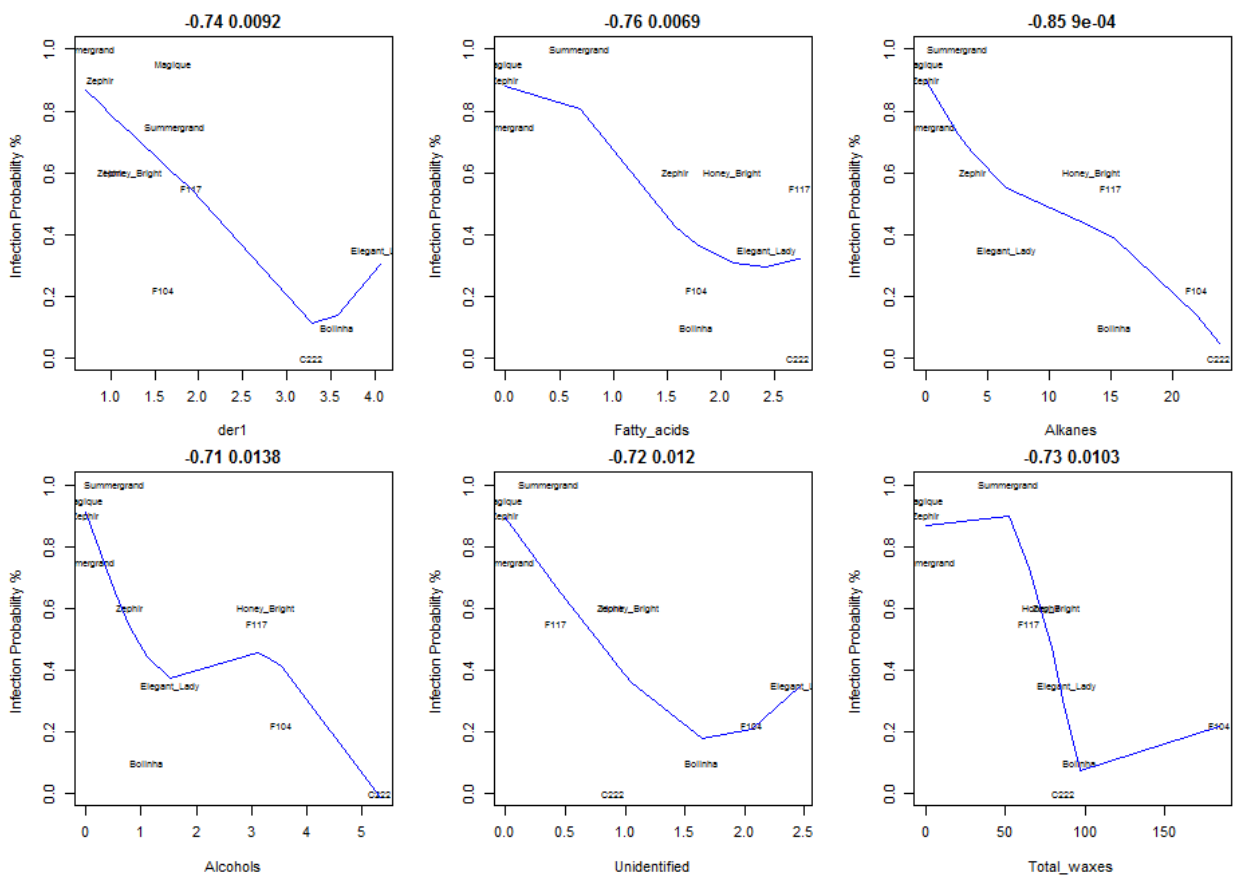


Figure 4 : Relations entre la probabilité d'infection et quelques composés de surface et de cires à maturité pour 9 cultivars. Le nom du composé d'une part et la corrélation et la pvalue associée d'autre part sont indiqués respectivement au-dessous et au-dessus de chaque graphe.



## **2 Facteurs de sensibilité et résistance à la pourriture brune: des progrès?**

Ce travail a permis d'explorer différents facteurs jouant un rôle potentiels dans l'augmentation de la sensibilité des fruits ou de leur niveau de résistance à l'infection par *M. laxa*.

### **2.1 Les 'cracks' : des portes ouvertes qui cicatrisent...**

Fréquemment les microfissures sont considérées comme des portes d'entrées principales aux monilioses à maturité. A l'approche de la maturité, les stomates semblent être des points de départ privilégiés pour la formation de ces fractures. Plusieurs photos faites au microscope montrent une fragilité particulière autour des stomates (Figure 1C). Nous n'avons pas pu quantifier ce lien, notamment parce qu'il est difficile d'estimer la surface de cracks d'un fruit, et d'autant plus celle d'une population de fruits. Mais il est surtout difficile de s'affranchir du double effet de la croissance du fruit sur l'apparition des cracks et sur la densité de stomates. Pour étudier cet aspect, il faudrait s'affranchir de l'effet de la taille du fruit et de la vitesse de croissance et donc étudier une collection de génotypes de nectarines uniquement, produisant de gros fruits avec une vitesse de croissance équivalente. Ce qui n'est pas facile à trouver...

