Diversité et adaptation aux fongicides des populations de Botrytis cinerea, agent de la pourriture grise

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TABLE DES MATIÈRES

Contexte général 6

Introduction générale 11

1. La diversité, base de l'évolution 11

2. Sélection naturelle et adaptation 13

2.1 Définitions 13

2.2 La sélection favorise l'adaptation locale 15

2.3 Les modes de sélection 15

2.4 Interaction entre sélection et dérive génétique 17

2.5 Interaction entre sélection et mutation 18

2.6 Interaction entre sélection et flux de gènes 19

2.7 Détecter et mesurer la sélection 22

3. Spéciation chez les champignons 25

3.1 Définitions 25

3.2 Critères d'espèce 25

3.3 Mécanismes d'isolement reproductif 27

3.4 Modes de spéciation 29

4. Modèle d'étude : *Botrytis cinerea*, un champignon pathogène polyphage 32

4.1 Nomenclature et systématique 32

4.2 Biologie et diversité de *Botrytis cinerea* 35

4.3 Lutte contre la pourriture grise 37

4.4 Résistance aux fongicides chez *Botrytis cinerea* 38

4.5 Mode d'action et résistance aux SDHIs 41

4.6 La résistance multidrogues chez *Botrytis cinerea* 42

5. Problématique et objectifs de la thèse 45

Chapitre I : Diversité et structuration des populations de pourriture grise 48

Article 1 : Etablissement de la nouvelle espèce *Botrytis pseudocinerea* 49

Article 2 : Structure et diversité des populations françaises de *Botrytis cinerea* 51

Chapitre II : Adaptation aux fongicides chez *Botrytis cinerea* 94

Article 3: Caractérisation des mécanismes de résistance aux inhibiteurs respiratoires chez *Botrytis cinerea*. 95

Article 4: Statut de la résistance aux anti-*Botrytis* au vignoble ou comment l'expérience française peut contribuer à améliorer les stratégies de lutte contre la pourriture grise 108

Article 5 : Signature de sélection et structure des populations de *Botrytis cinerea* recevant des applications de fongicides 110

Discussion générale 150

1. De grandes populations, diverses mais structurées 150
2. *Botrytis* cinerea, un modèle d'adaptation 154
3. De l'évolution à la protection des plantes : implications en pratique 157

Conclusions 159

Références 160

« *LA DECOUVERTE EST UN PLAISIR AUSSI SUBTIL ET INTERESSANT QUE LA CONNAISSANCE* »

Jacques Lamarche, Eurydice (1971)

CONTEXTE GÉNÉRAL

Les maladies des plantes sont à l'origine de pertes importantes en agriculture, tant quantitatives (pertes de rendements à la récolte ou au court du stockage) que qualitatives (production de toxines fongiques, d'arômes ou d'odeurs indésirables) (Oerke 2006). Dans les pays industrialisés, ces pertes s'élèvent à près de 40% et concernent toutes les étapes de la chaîne alimentaire. Leur niveau est bien plus élevé encore (50%) dans les pays en développement (Lepoivre 2007). Parmi les nombreux exemples de maladies ayant des conséquences historiques, on peut citer l'exemple de l'ergot du seigle, *Claviceps purpurea*, qui, au début du XVII^{ème} siècle, décima les armées du Tsar de Russie après qu'elles eurent consommé des farines de seigle infectées, permettant ainsi à l'empire ottoman de perdurer 200 ans encore. Un autre exemple est celui de l'introduction en Irlande de l'agent du mildiou de la pomme de terre *Phytophthora infestans*, qui, ravageant les récoltes, affama la population au point de provoquer une émigration historique vers le Nouveau Monde (Bain *et al.* 2010).

Figure 1 : L' « Angélus », par Millet (achevé le 6 décembre 1859), Paris, Musée d'Orsay.

L'auteur avait initialement intitulé sa toile « La mauvaise récolte de pommes de terre », montrant ainsi combien les esprits furent frappés à l'époque par les ravages exercés en Europe par le mildiou de la pomme de terre.

Plus récemment, un sondage à l'adresse de la communauté scientifique (Dean *et al.* 2012) a listé les agents de la pyriculariose du riz (*Magnaporthe grisea*) et de la pourriture grise *(Botrytis cinerea*) comme les deux espèces les plus dommageables pour l'agriculture actuelle, et de ce fait, présentant un intérêt scientifique majeur (Tableau 1). Enfin, en plus de cet intérêt fondamental pour la production alimentaire humaine, les champignons pathogènes peuvent également constituer une menace pour la biodiversité (Desprez-Loustau *et al.* 2007), par exemple en éliminant des espèces d'hôtes sensibles, à l'instar de *Cryphonectria parasitica* qui a causé la disparition quasi-totale du châtaignier en Amérique du Nord, à la fin du XIX^{ème} siècle (Milgroom *et al.* 1996).

1.	*Magnaporthe oryzae* (pyriculariose du riz)
2.	*Botrytis cinerea* (pourriture grise)
3.	*Puccinia* spp. (rouilles des céréales)
4.	*Fusarium graminearum* (fusariose des céréales)
5.	*Fusarium oxysporum* (fusariose des *Cucurbitaceae*)
6.	*Blumeria graminis* (oïdium des céréales)
7.	*Mycosphaerella graminicola* (septoriose du blé)
8.	*Colletotrichum* spp. (anthracnose)
9.	*Ustilago maydis* (charbon du maïs)
10.	*Melampsora lini* (rouille du lin)

Tableau 1 : Liste des 10 champignons pathogènes d'intérêt agronomique et scientifique (Dean *et al.* 2012)

Depuis les débuts de l'agriculture, l'homme utilise divers moyens pour protéger les plantes cultivées de leurs bioagresseurs. Les premières mentions, mal référencées, de l'activité biocide de l'arsenic et du soufre seraient évoquées par Homère et par Pline l'Ancien. En France, la lutte chimique se démocratise peu à peu à partir de la seconde moitié du XIX^{ème} siècle (notamment avec la découverte de la bouillie bordelaise) et prend véritablement son essor après la seconde guerre mondiale, lors de la montée en puissance de la chimie organique de synthèse moderne, contribuant ainsi à augmenter la quantité et la qualité des récoltes et à accomplir la « révolution verte » (Figure 2). Les conséquences souvent délétères des pesticides pour la santé humaine et pour l'environnement (par exemple, pollution importante des écosystèmes et problèmes de santé publique causés par le chlordécone, aux Antilles (Dallaire *et al.* 2012) ou l'intoxication chronique supposée des ruchers français par les insecticides néonicotinoïdes (Henry *et al.* 2012)) s'accompagnent également de problèmes de durabilité de leur efficacité. Ainsi, pour les fongicides, leur usage intensif a rapidement conduit à la sélection des souches résistantes (le premier cas rapporté à la fin des années 70s est celui de la résistance aux benzimidazoles, premiers fongicides de synthèse à cible biochimique unique, chez de nombreux champignons phytopathogènes). Cette résistance est d'autant plus facilitée, chez les champignons, que ces organismes possèdent des caractéristiques biologiques favorisant leur adaptation aux contraintes anthropiques (Stukenbrock *et al.* 2008) comme les fongicides : régimes de reproduction mixtes, grandes tailles de populations, grandes capacités de dispersion, temps de génération courts, contribuant ainsi à généraliser les résistances sur de larges territoires. D'un point de vue théorique, l'évolution des pathogènes aux fongicides constitue un cas d'école de sélection naturelle, observable *in natura* et sur une échelle temporelle humaine, voire parfois très courte (par exemple : chez *Zymoseptoria tritici*, l'agent de la septoriose du blé, la résistance aux strobilurines a été sélectionnée en seulement 4 ans ; Torriani *et al.* 2009). Elle s'oppose ainsi à l'évolution lente et graduelle la plus communément observée face aux pressions de sélection habituellement plus faibles auxquelles font face les organismes *in natura* (Fisher 1930). Il est donc crucial de comprendre et de quantifier les mécanismes évolutifs qui sous-tendent la sélection de la résistance aux fongicides pour pouvoir éventuellement la limiter.

Figure 2 : Sulfatage des vignes dans les années 1940, montrant la faiblesse des protections individuelles lors de l'utilisation de produits toxiques pour la santé humaine (http://www.choko.fr/spip.php?rubrique22).

La femme française, de l'entre-deux guerres aux années récentes, contribue activement à l'essor de la protection des plantes ;-).

Un autre intérêt majeur à ce type d'étude réside dans le fait que les considérations environnementales sont désormais devenues prégnantes, rendant les enjeux de la protection des plantes bien différents de ceux de l'après-guerre. En 2007, le gouvernement français a mis en place le « Plan Ecophyto » (http://agriculture.gouv.fr/ecophyto-2018,510), comme l'une des actions du Grenelle de l'environnement. Ce plan préconise de « réduire de 50% l'usage des pesticides, si possible », en l'espace de 10 ans. De manière concomitante, la communauté européenne, par sa Directive 2009/128CE sur les pesticides, a durci les critères de toxicité et d'écotoxicité, réduisant ainsi l'accès à l'homologation de nouveaux mais également d'anciens produits phytosanitaires. Enfin, le 18 décembre 2012, le gouvernement français a présenté son « plan agroécologie » (http://agriculture.gouv.fr/Conference-nationale-Stephane-Le), visant à prolonger le « Plan Ecophyto ». Dans ce contexte, l'enjeu devient donc la mise au point des stratégies de lutte efficaces, plus respectueuses de l'environnement et assurant un revenu suffisant aux agriculteurs. Les pesticides, et les fongicides en particulier, doivent être utilisés en moins grande quantité, mais dans des conditions qui maximisent leur efficacité et leur durabilité. Or, ces décisions réglementaires récentes peuvent induire une mutation vers des pratiques à plus fort risque de résistance (doses réduites, applications fractionnées, perte de diversité des modes d'action restant homologués). L'analyse du risque résistance de ces nouvelles pratiques prend alors une dimension décisive pour la durabilité de la lutte chimique. De manière opérationnelle et standardisée, ce risque est actuellement raisonné d'après sa décomposition en trois composantes, elles-mêmes découpées en classes correspondant aux différents niveaux de risque, et évaluées de manière théorique et empirique (Tableau 2). Enfin, la réalité du terrain a récemment démontré que l'évaluation du risque, telle qu'elle est menée actuellement, n'est pas totalement prédictive, puisque certaines résistances ont été détectées plus rapidement qu'attendu. Dans ce contexte, les études évolutives peuvent apporter des informations précieuses pour (1) améliorer la prédiction du risque résistance (notamment, en aidant à mieux comprendre quels traits biologiques de l'espèce fongique sont les plus favorables à l'apparition rapide de résistance) ; (2) mieux organiser la surveillance de l'évolution des résistances (en ciblant les périodes-clés pour l'observation, et en optimisant les effectifs et fréquences de prélèvement), telle qu'elle est décrite dans l'axe 5 du plan Ecophyto ; (3) concevoir et adapter des programmes de traitement à faible pression sélective (par exemple en utilisant des coûts sélectifs, ou fitness, associés aux résistances, ou en utilisant à bon escient les différentes intensités de sélection exercées par les différents modes d'action) ; et enfin (4) améliorer l'efficacité des méthodes de lutte complémentaires, par exemple la prophylaxie (en manipulant de façon optimale les flux de gènes naturels et les réservoirs de résistance).

C'est précisément dans ce contexte de prédiction et de gestion de la résistance aux pesticides que s'inscrit cette thèse. Le modèle biologique choisi, le champignon *Botrytis cinerea* (l'agent de la pourriture grise), représente un problème phytosanitaire majeur pour

Fungicide risk		Combined risk			Agronomic risk
Benzimidazoles Dicarboximides Phenylamides QoI	High=3	3 1.5 0.75	6 3 1.5	9 4.5 2.25	High=1 Med=0.5 Low=0.25
SDHIs SBI Anilinopyrimidines Phenylpyrroles	Med=2	2 1 0.5	4 2 1	6 3 1.5	High=1 Med=0.5 Low=0.25
Multisite MBI-R inhibitors SAR inducers	Low=0.5	0.5 0.25 0.125	1 0.5 0.25	1.5 0.75 0.3	High=1 Med=0.5 Low=0.25
		Low=1	Med=2	High=3	**Pathogen risk**
		Seed borne Soil borne Rust fungi	Eyespot *Rhynchosporium* *Septoria*	*Blumeria* *Botrytis* *Plasmopara* *Magnaporthe* *Venturia*	

Tableau 2 : Grille d'évaluation du risque résistance (Kuck *et al.* 2006).