Dans le lien densité de cracks et probabilité d'infection, il faut aussi considérer la capacité de cicatrisation des fruits. En effet, nous avons montré dans une étude préliminaire que la probabilité d'infection au niveau d'une blessure diminue lorsque l'infection est effectuée quelques heures après la blessure (Figure 3). Plus le temps passe, plus le fruit blessé devient résistant. L'identification des composés générés par le fruit à la suite de la blessure pourrait donner de nouvelles pistes sur les composés impliqués dans la résistance du fruit à *M. laxa*.

### **2.2 Les stomates : toujours un doute sur leur rôle**

Nous n'avons pas trouvé de corrélations entre le nombre de stomates et la probabilité d'infection au stade I petits fruits verts. Probablement cela est dû au fait que les stomates sont fonctionnels à ce stade et sont capables de se fermer. Cependant un doute sur leur rôle subsiste comme énoncé en début de ce chapitre.

### **2.3 Des composés secondaires de l'épiderme : potentiels facteurs inhibiteurs et inducteurs**

Les travaux réalisés dans les Chapitres 2 et 5 ont permis de mettre en évidence différents composés avec de potentiels effets inducteurs et inhibiteurs de la probabilité d'infection. Cependant il est primordial de garder en tête qu'un simple effet temporel peut être à l'origine des corrélations observées. En effet différents processus se déroulent en parallèle suivant le développement du fruit et se retrouvent fortuitement corrélés sans qu'il y ait des liens de cause à effet.

Aussi pour vérifier les corrélations observées, nous avons évalué un groupe de génotypes pour la probabilité d'infection et la quantité de composés de surface et cires à maturité (*Annexe 2*). Les résultats montrent un effet inhibiteur de différents composés (Figure 4). Parmi eux *der1* et les composés 'unidentified' confirment les résultats obtenus dans la cinétique du Chapitre 2. Par contre, en ce qui concerne les composés des cires, des relations inverses sont souvent détectées. Les résultats suggèrent que les relations positives observées au Chapitre 2 sont un artéfact de l'effet temporel inclus dans les données.

Pour conclure, des tests d'activité fongicide de ces composés doivent être effectués.

## **2.4 Un effet fort de l'environnement sur l'infection**

Nos résultats et ceux de la littérature convergent sur le fait que l'environnement exerce un effet fort sur la probabilité d'infection. Dans un tel cas, les études génétiques sont délicates et aboutissent à des résultats souvent décevants tant il est difficile de détecter le contrôle génétique du caractère. C'est d'autant plus difficile que la résistance à la moniliose semble sous le contrôle de divers facteurs à effets faibles. Ainsi dans les trois études génétiques réalisées, des résistances faibles ont été observées provenant notamment de génotypes sensibles comme *Elegant Lady*, *Zephir*, *Summergrand*.

Mais jusqu'à présent aucun facteur climatique précis influençant l'infection en condition de verger n'a été identifié. Nous n'avons pas trouvé de liaison entre les différents facteurs climatiques enregistrés dans le verger et l'instabilité observée entre années.

D'une façon plus générale l'effet de l'environnement du fruit sur l'infection est étudié par les agronomes et les épidémiologistes. Des solutions culturales sont proposées visant notamment à réduire la forte croissance des fruits (et l'apparition de cracks), modifier le micro-climat au sein de l'arbre et réduire l'inoculum dans le verger.

Ces constats encouragent pour le futur des recherches conjointes visant à associer des cultivars tolérants aux monilioses avec des pratiques culturales adaptées de façon à réduire notablement sans avoir recours à des traitements fongiques les risques d'infection.

## **3 Une maladie sous le contrôle génétique de quelques QTL à effets faibles**

Les résultats de notre étude montrent que les facteurs de résistance identifiés ont un effet faible et sont difficiles à détecter. Cependant, des QTL instables ou simplement putatifs trouvés dans une étude prennent toute leur importance quand ils s'avèrent être positionnés dans des régions identiques à des QTL détectés dans des études indépendantes. C'est le cas pour certains des QTL que nous avons détectés et notamment celui sur le LG1. Ceci suggère que, contrairement à ce qu'on pourrait penser, le nombre de gènes impliqués dans la résistance à la moniliose n'est probablement