La composante biologique prend en compte le risque intrinsèque lié à la biologie du pathogène (durée des cycles, dissémination des spores, résistance détectée à d'autres fongicides). La composante chimique évalue le risque de résistance inhérent à un mode d'action (une ou plusieurs cibles biochimiques, activité intrinsèque, rapidité d'acquisition des résistances connues). La composante agronomique prend en compte l'exposition du pathogène par le fongicide dans le système de culture (durée des rotations, contexte pédo-climatique, diversité des modes d'actions utilisés sur la culture…).

la culture de la vigne. De par ses contraintes climatiques et économiques, le vignoble champenois reçoit, par rapport aux autres vignobles français, les programmes de traitement anti-*Botrytis* les plus chargés, conduisant régulièrement à l'émergence des résistances chez l'agent pathogène, qui sont de fait principalement observées dans cette région. La surveillance ancienne de l'évolution des résistances, menée conjointement par le Comité Interprofessionnel des Vins de Champagne (CIVC) et l'INRA, ainsi que la publication annuelle de recommandations de limitation d'usage des principales familles d'anti-*Botrytis*, ont permis jusqu'à présent de maintenir un niveau d'efficacité satisfaisant pour l'ensemble des molécules. Cependant, l'émergence de la résistance aux SDHIs (inhibiteurs de la succinate déshydrogénase ; classés avec un risque moyen mais ayant cependant généré très rapidement des résistances en pratique pour d'autres pathogènes dans le monde), et la progression constante des souches multidrogues résistantes (MDR), sont suffisamment inquiétantes pour mériter une attention particulière de la part de la communauté scientifique. Ces résistances, et plus généralement, les résistances déjà installées au vignoble, procurent l'opportunité d'étudier les déterminants de leur sélection, tant au niveau individuel que populationnel, et de leur dissémination dans les populations. Cette recherche nécessitait en premier lieu de caractériser finement la structure et la diversité des populations. L'ensemble de ces travaux est présenté dans ce mémoire de thèse.

INTRODUCTION GENERALE

Montagne de Beaune, Côte d'Or

INTRODUCTION GÉNÉRALE

1. LA DIVERSITÉ, BASE DE L'ÉVOLUTION

La diversité désigne le degré de variation d'un groupe ou d'un ensemble d'objets. En biologie des populations, le terme diversité regroupe la pluralité des individus, distingués entre eux par la variation d'un caractère phénotypique (basé sur l'examen d'une caractéristique morphologique, physiologique, biochimique ou comportementale) ou génotypique (basé sur l'examen d'un ou plusieurs loci génétiques combinés).

Ainsi, dans « L'origine des espèces » (Darwin 1859), Darwin conclut, soutenu depuis par les évolutionnistes modernes, que l'évolution repose sur la variation des caractéristiques des organismes, s'appliquant à plusieurs échelles : (1) sur les différences entre individus au sein d'une population, et/ou (2) entre les populations et les espèces. L'évolution devient alors un processus en deux étapes, où la variation émerge au niveau individuel puis se transmet dans les populations de génération en génération, dans des proportions variables. Ainsi, caractériser et quantifier la variation génétique, identifier les sources de variation et les facteurs qui l'influencent constituent des enjeux majeurs de la biologie évolutive.

A l'échelle de la population, les changements de fréquences des différents variants, caractérisés par leurs allèles aux loci considérés, reflètent les processus évolutifs opérant dans un environnement donné. Le maintien des fréquences alléliques selon des proportions prédites mathématiquement par les lois de Mendel dans le cas idéal de l'équilibre de Hardy-Weinberg nécessite que (1) les gamètes se rencontrent de manière aléatoire, (2) la population soit de taille infinie, (3) le nombre d'allèles reste constant, (4) il n'y ait pas d'échange d'individus avec d'autres populations et (5) tous les individus produisent le même nombre de descendants. Autrement dit, toute violation d'une de ces hypothèses modifiera les fréquences alléliques au sein de la population. Les quatre grandes forces évolutives qui peuvent générer un écart à ces hypothèses sont:

- la dérive génétique, qui induit une évolution stochastique de la fréquence d'un allèle dans une population, pouvant entrainer la perte d'allèles par le simple effet du hasard,
- la mutation, qui correspond à une erreur dans la reproduction du message héréditaire (par exemple lors de la réplication ou par des crossing-over entre les bras chromosomiques), générant ainsi de nouveaux allèles,
- la migration, qui correspond à l'échange d'individus entre populations (ou flux de gènes) et donc à l'introduction de nouveaux allèles,
- la sélection, qui favorise ou défavorise un allèle dans un environnement donné selon sa valeur sélective (ou fitness, c'est à dire le nombre moyen de descendants laissés par l'individu portant cet allèle ; voir 2.2), modifiant ainsi les fréquences alléliques.

En plus de ces quatre forces évolutives, le mode de reproduction peut également modifier les probabilités de rencontre entre gamètes. Il n'existe aucune situation naturelle où l'un ou plusieurs, de ces facteurs, ne jouent un rôle C'est leur combinaison qui conditionne la trajectoire évolutive d'une population donnée (Figure 3).

De manière générale, la diversité génétique est donc générée par l'effet de (1) la mutation, qui crée de nouveaux allèles et (2) par la recombinaison génétique, causée par la sexualité ou la parasexualité, qui crée de nouvelles combinaisons d'allèles mais elle est également amplifiée par (3) la sélection naturelle diversifiante ou balancée (voir 2.3), qui favorisent le maintien de plusieurs allèles dans un environnement donné. A l'opposé, la diversité génétique sera diminuée sous l'effet de (1) la dérive génétique, qui fixe les allèles d'autant plus rapidement que la taille de la population est réduite, (2) la sélection négative, associée à des allèles délétères et (3) l'endogamie et l'inbreeding (consanguinité), qui réduisent la diversité des rencontres entre gamètes ou plus généralement, par tout mode de reproduction clonal. L'effet de la migration est variable selon l'échelle considérée : l'apport de nouveaux migrants au sein d'une population contribue à accroître sa diversité, alors que l'échange de migrants entre deux populations tend à homogénéiser leurs diversités respectives.

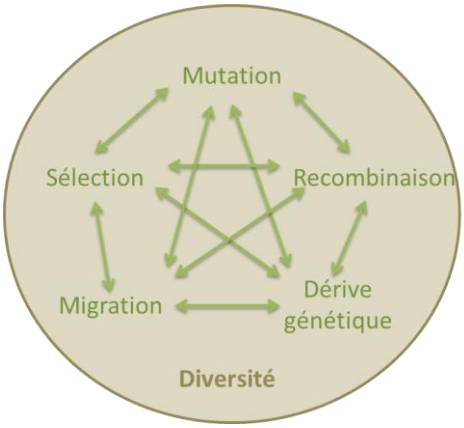

Figure 3 : Différents processus évolutifs intervenant dans le façonnement des populations.

Ainsi, l'action combinée des différentes forces évolutives modifie-t-elle les proportions des différents variants alléliques dans les populations, infléchissant leur trajectoire évolutive. L'action des différentes forces éloigne les populations de l'équilibre idéal de Hardy-Weinberg. L'analyse de cet écart à l'équilibre permet dans certains cas d'inférer les forces en jeu et d'estimer leur intensité. Dans le cas particulier où deux populations s'adaptent à des environnements écologiquement distincts, sous l'effet fort de la sélection, ces populations peuvent diverger au point de ne plus pouvoir se croiser (isolement reproducteur), conduisant à la différentiation de deux espèces.

Cette rapide synthèse des bases de la génétique des populations présente finalement les principales sources de variations entre individus au sein des populations et entre les populations et les espèces. La section suivante détaillera plus particulièrement comment la sélection naturelle crée et maintient du polymorphisme dans les populations, en interaction avec les autres forces évolutives.

2. Sélection naturelle et adaptation

2.1 Définitions

La sélection naturelle a été formalisée par Darwin en 1859 (Darwin 1859). Depuis, plusieurs définitions ont été proposées. La proposition de S. Wright, l'un des fondateurs de la génétique des populations, est une définition par défaut : « tout processus qui, au sein d'une population, modifie la fréquence des gènes de manière directionnelle, sans changement du matériel génétique (mutation) ou l'introduction (d'individus) depuis l'extérieur (immigration) » (Wright 1969). John Endler définit la sélection comme un « processus lors duquel, si une population montre une variation d'un caractère héritable, consécutive à la relation entre ce trait et sa fitness (voir 2.2), alors la fréquence de cette variation doit varier entre les classes d'âge et peut différer entre les générations » (Endler 1986). Cette définition plus moderne établit d'une part les différences entre phénotypes (leur fitness), et leur déterminisme héréditaire, et d'autre part leurs conséquences (en réponse à la sélection), sur l'évolution des populations. Autrement dit, la sélection naturelle correspondrait à toute différence remarquable de fitness entre des entités biologiques différentes, pouvant plus largement correspondre à des gènes, groupes de gènes, individus, populations ou taxa supérieurs. Il s'agit d'un processus à deux étapes, la première consistant dans la production de variation héritable, et la seconde dans le test de cette variation *via* les filtres sélectifs (Mayr 1984).

Ces définitions font par ailleurs intervenir la notion fondamentale de fitness absolue, correspondant à la valeur sélective d'un génotype, *i.e.* au nombre moyen de descendants par unité de temps qu'il laisse dans la population pour les générations à venir (Haldane 1924 ; Antonovics *et al.* 1986 ; Orr 2009). Cette définition reflète directement la capacité d'un organisme à survivre et à se reproduire dans son environnement. En pratique, dans une espèce à générations séparées (non chevauchantes), la valeur sélective est définie sur un cycle, de zygote à zygote (Gillespie 1998), ou d'adulte à adulte (Rousset 2004). La fitness absolue d'un génotype donné est alors le produit de la probabilité de survie de cet individu du stade zygote au stade adulte (phase zygotique), par l'espérance du nombre du zygotes produits par cet individu à l'âge adulte (phase gamétique) (Christiansen 1984; Olivieri 2010)(Figure 4). De manière opérationnelle, la mesure des composantes de fitness chez les champignons peut être

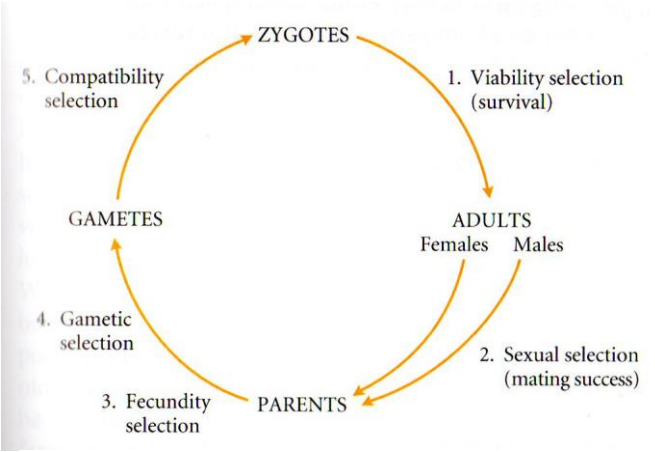

Figure 4 : Représentation simplifiée des composantes du cycle de vie d'un organisme pouvant être affectée pour leur fitness, soit dans la phase zygotique (1 à 3), soit dans la phase gamétique (4 à 5). D'après Christiansen 1984.

complexe et le choix des critères doit tenir compte de l'écologie de l'espèce, et en particulier du nombre de fois pendant son cycle de vie où il se reproduit (Pringle *et al.* 2002). La fitness relative d'un individu est sa fitness absolue normalisée par la fitness absolue de l'individu ayant la meilleure valeur sélective (ayant donc par définition une fitness relative valant 1) (Orr 2009). Enfin, l'ensemble des mutations génétiques défavorables dans une population, portées par les individus de moindre valeur sélective est appelée fardeau génétique (genetic load ; Muller 1950). Le fardeau génétique pointe un déficit d'adaptation dans une population. Il se mesure par l'écart relatif entre la fitness optimale d'une population dont tous les individus auraient le meilleur génotype possible et la valeur sélective réelle de la population. Le fardeau génétique a trois composantes : le fardeau de mutation (apparition de mutations délétères chez les individus), le fardeau de substitution (polymorphisme génétique transitoire qui se crée le temps qu'un allèle en remplace un autre) et le fardeau de ségrégation (maintien d'un polymorphisme génétique stable au cours du temps, du fait que sa ségrégation mendélienne, à chaque génération, fabrique des individus de valeur sélective moindre en faisant reparaître le génotype le moins optimal).

La sélection naturelle repose donc sur l'héritabilité des variations de la fitness (et en particulier de ses composantes). Dans une population soumise à sélection, la fitness moyenne (la moyenne des fitness absolues des individus) peut augmenter parce que les variances de fitness des allèles portés par les différents individus sont additives : c'est le théorème fondamental de Fisher sur la sélection naturelle (Fisher 1930 ; Figure 5), qui définit également un outil de mesure de la sélection :

$$\Delta\overline{W} = \frac{var_A\ (W)}{\overline{W}}$$

avec $\Delta\overline{W}$ correspondant à l'augmentation de la fitness moyenne due à la sélection naturelle, $var_A\ (W)$ correspondant à la variance génétique additive de la fitness, divisée par la moyenne de la fitness avant sélection $\overline{W}$.

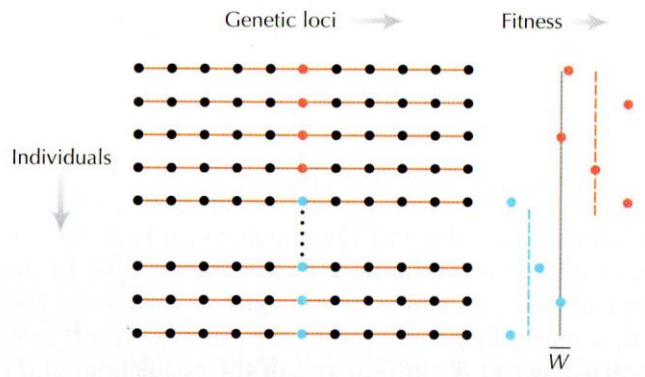

Figure 5 : Illustration du théorème de Fisher.

Chaque allèle au sein du génome a sa propre fitness (faible, en bleu, ou forte, en rouge), avec une variabilité inter-individuelle. C'est la fitness moyenne de chaque allèle qui détermine la variation de sa fréquence dans la population et qui contribue à modifier la variance génétique de la fitness, par son additivité. D'après Barton *et al*, 2007.

2.2 La sélection favorise l'adaptation locale

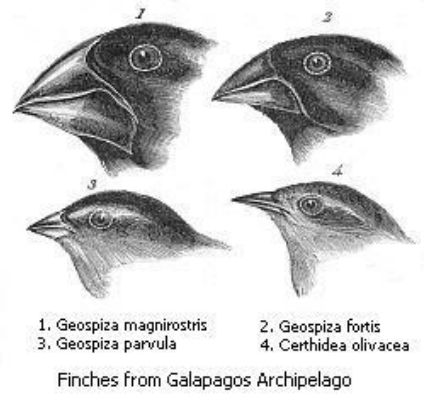

Figure 6 : Planche ornithologique illustrant quelques une des espèces de pinsons ramenées par C. Darwin lors de son expédition aux Galapagos sur le Beagle.

La forme du bec est adaptée au régime alimentaire de chaque espèce (http://fr.wikipedia.org/wiki/Pinson_de_Darwin).

En biologie évolutive, le terme adaptation fait référence soit au processus qui permet à un individu d'être adapté à un environnement donné, étant acquis que toute adaptation ne peut survenir sans variation génétique, soit au trait (phénotype) acquis par l'organisme et qui améliore son succès reproducteur (Futuyma 1998). En d'autres termes, le phénomène d'adaptation permet au trait phénotypique qui procure la meilleure fitness d'augmenter en fréquence dans un environnement donné. L'adaptation du bec des pinsons dits de Darwin (Figure 6) constitue l'un des exemples les plus célèbres (Darwin 1859). Plus récemment, au XIX^{ème} siècle, la dominance de la forme mélanisée de la phalène du bouleau, *Biston betularia*, a été expliquée par l'adaptation de cette espèce au changement de couleur des troncs de son hôte du fait de l'augmentation des activités industrielles, le phénotype sombre étant moins facilement prédaté (Cook 2000). L'adaptation peut donc se manifester de diverses manières, mais tous les traits ne résultent pas forcément d'une adaptation. Les méthodes permettant d'identifier et de caractériser des adaptations incluent généralement des études portant sur la forme et la fonction (d'un organe, par exemple), sur la relation entre fitness et variations au sein d'une espèce ou enfin sur la comparaison de traits entre les espèces et l'établissement de corrélations avec les caractéristiques de son environnement (Williams 1966).

2.3 Les modes de sélection

Lorsqu'il n'existe pas de relation entre phénotype et valeur sélective, on parlera de neutralité sélective. Cependant, le plus souvent, la relation entre phénotype et fitness peut être décrite d'une des manières suivantes (Barton *et al.* 2007 ; Kingsolver *et al.* 2007 ; Huneman 2009 ; Hurst 2009 ; Olivieri 2010 ; Figure 7).

La sélection est **directionnelle** (ou darwinienne, ou positive) si c'est toujours le même phénotype (le mieux adapté) qui est sélectionné (exemple : cas de la phalène du bouleau). Dans le cas particulier où la sélection directionnelle permet d'éliminer des allèles délétères, on parle de sélection purifiante (ou négative).

La sélection est **stabilisante** (ou normalisante) si un phénotype intermédiaire, ajusté au milieu, est avantagé par la sélection naturelle alors que les variants les plus

éloignés sont éliminés (exemple : nombre optimal d'œufs pour une ponte, permettant d'optimiser les ressources alimentaires). La sélection directionnelle agit donc sur la moyenne d'un trait, alors que la sélection stabilisante agit sur la variance, sans en changer la moyenne.

La sélection est **disruptive** (ou diversifiante) lorsqu'elle correspond au cas où la relation entre le phénotype et la valeur sélective n'est pas unimodale. Ce mode de sélection élimine les phénotypes intermédiaires (exemple : cas de prédateurs se spécialisant sur un nombre limité de proies).

La sélection **balancée** maintient du polymorphisme et peut correspondre à plusieurs processus évolutifs (voir 2.5). Dans ce cas, un allèle n'est jamais fixé ; il peut subir une sélection fréquence-dépendante (la valeur sélective d'un allèle est inversement proportionnelle à sa fréquence : il subit une sélection positive lorsqu'il est rare, puis une sélection négative lorsque sa fréquence devient trop élevée), de sorte qu'on ne peut le classer comme délétère ou avantageux (exemple de l'autoincompatibilité chez les plantes à fleurs ou du complexe majeur d'histocompatibilité des vertébrés).

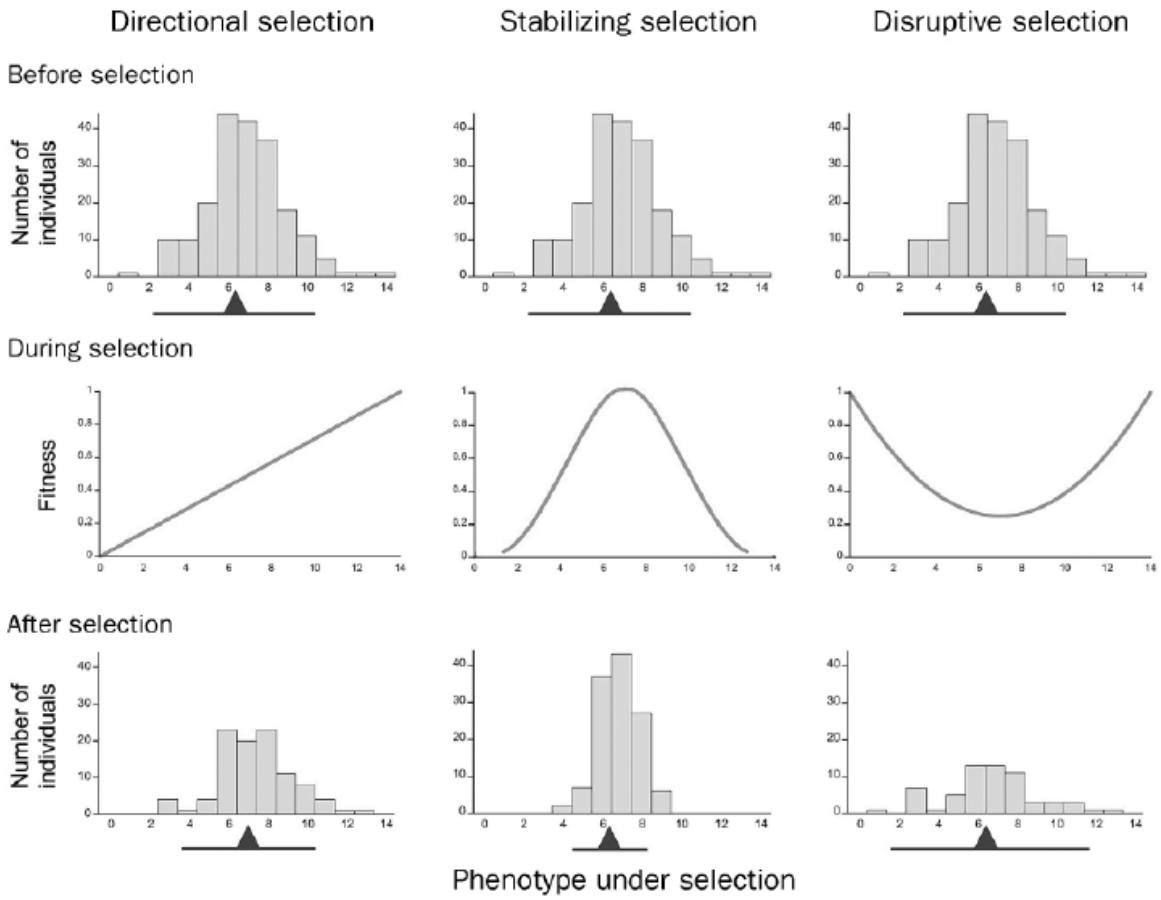

Figure 7 : Mode de sélection pour un caractère héritable, montrant la distribution du trait dans une population avant la sélection (en haut), la fitness des classes d'individus (au centre) et la distribution du trait juste après la sélection (en bas). Les triangles sous les histogrammes indiquent la moyenne du trait pour chaque population ; les barres indiquent la variation (± 2 déviations standard). D'après Kingsolver *et al.* 2007.

2.4 Interaction entre sélection et dérive génétique

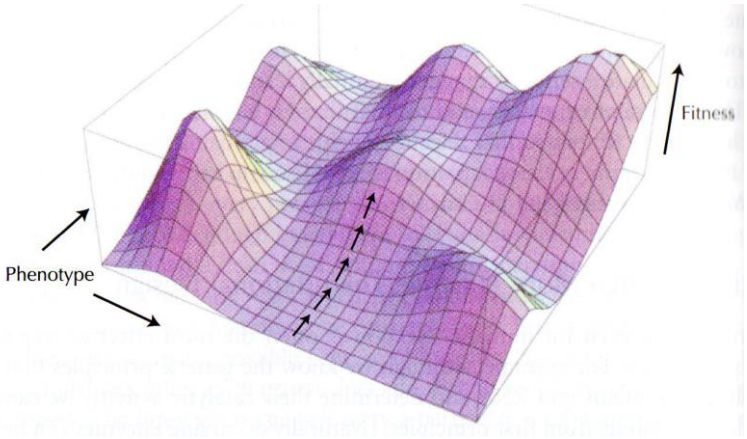

Figure 8 : Paysage adaptatif, montrant comment la fitness d'une population dépend de la fréquence et de la fitness des phénotypes individuels. Une série d'améliorations graduelles de la fitness (flèches) peut conduire à un optimal local de fitness, plutôt qu'à la fitness maximale. D'après Barton *et al.* 2007.

Le théorème fondamental de la sélection naturelle de Fisher (voir section 2.1) suppose que la nature est constituée de grandes populations, considérées comme infinies. Dans ce cas, l'effet de la sélection est dominant, et en général, la valeur sélective moyenne peut augmenter, comme décrit précédemment, dans un paysage adaptatif (Figure 8), jusqu'à une valeur d'équilibre locale, pour laquelle les fréquences alléliques ne varieront plus (Wright 1932). Cependant, beaucoup de travaux montrent que certaines populations sont de taille limitée, se rapprochant ainsi de l'hypothèse de S. Wright selon laquelle la dérive génétique (échantillonnage aléatoire des allèles d'une génération pour former ceux qui vont être retenus à la génération suivante) est plus importante que la sélection dans la trajectoire évolutive des populations fractionnées (Wright 1969). Cette hypothèse a donné lieu à une controverse historique entre les deux auteurs, et plus généralement, à un débat sur le long-terme entre « neutralistes » et « sélectionnistes », débat qui trouvera une issue dans les années 1950 avec la synthèse néo-darwinienne de la théorie de l'évolution (Huxley 1942).

La dérive peut d'abord avoir une influence sur l'efficacité de la sélection. Premièrement elle peut favoriser l'errance de l'allèle sélectionné autour du pic adaptatif optimal, selon une distribution probabiliste déterminée par le coefficient de sélection et la taille efficace de la population, maintenant ainsi la population dans un état sub-optimal de fitness, proche de, mais pas à l'équilibre. De plus, un allèle légèrement avantageux aura une plus faible probabilité de se fixer dans la population sous l'effet de la sélection, car il aura au contraire une plus forte probabilité d'être éliminé par l'effet de la dérive.

Par ailleurs, les évènements générant des goulots d'étranglement (bottleneck ; réduction brusque de la taille efficace d'une population) provoquent temporairement et fortement la dérive génétique. De manière générale, la dérive conduit à une perte des allèles rares et à l'augmentation en fréquence des allèles de fréquence intermédiaires. Ainsi, la seule dérive peut par exemple conduire à l'augmentation en fréquence d'allèles présentant une fitness réduite, voire délétère. La sélection, en agissant sur des populations ayant subi un goulot d'étranglement, peut alors modifier la trajectoire évolutive de la population en la

rapprochant secondairement d'un autre pic adaptatif. Ainsi, quand la dérive et la sélection agissent de concert, la population peut se déplacer d'un pic adaptatif à l'autre, ce qui n'est possible, lorsque la sélection agit seule, que dans le cas de la sélection balancée (Kimura 1983 ; Endler 1986 ; Barton *et al.* 2007). Il est donc parfois difficile, voire impossible, de prédire l'issue de l'évolution dans des populations subissant de fortes variations démographiques, même si elles subissent une sélection directionnelle.

2.5 Interaction entre sélection et mutation

Mutation et sélection ont par essence des principes antagonistes : la mutation crée de la variation, générée par les erreurs de réplication de l'ADN par exemple, alors qu'en général, la sélection élimine cette variation en favorisant un ou quelques phénotypes adaptés. Dans un environnement donné, le ou les quelques allèles porteurs des meilleures fitness devraient se fixer donc sous l'effet de la sélection naturelle, la variabilité observée étant générée par des mutations nouvelles, rares, voire délétères, rapidement éliminées sous l'effet de la sélection purifiante. Cette vision de l'école classique, défendue par Morgan et Muller (Morgan 1903), ne suffit cependant pas expliquer l'important polymorphisme constaté dans les populations, même si l'on considère l'effet de la dérive génétique dans les populations de petite taille. L'école de l'équilibre, menée par Dobzhansky (Dobzhansky 1937) cherche par contre à expliquer pourquoi la sélection peut maintenir un polymorphisme aussi important, notamment à cause de son interaction avec les autres forces évolutives (Futuyma 1998).