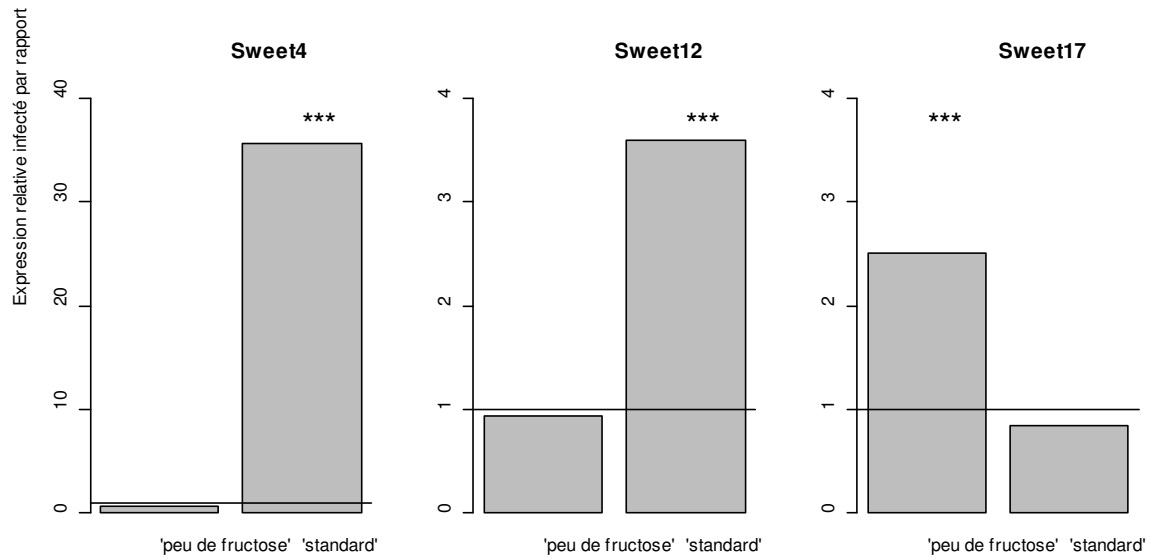


Figure 5. Comparaison de l'expression dans le fruit de 3 gènes de transporteurs (Sweet4, Sweet12 et Sweet17) chez 2 génotypes aux phénotypes fructose contrastés ('peu de fructose' versus 'standard'). L'expression dans les fruits à 18h, c'est-à-dire 10 heures après pulvérisation d'une suspension de spores de *M. laxa*, est exprimée par rapport à l'expression d'un fruit témoin non infecté prélevé à la même heure. Le gène Sweet17 est significativement surexprimé dans le fruit infecté chez le génotype 'peu de fructose' uniquement. Au contraire, les 2 gènes Sweet4 et Sweet12 sont surexprimés dans les fruits infectés chez le génotype 'standard' uniquement.

pas très important puisque des QTL identiques ont été détectés dans 3 descendances de fonds génétiques différents et pour 3 espèces de *Monilinia*... Ainsi la base génétique de la résistance à la maladie est au moins partiellement similaire entre les espèces de *Monilinia*.

Nous n'avons pu résister à explorer le lien entre le QTL de fructose, pour lequel un transporteur de la famille des Sweet (Sweet17) a été identifié comme meilleur gène candidat, et le QTL de probabilité d'infection détecté au même endroit. L'implication de certains de ces transporteurs dans des réponses à des stress biotiques a déjà été montrée (Chen et al, 2010; Chong et al, 2014) et Sweet17 semble intervenir dans la relation de symbiose d'un champignon et de la pomme de terre (Manck-Götzenberger & Requena, 2016). Ainsi nos résultats préliminaires montrent que l'expression des gènes des transporteurs de sucres de la famille des Sweet est modifiée dans le fruit après infection par *M. laxa* (Figure 5) mais dépend du génotype. Ces résultats sont en cours de confirmation une 2<sup>nd</sup>e année.

Nos résultats montrent que les cultivars Summergrand et Zephir portent des allèles favorables pour la résistance à la pourriture brune. Contrairement à ce qu'on pensait les allèles du parent sauvage *P. davidiana* contribuent souvent à augmenter la susceptibilité à la maladie. Aussi il semble important de caractériser nos ressources génétiques avec l'idée d'y découvrir des facteurs de résistance à effets faibles qui pourraient ensuite être cumulés dans un même matériel végétal. Le cumul de différents facteurs de résistance, liés à des composés secondaires, des sucres, des dérivés réactifs de l'oxygène, des protéines PR (pathogenesis-related)... devrait permettre d'atteindre des niveaux de résistance satisfaisant.

Le choix de la méthodologie de phénotypage adaptée à une telle recherche de résistances multiples n'est pas évident. Sans doute faut-il combiner deux méthodes consistant d'une part à caractériser le niveau de résistance des génotypes par des tests d'infection et d'autre part à identifier directement les différents facteurs de défense.

## PERSPECTIVES

A court terme, la priorité est de réaliser une 2<sup>ème</sup> année de phénotypage des composés de l'épiderme dans la BC2 pour confirmer les premiers résultats trouvés ici et la stabilité des QTLs face aux facteurs environnementaux. Outre l'intérêt potentiel de ces composés dans la résistance à la moniliose, ces composés sont également intéressants pour la santé humaine. La population étudiée dans ce travail de thèse apparaît comme un matériel de choix pour étudier ces composés.

L'exploration '*in vitro*' de l'effet inducteur ou inhibiteur des composés identifiés par corrélation, sur le développement du champignon, apparaît comme une suite logique à ce travail.

Par ailleurs, la diversité étudiée dans ce travail de thèse était restreinte aux génotypes utilisés comme parents de la descendance. Explorer la diversité dans un panel plus large telle qu'une

collection de ressources génétiques d'une part et phénotyper d'autres populations de croisement permettant d'étudier la variabilité créée par recombinaisons entre les gènes d'un parent semble une étape indispensable pour identifier de nouvelles sources d'allèles de résistance à la moniliose.