Un polymorphisme est maintenu si la fréquence des allèles rares, générés par la mutation et idéalement peu délétères, peut augmenter (Lynch *et al.* 1999). La description des interactions entre sélection et mutation revient donc à identifier les processus qui favorisent le polymorphisme. La superdominance (ou heterosis) permet le maintien des allèles rares puisque les hétérozygotes sont avantagés par une fitness accrue, en comparaison des homozygotes (exemple : cas de la drépanocytose) ; c'est le phénomène de vigueur hybride, largement utilisé par l'homme en sélection végétale et animale, qui s'oppose à la dépression d'hybridation (inbreeding depression) observée dans les populations consanguines, généralement de plus faible fitness (Barton *et al.* 2007). Cette hypothèse n'est cependant pas suffisante pour expliquer le maintien du polymorphisme, puisque même des espèces haploïdes ou auto-fertiles peuvent être diverses génétiquement. La sélection fréquence-dépendante peut maintenir le polymorphisme car dans ce cas, plus un phénotype est rare, meilleure est sa valeur sélective (avantage du rare, observé par exemple chez les espèces présentant un biais important de sex-ratio) (Futuyma 1998; Barton *et al.* 2007). Le polymorphisme est aussi maintenu lorsque deux organismes co-évoluent (cas des hôtes et de leurs parasites, en particulier des champignons phytopathogènes ; Van Valen 1973 ; Dawkins *et al.* 1979 ; Thompson 1994). Dans ce cas, chaque évolution d'une espèce

s'observe en réponse à l'évolution de l'autre (théorie de la Reine Rouge (Van Valen 1973 ; Salathe *et al.* 2008)). Enfin, le polymorphisme peut également être conservé en réponse aux fluctuations de l'environnement dans le temps ou dans l'espace (Felsenstein 1976 ; Hedrick 1986). Les fluctuations temporelles ralentissent généralement mais n'empêchent pas forcément la fixation d'un allèle, sauf dans le cas où les individus hétérozygotes seraient particulièrement adaptés à ces conditions fluctuantes. Les variations spatiales de l'environnement peuvent fournir une grande diversité de micro-habitats au sein de l'aire de distribution d'une espèce. Dans ce cas, l'espérance du maintien d'un polymorphisme sera plus forte si l'organisme est confronté à une multitude de niches écologiques au cours de son cycle de vie (« fine-grained environment », en opposition à « coarse-grained environment », lorsque l'organisme occupe une ou quelques niches). Elle sera également favorisée par une sélection « molle » (« soft selection »), qui implique que la survie d'un individu soit déterminée par son génotype, *i.e.* sa capacité à rentrer en compétition pour l'espace ou les ressources nutritionnelles de la niche écologique, indépendamment de la sélection naturelle, et non uniquement par la fitness de son génotype (par exemple, sensibilité ou résistance à un pesticide ; « hard selection »). Ces conditions favorisantes sont plus facilement rencontrées chez les animaux, qui peuvent sélectionner leur habitat, ou les plantes dont les graines sont peu dispersées en dehors de la niche favorable (Futuyma 1998). Tous ces phénomènes où la sélection participe au maintien du polymorphisme peuvent produire au final un patron d'évolution caractéristique de la sélection balancée.

A un locus donné, l'équilibre entre mutation (mesurée par son taux de mutation μ) et sélection (mesurée par le coefficient de mutation s) contre les hétérozygotes maintient une fréquence allélique à l'équilibre équivalente à μ/s (correspondant à un pic adaptatif). Un tel équilibre peut également être mesuré pour des traits quantitatifs (affectés par plusieurs mutations) mais peut-être perturbé par des interactions pléiotropiques (Barton *et al.* 2007 ; Olivieri 2010).

2.6 Interaction entre sélection et flux de gènes

La migration induit un déplacement d'individus dans l'espace et permet éventuellement d'introduire des allèles nouveaux (flux de gènes) dans des populations auparavant « naïves », augmentant ainsi leur diversité. Elle peut également limiter l'adaptation en introduisant des individus mal adaptés à leur nouvel environnement (Lenormand 2002) La fréquence de l'allèle introduit varie le long d'un gradient spatial, également appelé cline (Endler 1977). La notion de cline s'applique par extension à un patron de distribution d'un phénotype, lorsqu'on connait son déterminisme génétique.

Deux types d'évènements peuvent expliquer un patron de cline à un locus donné (Haldane 1948; Endler 1977):

- Un cline peut simplement témoigner du contact secondaire entre deux populations du même organisme ayant divergé, suite à des changements géologiques ou climatiques, par exemple (Figure 9A). Dans ce cas, la dérive génétique a permis la fixation d'allèles distincts dans les deux populations et la migration tend à homogénéiser leurs fréquences. En théorie, ce type de cline s'observe à tous les loci neutres, mais n'est pas stable dans le temps (il s'aplatit au cours du temps du fait de l'homogénéisation de la composition génétique des deux populations en contact à cause des flux de gènes).

- Un cline peut également être observé lorsque lorsqu'on rajoute l'effet de la sélection naturelle (Figure 9B). Dans ce cas, le cline sera stable pour les loci soumis à sélection (ou les loci neutres liés) si un équilibre s'établit entre l'effet homogénéisateur de la migration et l'effet diversifiant de la sélection. La sélection peut être de type endogène (indépendante de l'environnement), comme par exemple dans les zones hybrides où les individus des deux populations se reproduisent, créant des zones de tension (Barton *et al.* 1985; Bierne *et al.* 2003; Gay *et al.* 2008), ou de type exogène (dépendante de l'environnement), comme par exemple l'application d'un pesticide.

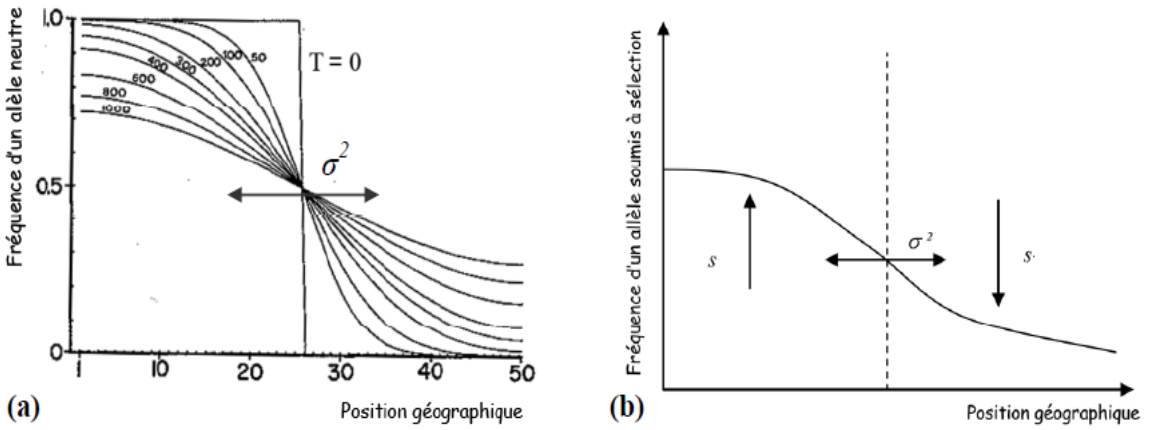

Figure 9 : Représentation de deux types de patterns de clines génétiques : (a) à un marqueur neutre, suite à un contact secondaire, à T=0 puis entre 50 à 1000 générations après le contact initial, et (b) à un marqueur soumis à sélection. L'intensité de la migration est notée σ^2 et la sélection est notée *s*. En zone traitée (à gauche), la sélection positive opère, alors que la sélection négative (à droite) contribue à diminuer les fréquences alléliques. D'après Endler 1977; Rieux 2011.

Le cline suppose une diffusion pas à pas des allèles. Il est caractérisé par les paramètres de la fonction mathématique qui le formalise, en particulier l'intensité de la pente en son centre et sa largeur, prédits par le paramètre de dispersion σ^2 (σ correspond à l'écart type des distances axiales de dispersion parents-descendants ou à encore la distance moyenne parcourue dans le cas d'une dispersion de type gaussienne) (Endler 1977), ainsi que par un ou plusieurs coefficients de sélection (Nagylaki 1975). La forme du cline est donc le reflet de l'équilibre local entre migration et sélection. Par exemple, une sélection positive plus intense que la migration génèrera un cline étroit et de forte pente. La modélisation

mathématique des clines permet d'inférer plusieurs de ces paramètres évolutifs, et donc de juger de leur importance respective dans un contexte donné. Cette méthode présente un intérêt évident pour décrire l'évolution des populations de bioagresseurs soumises à des pressions de sélection anthropiques, comme l'application de pesticides. Cependant, nous n'avons pu relever que deux exemples d'utilisation de cette méthode permettant de quantifier les flux de gènes et la sélection chez des bioagresseurs résistants. Le premier décrit le cas de l'adaptation du moustique *Culex pipiens* aux insecticides dans la région montpelliéraine (Lenormand *et al.* 1998a ; Lenormand *et al.* 1999 ; Lenormand *et al.* 2000) (Figure 10). Le second décrit l'évolution de la résistance aux strobilurines et aux benzimidazoles chez le pathogène du bananier *Mycosphaerella fijiensis* au Cameroun (Rieux *et al.*).

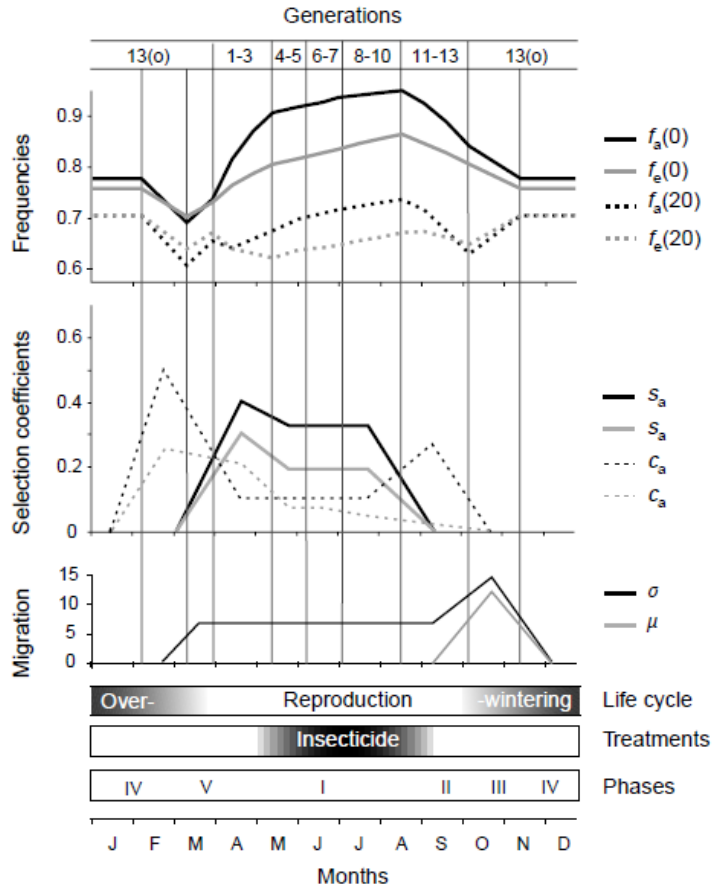

Figure 10 : Variation annuelle des clines fittés pour 2 loci (indices a et e) impliqués dans la résistance aux organophosphorés chez le moustique *Culex pipiens* collecté dans la région montpelliéraine soit sur les côtes (f(0)), soit à 20 km à l'intérieur des terres (f(20)). Les indices de sélection (*s* sélection insecticides, *c* coût de la résistance) et de migration (µ distribution moyenne et σ déviation standard, en km) sont également indiquées en fonction des saisons et du cycle de vie de l'insecte. D'après Lenormand *et al.* 1999.

2.7 Détecter et mesurer la sélection

Comme exposé dans les précédents paragraphes, la fréquence d'un allèle peut varier dans les populations sous l'effet des différentes forces évolutives. Il est donc important de pouvoir détecter et quantifier l'effet de la sélection naturelle, comparativement aux autres forces évolutives. Deux types de méthodes sont développés dans la littérature. Elles se basent soit sur le polymorphisme phénotypique, soit sur la diversité moléculaire (Futuyma 1998 ; Olivieri 2010).

Méthodes basées sur le phénotype (déterminé par un ou plusieurs allèles)

Ces méthodes se basent sur la variation de la mesure d'un trait phénotypique, ou sur la variation de fréquence du trait dans différents environnements. Elles peuvent également associer la valeur de la fitness du trait, mesurée expérimentalement (Kingsolver *et al.* 2007) :

- Mise en évidence de patrons de distribution spatiale, organisés non au hasard mais en relation avec des modifications de l'environnement (méthode des clines, voir 2.6) (Endler 1977)). La détection répétée de patterns de clines dans différentes situations géographiques conforte l'hypothèse de sélection. La formalisation mathématique du cline permet de mesurer les paramètres de sélection et de migration.

- Mise en évidence de patterns d'adaptation temporels en relation avec des modifications de l'environnement (exemple : cas du mélanisme de la phalène du bouleau ; 2.2).

- Recherche d'une corrélation entre la démographie des phénotypes ou génotypes et leur fitness (ou de composants de leur fitness mesurée expérimentalement), voire d'une fonction physiologique.