Enfin, les génotypes avec de solides niveaux de résistance à la pourriture brune identifiés au sein de la BC2 cumulent sans doute plusieurs allèles favorables. Ils sont des géniteurs portant sources de résistance à utiliser dans un programme d'amélioration à cette maladie.

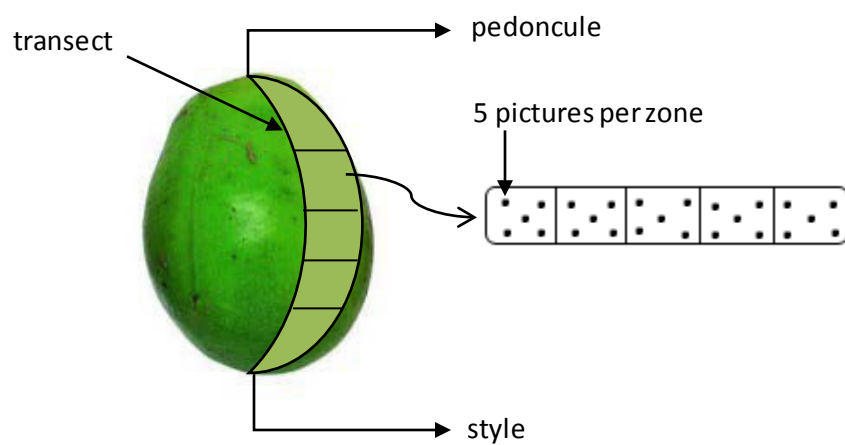
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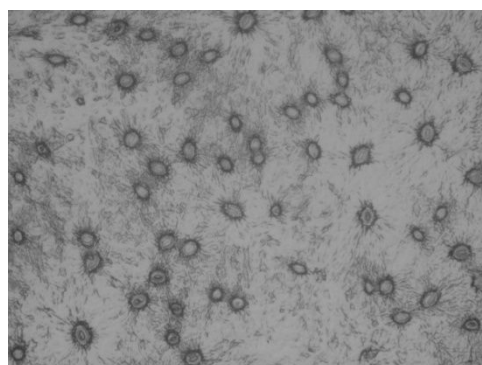
## ANNEXE 1

### Supplementary data of Chapter 3

Presentation of the preliminary experiment performed to set up a protocol suitable to estimate the mean number of stomata per fruit for the genotypes of the whole population. The objective was i) to compare counting all stomata under microscope and counting stomata on a fraction of the fruit surface on pictures, which allow gaining time and delaying counting from harvest, ii) to define the best zone of the fruit to consider to reduce sampling and counting.



**Figure 1.** Sampling prints

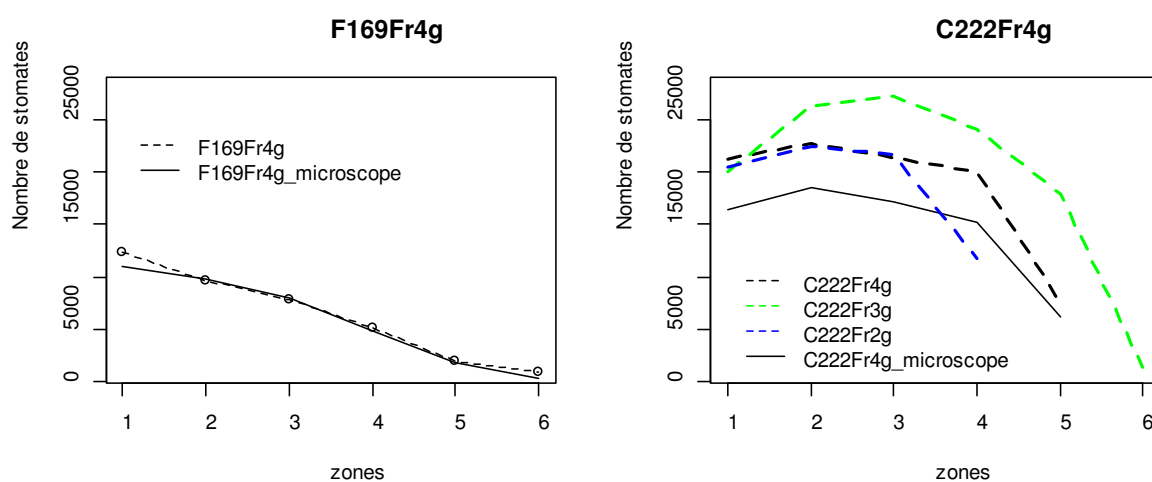


from an entire fruit.

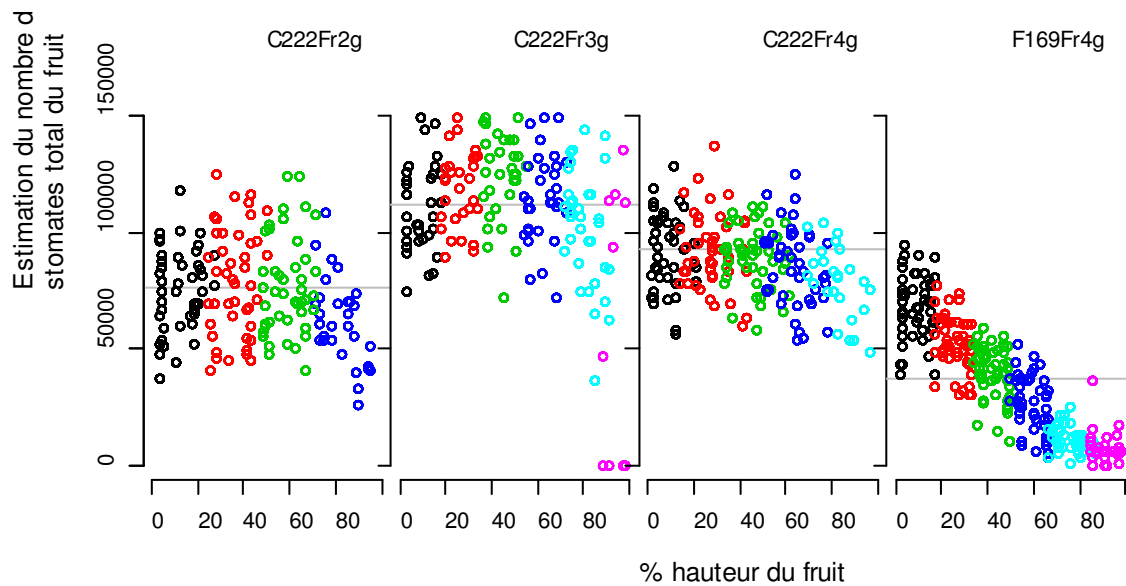
**Figure 2.** Picture of imprinting of stomatas (magnification x10)

**Table 1.** Summary of the results from the preliminary experiment performed to set up a protocol suitable to estimate the mean number of stomata per fruit for the genotypes of the whole population. Two methods were assessed for 2 of the 4 fruits studied.

genotype	fruit mass (g)	number of pictures	Fruit surface (mm <sup>2</sup> )	% of the fruit surface observed with the pictures	Total number of stomata estimated from the pictures	Total number of stomatas counter under microscope
C222	4.20	205	1159	15.9	92764	73707
C222	3.90	185	1217	13.7	117069	-
C222	2.02	165	722	20.6	76374	-
F169	4.23	275	1213	20.4	37781	35879