Une fois ces paramètres mesurés, ils peuvent être utilisés pour calculer l'un des estimateurs de sélection détaillés dans le tableau 3 (Brodie *et al.* 1995).

Parameter[a]	Symbol	Determined by:	Formula	Interpretation
Opportunity for selection[14,15]	I	Variance in relative fitness.	$\mathrm{VAR}\left(\frac{W}{\overline{W}}\right)$	Upper limit of the strength of selection.
Intensity of selection[7,8,b]	V	Variance in expected relative fitness [$\hat{f}_w(z)$], where $\hat{f}$ is estimated from nonparametric regression.	$\mathrm{VAR}\left(\frac{\hat{f}(z)}{\overline{W}}\right)$	Overall strength of selection (linear and nonlinear combined).
Selection differential – linear selection[4,6,14]	S_i	Difference in trait means ($\overline{z}_i$) before and after selection, or covariance between relative fitness and trait.	$(\overline{z}_i)_{\text{after}} - (\overline{z}_i)_{\text{before}}$ $\mathrm{COV}(w,z)$	Total change in the mean phenotype within a generation (total linear selection).
Selection differential – univariate nonlinear selection[4,6,14]	C_{ii}	Difference in trait variance before and after selection, or covariance between relative fitness and squared deviation ($\tilde{z}^2$).	$\mathrm{VAR}(z_i)_{\text{after}} - \mathrm{VAR}(z_i)_{\text{before}} + S_i^2$ $\mathrm{COV}(w,\tilde{z}^2)$	Total change in the variance of a trait within a generation after adjusting for directional selection (total nonlinear selection).
Selection differential – bivariate nonlinear selection[4,6,14]	C_{ij}	Difference in trait covariance before and after selection, or covariance between relative fitness and cross-product of deviations ($\tilde{z}_i\tilde{z}_j$).	$\mathrm{COV}(z_i,z_j)_{\text{after}} - \mathrm{COV}(z_i,z_j)_{\text{before}} + S_iS_j$ $\mathrm{COV}(w,\tilde{z}_i\tilde{z}_j)$.	Total change in the covariance of two traits within a generation after adjusting for directional selection (total nonlinear selection on a combination of traits, i.e. correlational selection).
Selection gradient – linear selection[4,6,9,14]	β_i	Multiple regression (purely linear model).	Partial regression coefficient for $\tilde{z}_i$.	Partial change in the phenotypic mean (direct linear selection).
Selection gradient – univariate nonlinear selection[4,6,9,14]	γ_{ii}	Multiple regression (full model including linear, squared and cross-product terms).	Partial regression coefficient of squared term ($\tilde{z}_i^2$).	Partial change in the variance of a character (direct nonlinear selection).
Selection gradient – bivariate nonlinear selection[4,6,9,14]	γ_{ij}	Multiple regression (full model including linear, squared and cross-product terms).	Partial regression coefficient of cross-product term ($\tilde{z}_i\tilde{z}_j$).	Partial change in the covariance between two characters (direct nonlinear selection on a combination of traits).

[a]Parameters estimated from statistical procedures may or may not correspond directly to the qualitative aspects of the processes of interest (Box 1). However, in all cases, the statistical measures are more general than the qualitative descriptors and therefore embody classical processes.
[b]A second measure of the 'intensity of selection' (i) is the selection differential measured on traits standardized to $\overline{z}=0$, $\mathrm{sd}=1$.

Tableau 3 : Paramètres, et leur formalisation mathématique, permettant de mesurer quantitativement la sélection positive exercée sur un trait. *Z* indique la valeur phénotypique du trait et *W* la fitness associée. D'après Brodie *et al.* 1995.

Méthodes basées sur la diversité moléculaire

Ces méthodes exploitent soit le polymorphisme de fréquence des marqueurs neutres, soit le polymorphisme moléculaire de gènes potentiellement soumis à sélection positive :

- Mesure de variations de fréquences alléliques par rapport à l'équilibre d'Hardy-Weinberg. Bien que grossières, car les variations de structure allélique peuvent être causées par d'autres facteurs, ces méthodes consistent à repérer les loci se comportant différemment des autres. Ce comportement peut porter sur différents paramètres génétiques : déficit ou excès en hétérozygotes relativement aux fréquences attendues sous équilibre panmictique (F_{IS}), variance plus grande ou plus faible des fréquences alléliques entre générations ou entre populations (F_{ST}), taux de polymorphisme anormalement élevé ou faible (mesuré par exemple par la richesse allélique ; Olivieri 2010).

- Mesure de la variation nucléotidique de gènes potentiellement soumis à sélection, principalement par deux indicateurs (Nielsen 2005 ; Aguileta *et al.* 2009) :

  - détection de traces « anciennes » de sélection, par la mesure de divergence adaptative $\omega=d_N/d_S$ (taux de substitutions non-synonymes par rapport au taux de substitutions synonymes) (Yang *et al.* 2000)

  - détection de traces récentes ou contemporaines de sélection, par la mesure du polymorphisme ou différence (le « *D* » de Tajima ; Tajima 1989) entre deux estimateurs, l'un équivalant à l'hétérozygotie, peu sensible à la présence d'allèles rares, et le second très influencé par la présence d'allèles rares. Sous l'hypothèse d'équilibre neutre, *D* vaut en espérance 0. Cependant, le *D* de Tajima est également sensible aux fluctuations démographiques, qui ont également un impact sur le changement de fréquence des allèles rares. La plupart des populations réelles n'étant pas à l'équilibre démographique, on corrige pour cet effet de la démographie en mesurant en pratique l'écart entre le *D* moyen pour des loci neutres, voire pour un génome, et les loci potentiellement sélectionnés.

Les méthodes précédentes gagnent désormais énormément en puissance avec la généralisation des approches génomiques, car la recherche de loci « outliers » peut être effectuée le long des génomes entiers (approche « genome scan » ; Nielsen 2005). La détection des loci sous sélection est donc plus exhaustive et plus précise, les tests d'écarts à la variation moyenne observés aux autres loci étant beaucoup plus puissants.

Cette section a détaillé la sélection naturelle, comme cause de l'évolution au sein des populations et des espèces, en interaction avec les autres forces évolutives. Bien que ces phénomènes opèrent sur des pas de temps relativement longs, ils constituent les moteurs de la microévolution, en opposition à la macroévolution qui concerne l'origine et la diversification des taxons supérieurs. La spéciation constitue le maillon intermédiaire en micro et macroévolution et fait l'objet de la section suivante.

3. SPÉCIATION CHEZ LES CHAMPIGNONS

3.1 DÉFINITIONS

S'il est entendu que l'espèce constitue une unité de décompte de la biodiversité, sa définition n'est pas triviale, du fait qu'elle a été souvent confondue avec les critères d'espèces (voir 3.2 ; Mayden 1997) et qu'elle a évolué au cours du temps, faisant ainsi l'objet d'une polémique abondante. Avant l'acceptation de la théorie de l'évolution, la définition d'espèce lie intimement la notion de ressemblance et de descendance puisqu'elle correspond à un « ensemble d'individus qui engendrent, par la reproduction, d'autres individus semblables à eux-mêmes » (définition du botaniste Ray, utilisée par Linné pour l'établissement de la systématique moderne). Les évolutionnistes ne conservent dans leur définition que la notion de descendance, puisqu'ils montrent par ailleurs que la variation est entre autres le résultat de l'expression de la sélection naturelle et que celle-ci est l'un des moteurs de la transformation des espèces au cours du temps. Ils introduisent ainsi la notion de réseau généalogique du vivant, où les organismes ont la propriété de donner naissance, à partir de fragments matériels d'eux-mêmes, à de nouveaux organismes (descendants) qui leur ressemblent imparfaitement et qui sont également capables de se reproduire (Ravigné 2010). La dissociation des exigences de conformité à la théorie de l'évolution et d'opérationnalité conduisent à la définition moderne : l'espèce est une sous-partie du réseau généalogique, définitivement divergente du reste du réseau (c'est à dire n'échangeant plus de gènes avec lui). Elle est délimitée dans le temps par un évènement de spéciation (origine) et par un évènement d'extinction ou un nouvel évènement de spéciation (De Queiroz 1998 ; Samadi *et al.* 2006 ; Figure 11).

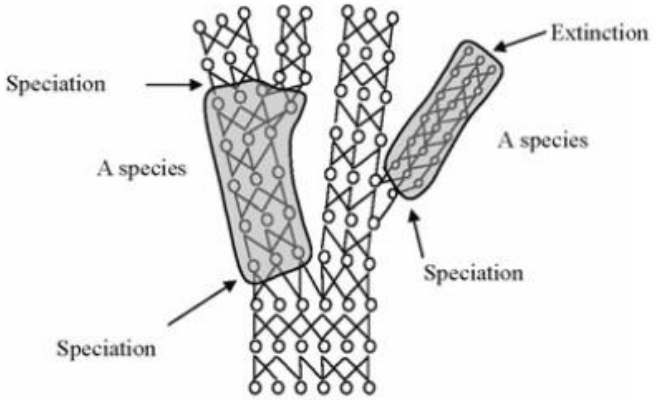

Figure 11 : Partition en espèces d'un réseau généalogique fictif.

Les espèces sont indiquées dans des ensembles grisés Les cercles représentent les individus et les traits les relations de parenté entre ces individus. D'après Samadi *et al.* 2006.

3.2 CRITÈRES D'ESPÈCE

Dans ce contexte, tout critère qui permet de montrer qu'un groupe d'individus appartient à une communauté de reproduction ayant une histoire évolutive autonome est donc pertinent. De manière opérationnelle, le choix du critère d'observation dépendra

surtout de la biologie de l'organisme. Vingt-deux critères d'espèces (ou « species concepts »), ainsi que des méthodes dérivées, sont répertoriés dans la littérature (Tableau 4 ; Mayden 1997 ; De Queiroz 1998). Il est communément admis que plusieurs critères sont souvent nécessaires, car complémentaires, pour délimiter les espèces, car leurs frontières sont parfois confuses (Sites *et al.* 2004).

1. Agamospecies (ASC)	14. Morphological (MSC)
2. Biological (BSC)	15. Non-dimensional (NDSC)
3. Cohesion (CSC)	16. Phenetic (PhSC)
4. Cladistic (ClSC)	17. Phylogenetic (PSC)
5. Composite (CpSC)	1. Diagnosable Version (PSC_1)
6. Ecological (EcSC)	2. Monophyly Version (PSC_2)
7. Evolutionary Significant Unit (ESU)	3. Diagnosable and Monophyly Version (PSC_3)
8. Evolutionary (ESC)	
9. Genealogical Concordance (GCC)	18. Polythetic (PtSC)
10. Genetic (GSC)	19. Recognition (RSC)
11. Genotypic Cluster Definition (GCD)	20. Reproductive Competition (RCC)
12. Hennigian (HSC)	21. Successional (SSC)
13. Internodal (ISC)	22. Taxonomic (TSC)

Tableau 4 : Liste des 22 concepts d'espèce (SC) référencés dans la littérature. D'après Mayden 1997.

Parmi ces méthodes d'observation, certains sont d'un intérêt particulier pour l'étude des champignons (Kohn 2005; Giraud *et al.* 2008a; Ravigné 2010):

- Le critère biologique est sans doute le plus connu. Il repose sur l'interfécondité de deux individus et de leur descendance. L'incapacité de deux organismes (de sexes ou de types sexuels opposés) à se reproduire constitue la preuve définitive de leur divergence par la mise en place d'un isolement reproductif (Mayr 1963). Cependant, ce critère trouve ses limites expérimentales pour les espèces asexuées ou pour lesquelles le croisement ne peut être obtenu en conditions contrôlées. Dans ce cas, ce critère peut être prolongé en mesurant des flux de gènes inter- et intra-groupes (Sites *et al.* 2004) ;

- Le critère morphologique (ou phénétique) repose sur la ressemblance des individus. Bien qu'intuitif chez beaucoup d'espèces, ce critère n'est pertinent que dans la mesure où il traduit l'apparentement des organismes. Les caractères choisis doivent donc être héritables, et non soumis à des interactions environnementales. Par ailleurs, la fixation des caractères n'étant pas instantanée, des caractères ancestraux (ou plésiomorphes) peuvent s'observer chez deux espèces récemment différenciées. Chez les champignons, connus pour leur grande variabilité phénotypique, les caractères utilisables pour distinguer les espèces concernent souvent les spores (sexuées ou asexuées), les organes reproducteurs, ou l'allure générale du mycélium ;

- Le critère phylogénétique repose sur la ressemblance des individus par descendance, avec apport de modifications. Ce critère cherche à retracer « l'arbre généalogique » des espèces (souvent, en utilisant les séquences nucléotidiques de gènes particulièrement adaptés pour les études phylogénétiques, notamment par leur « vitesse » d'évolution) en considérant que seuls les caractères dérivés partagés (ou

synapomorphies) permettent de situer les branchements de l'arbre (Hennig 1966). Ce critère atteint ses limites lorsque le caractère dérivé n'est pas encore fixé, même si les deux espèces sont déjà séparées. Ce critère est particulièrement utilisé chez les champignons, notamment parce que ces organismes constituent souvent des cas limites pour les deux critères précédents (difficulté des croisements *in vitro*, plasticité phénotypique). Parmi d'autres outils récemment mis au point par la communauté d'évolutionnistes moléculaires, la base de données « Funybase » présente une sélection de gènes particulièrement adaptés pour l'application de ce critère chez des espèces fongiques (Marthey *et al.* 2008). Le critère de concordance phylogénétique est une extension du précédent. Il repose sur la comparaison multiple de phylogénies (Taylor *et al.* 2000) et s'est avéré particulièrement utile pour différencier des complexes d'espèces cryptiques chez les champignons (Dettman *et al.* 2003 ; Fournier *et al.* 2005 ; Le Gac *et al.* 2007a) ;

- Le critère écologique repose sur la similitude des exigences écologiques de deux groupes (contraintes nutritionnelles, type trophique, adaptations aux contraintes climatiques…). Ce critère est particulièrement pertinent dans le cas des champignons, capables de coloniser une grande variété de niches écologiques (altitudes, pression et températures extrêmes, larges spectres d'hôtes) grâce à la diversité de leurs styles de vie (parasitisme, nécrotrophie, biotrophie, symbiose).