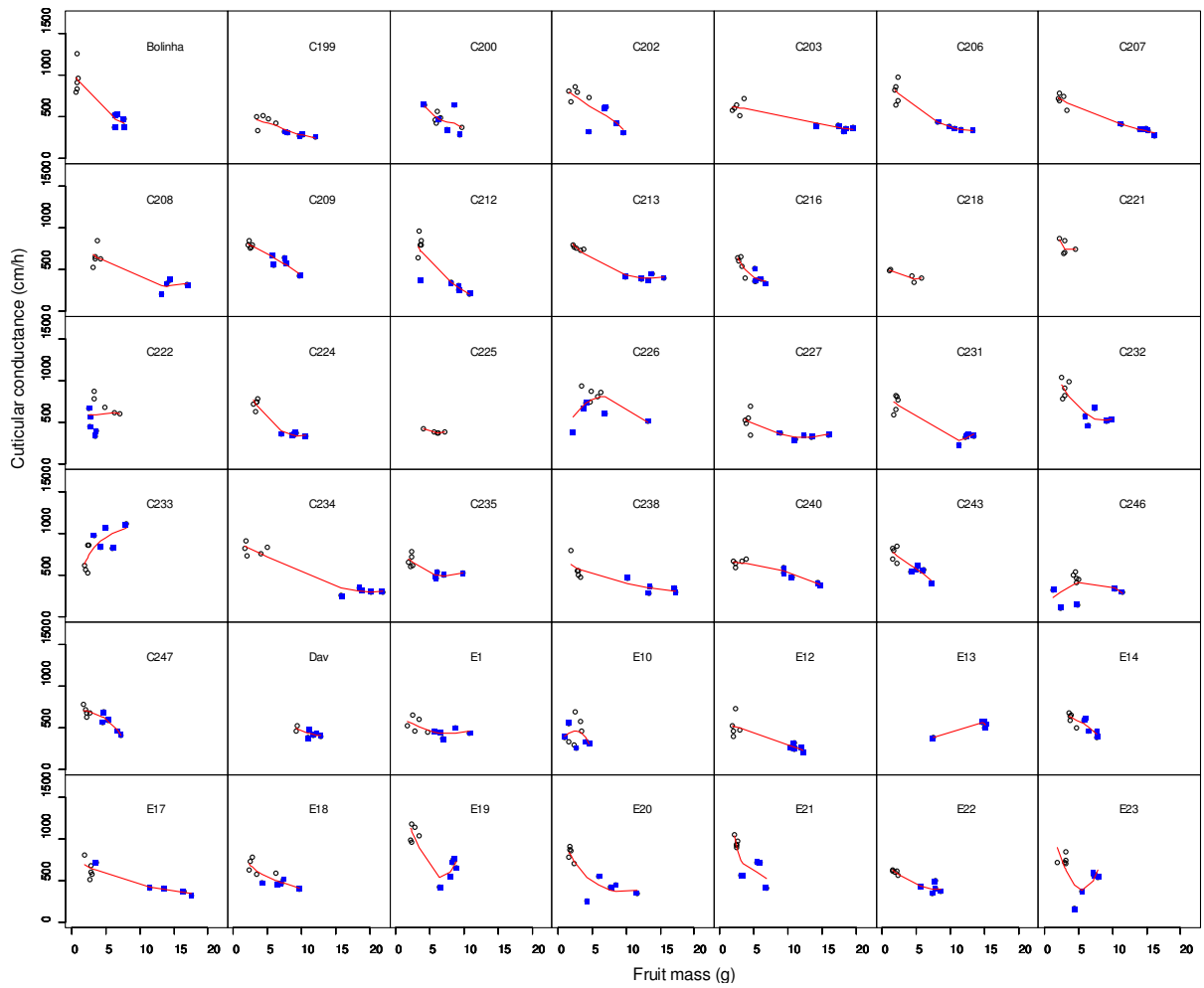


**Figure 3.** Number of stomata per zone estimated by the two methods (solid line: all stomata counted under a microscope; dashed: counting method from the pictures). a) the fruit from the genotype F169, b) the 3 fruits from the genotype C222. The estimation via pictures is a good approximation of the result obtained counting all stomata under microscope.



**Figure 4.** Estimation of the total number of stomata of a whole fruit from the average density of stomata in each single zone. The color of the dots represents the different sampling zones from the pedoncule to the style. The gray line is the total number of stomata of fruit estimated on the basis of all areas. The middle zone of the fruit (equator) gives the best approximation of the total number of fruit stomata.





**Figure 5.** Relationships between fruit mass and cuticular conductance for 42 genotypes. In black and blue, data from 1<sup>st</sup> and 2<sup>nd</sup> harvests, respectively. Red line is the smoothed line.

## ANNEXE 2

### Analysis of the diversity of infection probability of a small collection of peach cultivars in link with surface compounds at maturity

#### Introduction

The evolution of surface compounds and the differences in infection probability observed in Chapter 2 between three cultivars, Summergrand, Zephir and Magique enabled the identification of relationships between infection probability and some compounds at maturity. These relationships were explored again at maturity on a wider set of cultivars.

#### Material and Methods

We use the same methods to quantify the surface compounds as used in Chapter 2. Six genotypes were evaluated in addition to the ones studied in Chapter 2.

#### Results

There is a diversity of resistance to brown rot at maturity stage between the cultivars (Figure 1).

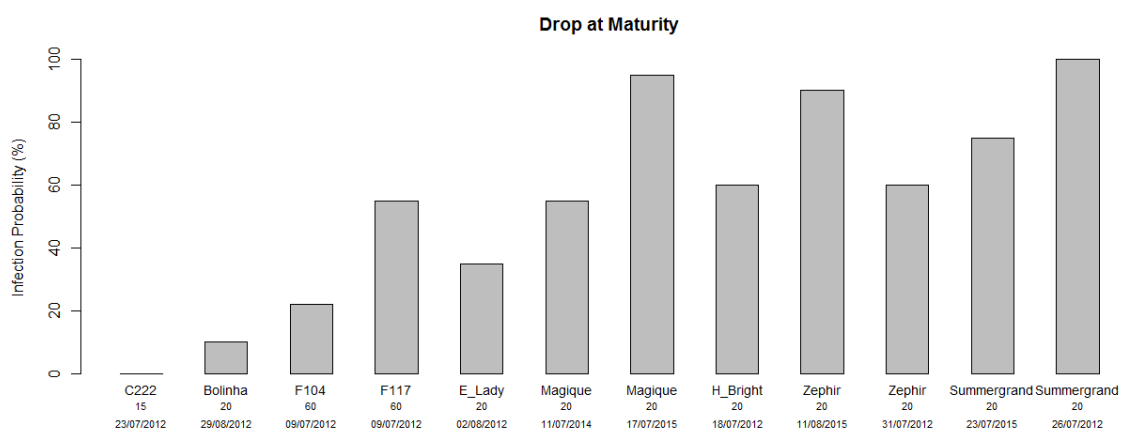


Figure 1. Infection probability of peach and nectarine cultivars at maturity stage.

No significant positive correlation was found between surface compounds and infection probability at maturity stage for the cultivars. On the contrary, significant correlations were. Concerning compounds detected by HPLC analysis, a significant correlation was observed between der1 and the infection probability (Figure 2). Concerning the analyses performed by GC, a significant effect was

observed for the group of waxes, alkanes, fatty acids, fatty alcohols, unidentified and for the total waxes. The more significant relationships were detected for the alkane group. Among the alkanes, negative correlations of 0.74, 0.76 and 0.76 were found for C23 – tricosane, C25 – pentacosane and C27 – heptacosane, respectively.

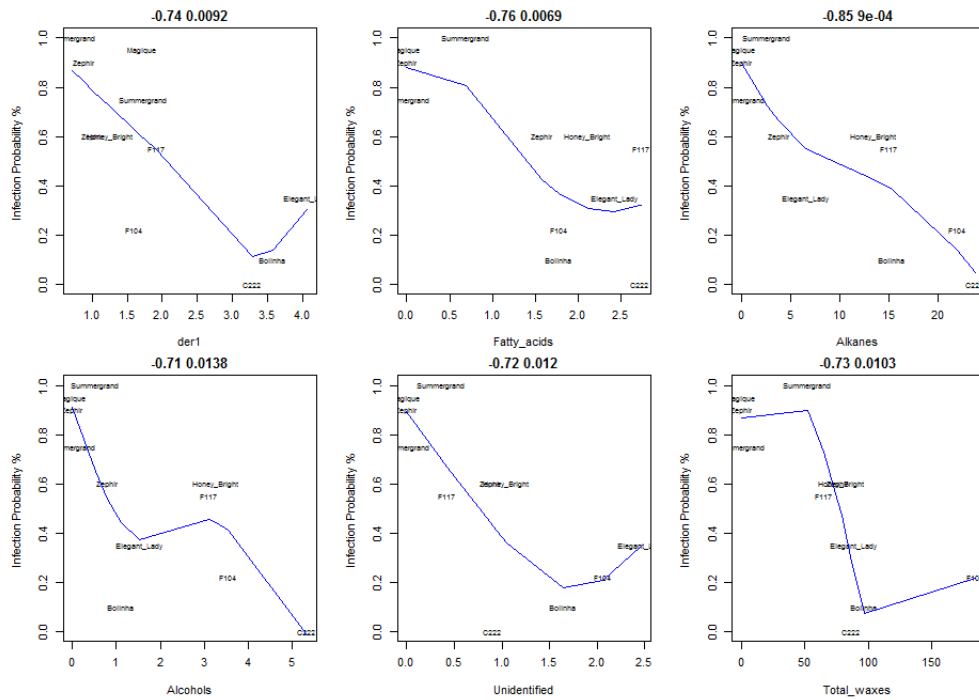


Figure 2: Diversity of response in infection probability (y-axis) and surface compounds (x-axis) in  $\mu\text{g}/\text{cm}^2$  for a group of peach cultivars.

## **Study of the genetic variability of peach in susceptibility to brown rot during fruit development in relation with changes in physical and biochemical characteristics of the fruit**

Brown rot (BR) in peach fruit caused by the fungus *Monilinia spp.* is a common disease that can provoke as much as 30 to 40% losses of crop. Currently, all cultivated peaches are more or less sensitive to BR. No other alternative than chemical treatment is available, hence fungicide applications are required until pre-harvest. Such applications are damaging the environment and may let residues in fruits. A review of literature was accomplished to compile the knowledge scattered in the literature from many years.

The aim of this study is to investigate the factors of resistance of the fruit to *M. laxa* at different stages of fruit growth and their genetic control by studying contrasted genotypes and an interspecific peach progeny.

The first focus was made on few cultivars to study the evolution of sensibility of fruits to *M. laxa* during their development in relation with structural and biochemical characteristics of the fruit, e.g. cuticular conductance, micro-cracks and fruit surface compounds. Some compounds were detected for the first time on peach fruit. The results confirmed that during the stage I immature fruits are susceptible to BR. Fruit cuticular conductance was high probably due to high density of stomata and thin cuticle in formation. In contrary, at pit hardening stage fruits were resistant, cuticular conductance was low and the levels of surface compounds exhibit a peak. When maturity approaches, fruit become susceptible again. With rapid development of the fruit during this stage, the surface compounds were diluted and micro-cracks often appear which resulted in high cuticular conductance.

At stage I we explored the different physical characteristics of the immature fruit in relation with susceptibility to *M. laxa*. A hundred of individuals of an interspecific peach progeny called BC2 were characterized through laboratory infection, monitoring of fruit transpiratory losses and estimating stomata density (only for nectarines). Unexpected symptoms (not progressing 'clear spot') were observed. The cuticular conductance was significantly linked to the likelihood of infection, but the stomata number had no effect on the likelihood of infection. QTL controlling fruit resistance to BR, cuticular conductance and stomata number have been identified and some co-locations observed.

At maturity stage we investigated the genetic control of BR resistance together with biochemical compounds of fruit epidermis. For three years, mature fruits from the BC2 progeny were infected with two modalities of infection: spray until runoff in the orchard to measure infection probability and drop in the laboratory conditions in order to observe the characters of beginning, progression and speed of infection. The BC2 progeny displayed high variability for BR resistance. Despite low stability between years, genotypes with high level of resistance were identified. In addition in 2015, we explored the variation in epidermis compounds of fruit within the BC2 progeny. Phenolic compounds, terpenoids and derivatives were quantified by HPLC. The relationship between BR resistance and presence and/or levels of certain epidermis compounds and the genetic control of these compounds were investigated.