3.3 Mécanismes d'isolement reproductif

La condition *sine qua non* à la spéciation chez les individus se reproduisant de manière sexuée est la rupture du flux de gènes entre espèces, autrement dit la mise en place d'un isolement reproducteur. Il existe deux types d'isolement reproducteur, suivant le moment où ils opèrent dans le cycle de vie : l'isolement pré-zygotique (avant la rencontre des gamètes), et l'isolement post-zygotique (après la rencontre des gamètes), mais également en fonction de leur dépendance à l'environnement (Figure 12 ; Giraud *et al.* 2008a ; Ravigné 2010).

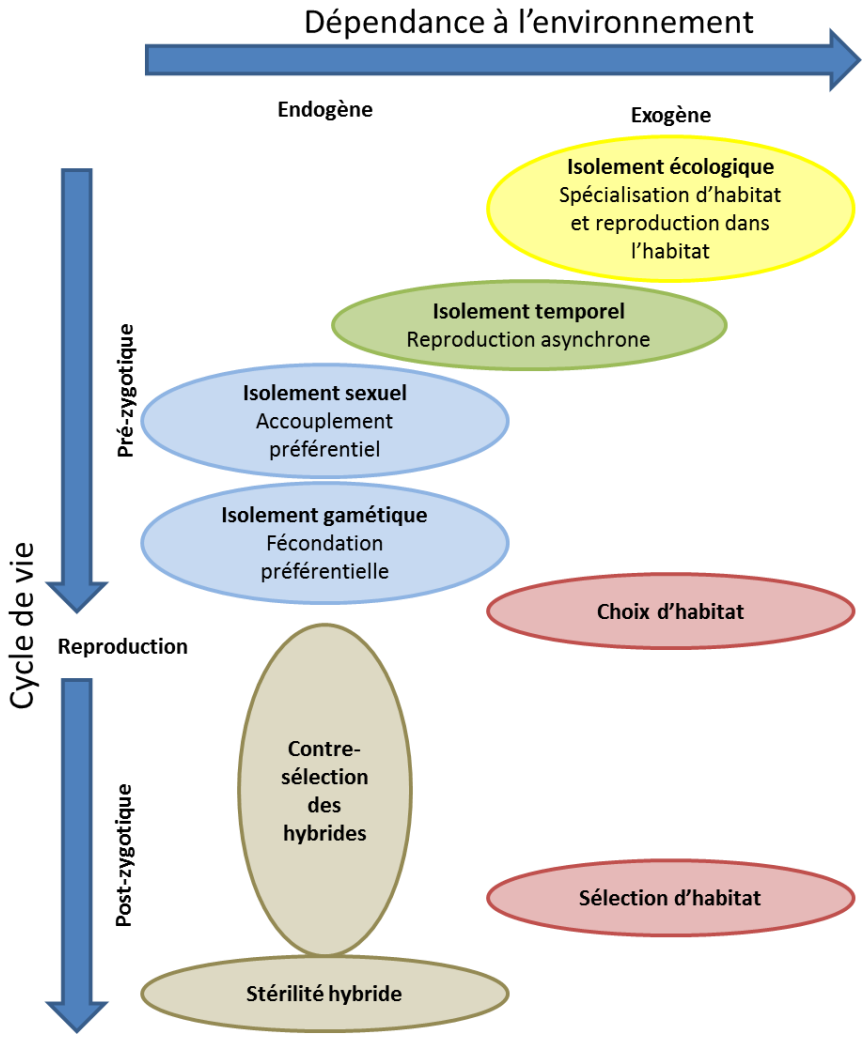

Figure 12 : Classification des mécanismes d'isolement reproductif en fonction de leur dépendance à l'environnement et du stade du cycle de vie au moment où ils interviennent. D'après Ravigné 2010.

Chez les champignons, de nombreux mécanismes interviennent avant la fécondation et empêchent la formation même des hybrides. L'isolement pré-zygotique peut ainsi être causé par :

- Un isolement écologique, lié à l'habitat, *i.e.* la niche écologique sur laquelle l'espèce est spécialisée. Chez les champignons parasites des plantes, les espèces sont souvent spécialistes d'un hôte donné. Ne peuvent se croiser entre eux que des individus spécialisés, c'est-à-dire ayant réussi à infecter et à croître sur la même espèce d'hôte (Giraud 2006a ; Giraud *et al.* 2006 ; Peever 2007) ;

- Un isolement écologique, lié à des vecteurs (généralement insectes), qui par exemple ne transportent les spores de champignons parasites qu'au sein d'une même espèce d'hôte (Bultman *et al.* 2003 ; van Putten *et al.* 2007) ;

- Un isolement temporel (allochronie), lié à un décalage des périodes de reproduction entre les deux groupes, potentiellement en adaptation au milieu ou à l'infection par des parasites qui déphasent le cycle de vie de leur hôte (Murphy *et al.* 2006 ; Kiss *et al.* 2011) ;
- Un isolement sexuel, lié à l'autogamie (« selfing ») qui, par définition, limite les croisements interspécifiques (Giraud *et al.* 2008b) ;
- Un isolement gamétique, lié à l'appariement préférentiel (« assortative mating ») résultant d'une rencontre dirigée entre des individus — ou des gamètes — capables de discriminer des partenaires con- ou hétérospécifiques.

L'isolement post-zygotique n'empêche par la rencontre des gamètes ni la formation d'un zygote. Les bases de ce type d'isolement sont bien connues chez les êtres vivants (Coyne *et al.* 1998 ; Noor *et al.* 2006). Les hybrides sont non viables ou infertiles donc de plus faible valeur sélective que les parents, voire nulle. Cependant, ces hybrides peuvent présenter une heterosis (vigueur hybride) sur un ou plusieurs caractères bien que la valeur sélective soit globalement réduite (exemple de la mule, croisement entre le cheval et l'âne). La fixation des incompatibilités génétiques implique le franchissement de vallées adaptatives, ce qui resterait un processus trop rare par rapport aux observations de terrain. Une hypothèse plus parcimonieuse propose que les allèles responsables de la dépression d'hybridation soient avantageux dans leur fond génétique habituel, mais délétères lorsqu'ils sont mélangés aux allèles d'une autre espèce (effets de l'épistasie ; modèle de Dobzhansky-Muller ; Orr *et al.* 2001). Ce mécanisme s'observe dans les croisements hétérospécifiques de champignons où les structures sexuées sont produites, mais sont parfois anormales, vides d'ascospores, ou produisent très peu de gamètes L'isolement post-zygotique peut également être lié à des facteurs écologiques. Dans ce cas, les hybrides présentent des traits intermédiaires à ceux des parents et sont fertiles dans un environnement protégé (*in vitro* par exemple), mais totalement non adaptés à l'environnement parental (Le Gac *et al.* 2007b).

3.4 Modes de spéciation

Le mode de spéciation correspond à la manière dont l'isolement reproducteur, générant la spéciation, se met en place. De manière intuitive, il semble évident que des barrières géographiques (montagnes, océans) puissent stopper des flux de gènes entre populations isolées, amenant à une spéciation allopatrique (Mayr 1963). Dans le cas des parasites, les vecteurs peuvent constituer un facteur supplémentaire de spéciation allopatrique. Ils constitueraient une barrière extrinsèque car les insectes reconnaissent les traits des plantes hôtes et pas des pathogènes (Giraud 2006b). Dans ce contexte, les

champignons sont longtemps apparus comme des exceptions car beaucoup d'espèces ont une répartition géographique large, voire mondiale, du fait notamment de traits favorisant, comme la forte production de spores dispensables sur de longues distances chez les pathogènes aériens (Brown *et al.* 2002 ; Finlay 2002). Cependant, plusieurs cas de spéciation allopatrique ont été décrits grâce à des approches de phylogénie multiple chez les champignons, par exemple chez des espèces cryptiques occupant des aires de répartition distinctes et séparées par des barrières géographiques (voir Taylor *et al.* 2006 pour les aspects théoriques et Dettman *et al.* 2003 ; O'Donnell *et al.* 2004 ; Kohn 2005; Kuehne *et al.* 2007 pour quelques exemples).

La spéciation sympatrique implique qu'il n'y ait pas de barrière extrinsèque au flux de gènes, *i.e.* que la probabilité de croisement entre deux individus dépende uniquement de leurs génotypes (Kondrashov *et al.* 1986). Ce type de spéciation semble moins intuitif que le précédent. En effet, dans un environnement donné et pour une espèce sexuée, la recombinaison génétique aura tendance à contrecarrer l'effet de la sélection naturelle sur deux populations qui se différencient, en cassant les déséquilibres de liaison entre allèles adaptatifs (gènes de l'isolement reproducteur, gènes de l'adaptation au microenvironnement) et en favorisant la divergence des loci non soumis à la sélection diversifiante. En théorie, un seul migrant par génération suffit à empêcher la différentiation de deux populations (Slatkin 1987). Plusieurs modèles théoriques ont montré que la manière la plus simple de prévenir l'effet de la recombinaison génétique, et ainsi de permettre la spéciation allopatrique sous l'effet de la sélection, était que les mêmes gènes puissent contrôler de manière pléiotropique soit l'adaptation à la niche écologique et l'appariement préférentiel, soit l'adaptation et le choix de l'habitat, dans le cas où la reproduction sexuée a lieu dans la niche écologique. Dans ce cas, l'adaptation à une niche écologique constitue un « trait magique », qui suffit à limiter le flux de gènes en sympatrie (Gavrilets 2004). Les champignons ne choisissent pas activement leur habitat ; les parasites sont déposés de manière passive sur leur hôte. Par contre, la reproduction sexuée se déroule sur l'hôte pour la majorité des espèces Ascomycètes, après une phase de développement mycélien. Les champignons constituent donc de bons candidats pour rechercher ces « traits magiques » leur permettant à la fois de s'adapter à leur hôte et de se reproduire dans cet habitat. Dans ce cas particulier, l'appariement préférentiel se produit *de facto*, en conséquence de l'adaptation à l'hôte, et est gouverné simplement par les gènes induisant cette adaptation (Giraud 2006a; Giraud *et al.* 2006). Ce type de spéciation a été démontrée chez quelques espèces de champignons, bien que complexe à mettre en évidence car il est souvent difficile de la dissocier d'une phase initiale d'allopatrie : *Aschohyta* des légumineuses (Peever 2007), *Mycosphaerella graminicola* sur graminées sauvages (Stukenbrock *et al.* 2007).

La spéciation par hybridation a également été montrée à plusieurs reprises chez les champignons. Elle repose sur le fait que de nombreuses espèces de champignons ne sont pas complètement inter-stériles (en particulier des espèces proches phylogénétiquement),

ce qui leur laisse la possibilité de s'hybrider. Les hybrides allopolyploïdes possèdent un niveau de ploïdie supérieur à celui des parents (exemples de *Botrytis allii* sur oignon, (Staats *et al.* 2005) ou des symbiotes de graminées *Neotyphodium spp.* (Moon et al. 2004) ; voir aussi la revue de (Olson *et al.* 2002)). En combinant les traits parentaux, certains hybrides présentent des avantages sélectifs qui leur permettent d'exploiter de nouvelles niches écologiques. L'allopolyploïdie induit ainsi un isolement reproducteur, par incompatibilité des caryotypes, et l'adaptation à un nouvel environnement (Mallet 2007). Les hybrides allodiploïdes (ou homoploïdes) présentent un niveau de ploïdie identique à ceux des parents et sont hétérozygotes, ce qui leur permet également de s'adapter à de nouvelles niches écologiques (exemple de l'agent de la rouille *Melampsora x columbiana* capable d'infecter un peuplier hybride des deux espèces d'hôtes infectées par les espèces fongiques parentales (Newcombe *et al.* 2000). Chez ces hybrides, l'hétérozygotie à de nombreux loci serait maintenue du fait de son avantage adaptatif, en dépit des possibilités de rétrocroisement avec les parents (Giraud *et al.* 2008a).

La spéciation chromosomique repose sur le réarrangement des chromosomes, induisant l'isolement reproducteur, même si ce mécanisme est relativement rare, du fait que la faible fitness des hétérozygotes réduit leur probabilité de fixation dans les populations (White 1978). Ce mode de spéciation semble cependant plus facilement observable chez les champignons, qui pratiquent largement la reproduction asexuée et l'autogamie, ce qui permet aux mutants « arrangés » pour leur caryotype de survivre dans les populations. Ce mécanisme a été notamment observé chez l'Ascomycète *Sordaria macrospora* et le Basidiomycete *Coprinus cinereus* (Zolan *et al.* 1994 ; Poggeler *et al.* 2000).