BR of peach fruit is a complex problem which is still far from resolved. Progress has been made in the knowledge of structural and biochemical characteristics involved in BR resistance and regions of the genome that could confer certain disease tolerance have been detected. Further work is needed to develop molecular markers for marker assisted selection. The results obtained suggest that solutions for the future lie in associations of tolerant cultivars \_ less susceptible to micro-cracks and with high content of epidermis compounds potential inhibitor of the fungus development \_ with cultural practices reducing both risks of fruit cracking and occurrence of micro-climatic conditions favorable to BR spread and sporulation.

## **Etude de la variabilité génétique de la sensibilité à la pourriture brune au cours du développement du fruit chez la pêche en lien avec l'évolution des caractéristiques biochimiques du fruit**

La pourriture brune des fruits (BR), causée par les champignons du genre *Monilinia*, est une maladie courante qui peut provoquer jusqu'à 30 à 40% de pertes de récolte chez la pêche. Actuellement, toutes les pêches cultivées sont plus ou moins sensibles à la moniliose. Aucune alternative aux traitements chimiques n'est disponible à ce jour, ce qui rend nécessaire les applications de fongicides jusqu'au stade pré-récolte. Ces applications sont préjudiciables pour l'environnement et peuvent laisser des résidus sur les fruits. Nous avons réalisé une revue de la littérature pour compiler les connaissances disponibles sur le couple pêcheur-monilioses.

Le but de cette étude est d'étudier les facteurs de résistance du fruit à *M. laxa* à différents stades de croissance des fruits chez la pêche et de déterminer leur contrôle génétique par l'étude de génotypes contrastés et d'une descendance interspécifique.

Nous avons tout d'abord focalisé notre étude sur quelques cultivars pour étudier l'évolution de la sensibilité des fruits à *M. laxa* au cours de leur développement en relation avec les caractéristiques structurales et biochimiques des fruits, par exemple la conductance cuticulaire du fruit, les micro-fissures de l'épiderme et des composés de surface des fruits. Certains composés ont été détectés pour la première fois chez la pêche. Les résultats ont confirmé que lors de la phase I les fruits immatures sont sensibles à la moniliose. La conductance cuticulaire des fruits était élevée probablement parce que la densité de stomates est forte et la cuticule en formation est mince. En revanche, au stade de durcissement du noyau, les fruits étaient résistants. A ce stade la conductance cuticulaire était faible et les niveaux de composés de surface présentaient un pic de teneurs. A l'approche de la maturité, les fruits sont devenus sensibles de nouveau. Avec le développement rapide du fruit au cours de cette étape, les composés de surface ont été dilués et des micro-fissures sont sans doute apparues entraînant une augmentation de la conductance cuticulaire.

Au stade I, nous avons exploré les différentes caractéristiques physiques du fruit immature en relation avec la sensibilité à *M. laxa*. Une centaine de génotypes d'une descendance interspécifique de pêcheurs appelée BC2 a été caractérisée par une infection au laboratoire, un suivi de pertes transpiratoires des fruits et une estimation de la densité de stomates (uniquement pour les nectarines). Des symptômes inattendus (une 'tache claire' qui ne progresse pas) ont été observés. La conductance cuticulaire était significativement liée à la probabilité d'infection, par contre le nombre de stomates n'a montré aucun effet sur la probabilité d'infection. Des QTL contrôlant la résistance des fruits à la moniliose, à la conductance cuticulaire et au nombre de stomates ont été identifiés et des co-localisations observées.

A la maturité, nous avons étudié le contrôle génétique de la résistance à la moniliose conjointement avec des composés biochimiques de l'épiderme des fruits. Pendant trois ans, les fruits de la descendance BC2 ont été infectés avec deux modalités d'infection artificielle avec une suspension de spores de champignon: une pulvérisation au verger pour mesurer la probabilité d'infection et un dépôt d'une goutte dans des conditions contrôlées en laboratoire afin d'estimer le délai d'apparition des symptômes, la progression de la lésion et la vitesse de progression de la lésion. La descendance de BC2 a affiché une forte variabilité de résistance à la moniliose. Malgré une faible stabilité entre les années, des génotypes à haut niveau de résistance ont été identifiés. De plus en 2015, nous avons exploré la variation des composés de l'épiderme des fruits au sein de la descendance de BC2. Les composés phénoliques, les terpènes et dérivés ont été quantifiés par HPLC. La relation entre la résistance à la moniliose et la présence et / ou les niveaux de certains composés de l'épiderme et le contrôle génétique de ces composés ont été étudiés.

La moniliose des fruits de pêche est un problème complexe qui est encore loin d'être résolu. Des progrès ont été accomplis dans la connaissance des caractéristiques structurales et biochimiques impliquées dans la résistance et des régions du génome qui pourraient conférer une certaine tolérance à la maladie ont été détectées. Des travaux supplémentaires sont nécessaires pour développer des marqueurs moléculaires pour la sélection assistée par marqueurs. Les résultats obtenus suggèrent que des solutions pour l'avenir résident dans l'association de cultivars tolérants \_ moins sensibles aux micro-fissures et à haute teneur en composés épidermiques potentiellement inhibiteurs du développement du champignon \_ avec des pratiques culturales réduisant les risques de fissuration des fruits et d'apparition de conditions climatiques favorables à la propagation de la moniliose.