Les sections précédentes montrent donc que les champignons constituent d'excellents candidats pour l'étude de la sélection naturelle et de la spéciation puisqu'ils présentent de nombreux caractères favorisants : grandes tailles de population, diversité des modes de reproduction, grandes capacités migratoires, diversité des types trophiques. La dernière section illustre ces traits chez le champignon choisi comme modèle d'étude pour cette thèse ainsi que sa plasticité phénotypique envers les fongicides.

4. MODÈLE D'ÉTUDE : *BOTRYTIS CINEREA*, UN CHAMPIGNON PATHOGÈNE POLYPHAGE

4.1 NOMENCLATURE ET SYSTÉMATIQUE

Botrytis cinerea Pers. Ex Fr. (1832) est un champignon Ascomycète responsable de la pourriture grise de nombreuses plantes cultivées et sauvages. L'étymologie de son nom fait référence directement à sa morphologie : « *Botrytis* » signifie « en forme de grappe », indiquant ainsi la morphologie des conidiophores, et « *cinerea* » renvoie à la couleur gris-cendrée de la sporulation. Le genre *Botrytis* a été décrit pour la première fois en 1729 par *Pier Antonio Micheli* qui la répertorié dans le « *Nova Plantarum Genera* » puis de manière définitive par Hennebert, comme la majorité des espèces du genre (Groves *et al.* 1953 ; Hennebert 1973). Le nom *Botrytis cinerea* a été proposé par Elias Magnus Fries, le botaniste suédois qui, à la suite de Linné, fut le fondateur de la systématique des champignons. Cet Ascomycète appartient à la classe des Léotiomycètes, ordre des Héliotiales et la famille des Sclerotiniaceae (Beever *et al.* 2004). La forme parfaite (ou sexuée) est nommée *Botryotinia fuckeliana* (Whetzel 1945). Récemment, il a été proposé par des taxonomistes des champignons d'abandonner la double nomenclature (téléomorphe *vs* anamorphe), au profit du « *One fungus, one name* » (Wingfield *et al.* 2012). Dans le cas de *B. cinerea*, le nom définitif de l'espèce n'a pas encore été choisi par la communauté.

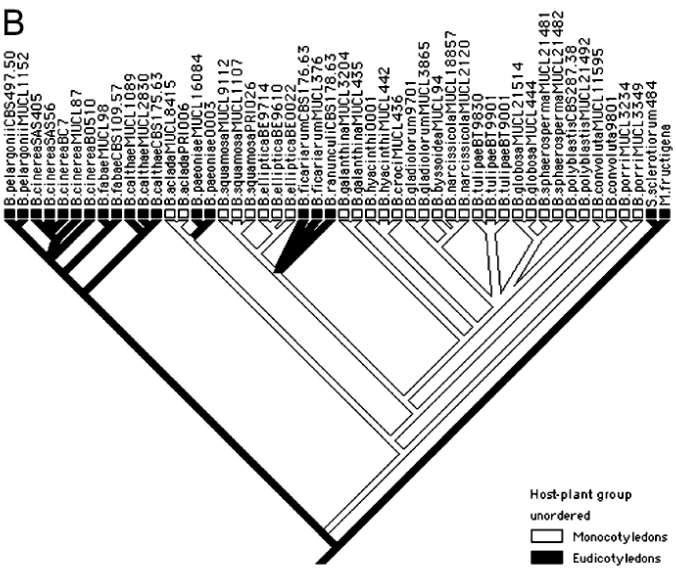

Figure 13 : Arbre phylogénétique (combinaison par la méthode de parcimonie des topologies obtenues pour les gènes *RPB2, G3PDH* et *HSP60*) montrant les relations entre les différentes espèces du genre *Botrytis.* En blanc : hôte monocotylédone ; en noir : hôte dicotylédone. L'espèce *B. allii* est un hybride entre *B. byssoidea* et *B. aclada*. D'après (Staats *et al.* 2005)

Le genre *Botrytis* est très proche du genre *Sclerotinia* (83 % d'identité protéique moyenne entre les deux génomes) (Amselem *et al.* 2011). Il compte 22 espèces et un hybride (Figure 13). La classification ancienne, basée essentiellement sur des caractères morphologiques et les spectres d'hôtes a été révisée récemment par une approche de généalogie multiple (Staats *et al.* 2005). Cette classification confirme que le genre peut être séparé en deux clades, le premier contenant 4 espèces colonisant des plantes dicotylédones, et le second contenant 18 espèces, pathogènes soit de dicotylédones (3), soit de

monocotylédones (15). La comparaison de la phylogénie des hôtes et de leur parasite suggère qu'ils n'auraient pas co-évolué conjointement, mais que le genre *Botrytis* se serait diversifié par sauts d'hôtes successifs, par l'acquisition de nouveaux déterminants de pathogénie. La perte de reproduction sexuée a été observée au moins trois fois au sein du genre.

Les travaux de la fin des années 90 ont montré que l'espèce *B. cinerea* ne constituait pas une entité monomorphe, mais qu'elle pouvait être subdivisée en au moins deux groupes inféodés à des espèces cryptiques : un groupe dit *transposa,* car les souches de ce groupe contiennent au moins l'un des deux éléments transposables *Boty* ou *Flipper*, et un groupe *vacuma*, qui ne contient pas ces ceux éléments (Diolez *et al.* 1995 ; Giraud *et al.* 1997 ; Giraud *et al.* 1999). Cette nomenclature est toujours largement utilisée de par le monde pour étudier la diversité des populations de *B. cinerea* (Munoz *et al.* 2002 ; Ma *et al.* 2005 ; Esterio *et al.* 2011 ; Samuel *et al.* 2012). Malheureusement, des travaux ultérieurs, utilisant d'autres marqueurs comme le polymorphisme du gène d'histocompatibilité *Bc-hch* et de 8 microsatellites spécifiques de *B. cinerea* (Fournier et al. 2002 ; Fournier et al. 2003), ainsi qu'une phylogénie multiple (Fournier *et al.* 2005 ; Fournier *et al.* 2008) ont montré qu'il existait bien deux espèces cryptiques, mais que leur partition ne recouvrait pas la partition *vacuma* / *transposa* : l'espèce appelée provisoirement « groupe I » (nouvelle espèce ; nom proposé *B. pseudocinerea*) semble ne contenir que des souches *vacuma*, alors que l'espèce « groupe II » (*B. cinerea sensu stricto)* contient à la fois des souches *vacuma* et *transposa*. Par ailleurs, l'espèce « groupe I » a été caractérisée comme naturellement résistante au fongicide fenhexamid (inhibiteur de la C4-déméthylation des stérols), à cause d'un mécanisme original permettant à cette espèce de métaboliser cette molécule toxique (Billard 2011). Cette espèce est également hypersensible aux amines, une autre classe d'inhibiteurs de la biosynthèse des stérols. Cependant, la nouvelle espèce (groupe I), n'avait à ce jour jamais été décrite précisément comparativement à *B. cinerea sensu stricto* et la position respective de ces deux espèces considérées jusqu'alors comme jumelles n'avait pas été déterminée dans l'arbre phylogénétique du genre. Avant son dépôt récent dans une collection internationale, la nouvelle espèce n'avait donc pas d'existence officielle. Enfin, très récemment, un nouveau clade semble avoir été identifié et serait proche de *B. cinerea* et *B. fabae*, sans que la phylogénie puisse totalement en trancher la topologie. Il serait inféodé au fraisier (nommé pour cela « groupe *s* » comme « strawberry » ; Leroch *et al.* 2013).

Figure 14 : Symptomatologie et morphologie de *Botrytis cinerea*. A : Attaque en serre de tomate. La proximité des plantes et la litière infectée au sol favorisent les épidémies. B : Chancre sur tige de tomate. C : Tâches fantôme sur tomate, correspondant à des infections avortées. D : Surinfection sur tomate cerise infectée par le mildiou. *B. cinerea* est également un parasite de faiblesse. E : Jeune fruit de concombre infecté. F : Infection sur mûres cultivées. G et H : Mycélium sporulant sur grappe de raisin. I : Infection sur feuille de *Pelargonium*, après contact d'une fleur infectée. J : Plant de cyclamen dont les pétioles ont pourri sous l'action de *B. cinerea*. K : Mycélium sporulant (macroconidies). L : Conidiophore en bouquet, portant les macroconidies. M et N : Rameaux de vigne, porteurs de sclérotes (forme de conservation du champignon). Au printemps, la sporulation des sclérotes permet les inoculations primaires. O : Apothécies, organes de la reproduction sexuée. P : Asques contenant les ascospores issues de la reproduction sexuée. Photographies personnelles.

4.2 Biologie et diversité de *Botrytis cinerea*

B. cinerea est un champignon nécrotrophe, attaquant des tissus vivants mais également capable de saprophytisme. On lui dénombre environ 270 espèces de plantes dicotylédones sauvages (notamment des *Rosaceae* sauvages), mais également cultivées (*Solanaceae, Cucurbitaceae, Vitaceae, Rosaceae, Fabaceae* principalement ; Figure 14), pour lesquelles il cause une macération, accompagnée d'une sporulation gris-cendré sur tiges, feuilles, fleurs et/ou fruits, pouvant aller jusqu'à la perte totale de la récolte. En vigne, il représente la 3ème maladie pour son importance économique, après le mildiou *Plasmopara viticola* et l'oïdium *Erysiphe necator*. Outre les pertes quantitatives à la récolte, *B. cinerea* perturbe la qualité de la vinification par la « casse oxydasique », conduisant à une modification de la couleur, une dégradation des arômes (goûts moisi-terreux), une diminution du degré alcoolique et une accumulation de glycérol, de glucanes et d'acide glucuronique (Dubos 2002). Sous certaines conditions climatiques et de physiologie de la vigne, *B. cinerea* délaisse le faciès « gris » pour le faciès dit « noble » (pourriture noble), permettant la dessiccation progressive des baies et la concentration des arômes typiques des vins liquoreux comme le Sauternes ou le Tokaj. Des travaux récents n'ont pas montré de différentiation génétique entre les deux types de souches, confirmant ainsi le déterminisme environnemental du faciès noble (Fournier *et al.* 2013). De manière générale, *B. cinerea* est connu au laboratoire pour sa grande diversité morphologique, allant de la couleur du mycélium, la production de spores, l'allure du mycélium aérien jusqu'à la production et l'allure de sclérotes (Martinez *et al.* 2003).

Sur vigne, le champignon se conserve sous forme de mycelium haploïde dormant ou condensé et mélanisé (sclérotes) sur les bois d'hivernage (Figure 15). Sous les conditions favorables du printemps, les sclérotes germent, produisent un mycelium capable de perforer la cuticule végétale grâce à ses appressoria. Ce mycélium primaire différencie des conidies (spores asexuées), responsables des premières contaminations, en particulier sur pièces florales sénescentes (contaminant également la jeune baie), et parfois sur feuilles. Ces premières sporées sont responsables des contaminations secondaires (dissémination par le vent ou la pluie, mais également par certains Lépidoptères (Fermaud *et al.* 1992)). La cinétique et la distribution de la dissémination des conidies ne sont pas connues, mais elles sont inversement proportionnelles à l'augmentation de l'humidité relative de l'air (Dubos 2002). Plusieurs cycles de reproduction asexuée peuvent se dérouler dans la saison (environ une semaine dans des conditions optimales : température (15-20°C) et d'humidité supérieure à 90%) ; Dubos 2002). L'infection par *B. cinerea* est favorisée par les blessures des baies (grêle, morsures de guêpes, dégâts d'oiseaux) et la grappe est généralement colonisée de proche en proche après la contamination d'une seule baie (Elmer *et al.* 2004). La reproduction sexuée est supposée se produire en hiver, et résulte de la fusion entre les microconidies (spermaties) produites par le mycélium, induit par les conditions hivernales, et les gamètes femelles (ascogones) produits dans les sclérotes. *In vitro*, la production des

organes de reproduction sexuée (apothécies) et des ascospores produit une descendance entre 3 à 6 mois après la mise en contact des gamètes de souches de types sexuels opposés (espèce hétérothallique ; Faretra *et al.* 1987 ; Faretra *et al.* 1988), ce qui permet d'étudier la génétique de traits d'intérêt chez cette espèce. *In natura*, l'observation d'apothécies est extrêmement rare (deux mentions : l'une en Suisse, et l'autre en Nouvelle-Zélande), ce qui suggère une reproduction sexuée cryptique, bien que l'existence de recombinaison régulière ait été démontrée par des preuves indirectes (Giraud *et al.* 1997 ; Beever *et al.* 2004 ; Fournier *et al.* 2008).

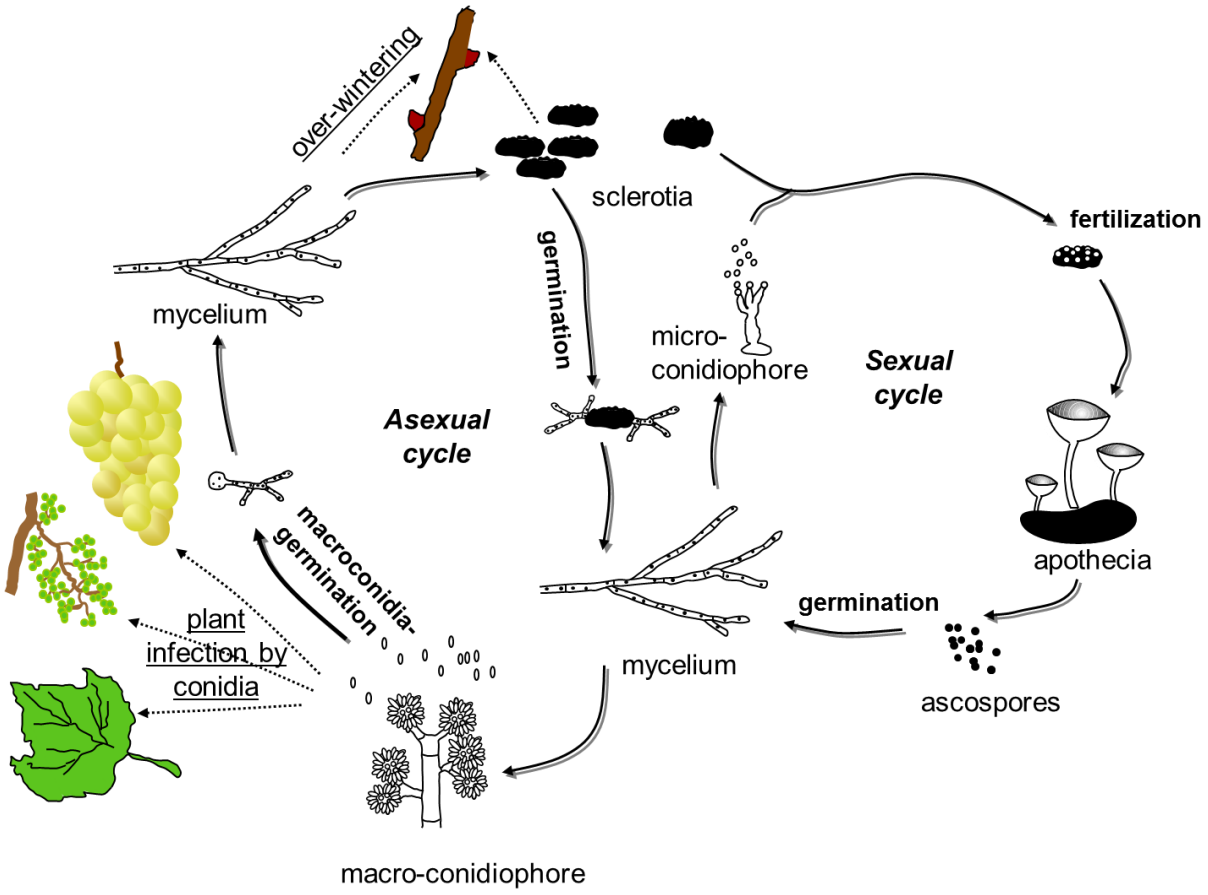

Figure 15 : Cycle de vie de *Botrytis cinerea* sur vigne. Adapté de Fillinger *et al.* 2007.

Les populations de pourriture grise sont décrites dans la littérature comme génétiquement très diverses, d'après différents types de marqueurs neutres (AFLP, RAPD, RFLP, SSR, éléments transposables), dans différents pays du monde (France, Chili, Californie, Canada, Bangladesh, Croatie, Macédoine, Grèce, Espagne, Italie, Australie, Tunisie, Allemagne, Hongrie, Grande-Bretagne, Serbie, Iran) et sur différents hôtes : vigne, tomate, concombre, fraise, mûre, groseille, kiwi, pois-chiche, pastèque, fève notamment (Giraud *et al.* 1997 ; Alfonso *et al.* 2000 ; Baraldi *et al.* 2002 ; Munoz *et al.* 2002 ; Fournier *et al.* 2003 ; Moyano *et al.* 2003 ; Topolovec-Pintaric *et al.* 2004 ; Ma *et al.* 2005 ; Calpas *et al.* 2006 ;

Isenegger *et al.* 2008a ; Isenegger *et al.* 2008b ; Karchani-Balma *et al.* 2008 ; Kretschmer *et al.* 2008 ; Vaczy *et al.* 2008 ; Decognet *et al.* 2009 ; Mirzaei *et al.* 2009 ; Tanovic *et al.* 2009 ; Munoz *et al.* 2010 ; Rajaguru *et al.* 2010 ; Valiuskaite *et al.* 2010 ; Esterio *et al.* 2011 ; Fekete *et al.* 2012 ; Kuzmanovska *et al.* 2012 ; Samuel *et al.* 2012 ; Leroch *et al.* 2013). Parmi les quelques travaux étudiant le pouvoir structurant de plusieurs facteurs, l'adaptation à la plante hôte est démontrée comme le facteur différenciant le plus efficacement les populations, sauf dans une situation (Ma *et al.* 2005). Une faible structuration par la géographie est régulièrement mais pas systématiquement démontrée à l'échelle d'un pays, selon notamment la présence de barrières physiques (Karchani-Balma *et al.* 2008). La différentiation géographique est cependant visible à l'échelle continentale (Asie-Océanie), par une analyse assignant systématiquement les individus dans des clusters correspondants aux pays de collecte, indépendamment de la présence des transposons (Isenegger *et al.* 2008a). Enfin, un cas d'adaptation aux conditions de stockage (réfrigéré *vs* non réfrigéré) a été décrit à partir de souches isolées de kiwi (Baraldi *et al.* 2002).

4.3 Lutte contre la pourriture grise

Plusieurs méthodes sont utilisables pour lutter contre la pourriture grise (Note nationale « Maladies de la vigne » 2013, http://www.afpp.net/apps/accueil/autodefault.asp?d=5121). La prophylaxie consiste à éliminer les sources d'inoculum primaire, constituées par les déchets végétaux et les bois de l'année contaminés, à diminuer la vigueur de la vigne (par la maîtrise de la fertilisation azotée, le choix du porte greffe et du clone, et la gestion de l'enherbement) et enfin à augmenter l'aération des grappes par des éclaircissages, effeuillages et rognages réguliers. La lutte contre les insectes vecteurs permet également de réduire les contaminations secondaires. La sensibilité variétale est essentiellement liée à la compacité de la grappe, la production de phytoalexines stilbéniques, l'épaisseur de la cuticule, la quantité de lenticelles de la baie, et la précocité du stade de sensibilité de la baie (Dubos 2002 ; Kretschmer *et al.* 2007). Les cépages champenois (Chardonnay, Pinot noir, Pinot meunier) utilisés pour leur typicité aromatique, sont réputés très sensibles à sensibles, ce qui limite l'intérêt de la lutte variétale dans cette région. La lutte biologique repose sur l'utilisation de champignons filamenteux antagonistes, comme *Trichoderma, Gliocladium* et *Ulocladium*, de levures des genres *Pichia* ou *Candida* ou de bactéries des genres *Bacillus et Pseudomonas* (Dubos et al. 1982 ; Elad et al. 2004). En France, deux préparations à base de *Bacillus* ou d'*Aureobasidium* sont homologuées. Ces micro-organismes agissent par compétition pour l'espace ou les ressources nutritionnelles à la surface de la plante, par parasitisme direct des spores, par stimulation des défenses naturelles de la plante ou par sécrétion de molécules à activité fongicides (par exemple, la pyrrolnitrine, analogue naturel du fongicide fludioxonil, produit par un *Pseudomonas* ; Ajouz *et al.* 2010)(Elad *et al.* 2004).

La lutte chimique reste utilisée dans de nombreux vignobles où le risque épidémiologique est important, en Champagne et en Bourgogne, par exemple. Les programmes de traitement varient actuellement de 0 (vignobles du Sud) à 3 traitements à base d'anti-*Botrytis* par campagne. Dans ce dernier cas, les traitements ont lieu au stade A (chute des capuchons floraux, ce qui limite l'infection des pièces florales par les contaminations primaires), au stade B (fermeture de la grappe, ce qui limite les infections du rachis et protège l'intérieur de la grappe) et au stade C (véraison, stade de réceptivité maximale de la baie). Huit modes d'actions sont utilisables pour constituer les programmes de traitement (Figure 16). Cette grande diversité autorise des stratégies d'alternance annuelles, voire pluriannuelles, chaque mode d'action n'étant autorisé que pour une seule application par saison (Note nationale « Maladies de la vigne » 2013 ; http://www.afpp.net/apps/accueil/autodefault.asp?d=5121).

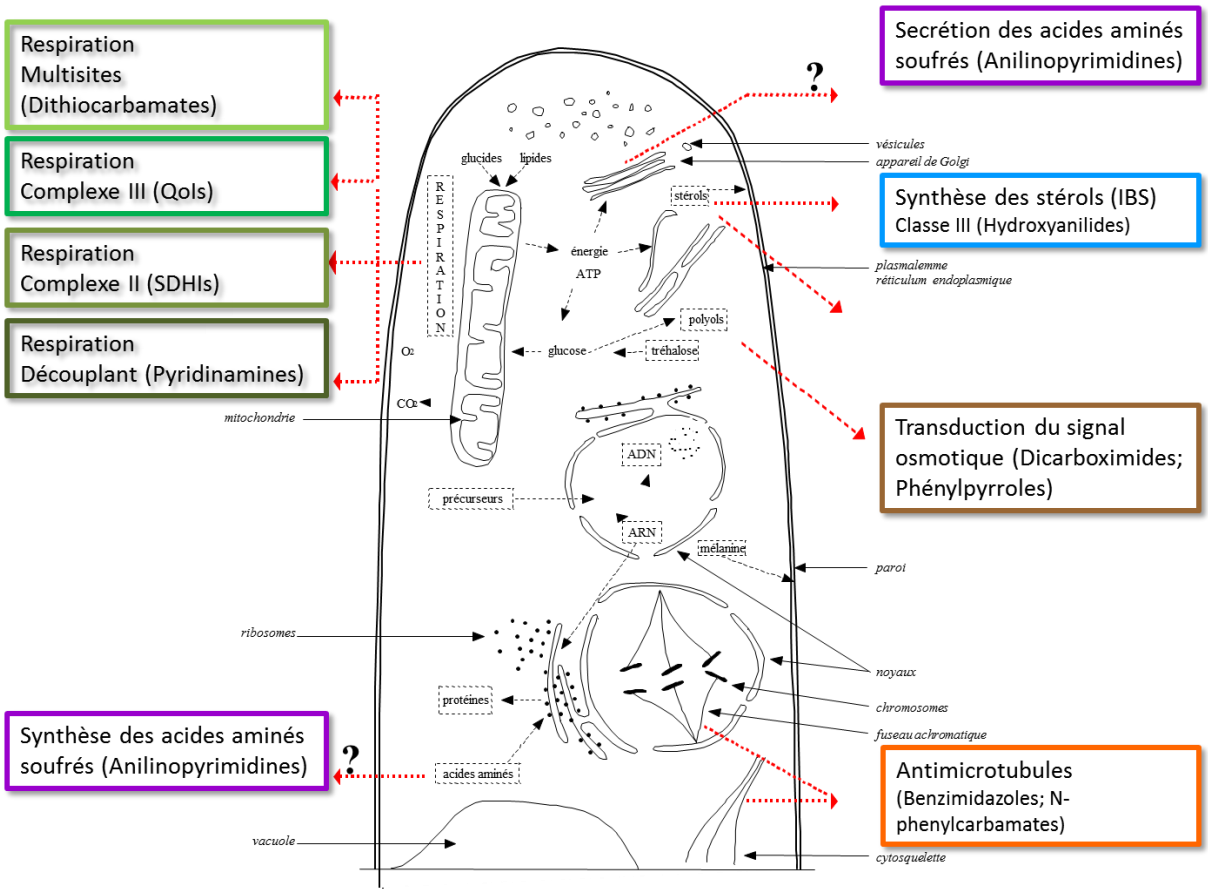

Figure 16 : Mode d'action des principales familles de fongicides anti-*Botrytis.* Adapté de Lepoivre, 2007.

4.4 Résistance aux fongicides chez *Botrytis cinerea*

La résistance acquise aux fongicides est définie au niveau individuel par l'organisation européenne de protection des plantes (OEPP) comme une « réduction stable de la sensibilité d'un champignon à un produit fongitoxique, résultant d'un changement génétique » (Anonymous 1988). Autrement dit, la croissance et le développement des souches

résistantes ne sont plus inhibées à des concentrations en fongicide qui affectent fortement les souches sensibles, *i.e.* les souches résistantes se sont adaptées à un nouvel environnement soumis à des traitements réguliers, dans lequel leur fitness est améliorée (Milgroom *et al.* 1989). En ce sens, la résistance acquise aux fongicides est donc bien un exemple de microévolution darwinienne, opérant à une échelle humaine. En pratique, lorsque la fréquence des souches résistantes atteint un certain seuil et que les facteurs de résistance (mesurés par le ratio CI_{50} souche résistante / CI_{50} souche de référence, calculé à partir des mesures des doses de fongicides qui inhibent de 50% la croissance du champignon) sont suffisamment élevés, l'efficacité des fongicides peut être affectée au champ (résistance en pratique). La résistance acquise est donc une condition nécessaire mais pas suffisante à la résistance en pratique, puisque celle-ci dépend de la biologie de l'espèce, du fongicide (mécanisme de résistance) et de l'usage que l'on en fait (voir la grille de risque proposée dans le tableau 2). En général, la résistance aux fongicides est causée par des mutations affectant la structure de la protéine cible des fongicides (Leroux *et al.* 2002). Cependant, d'autres mécanismes sont connus, comme la surexpression de la protéine cible (exemple de la surexpression du gène *cyp51*, cible des fongicides triazoles, chez *Mycosphaerella graminicola* ; Cools *et al.* 2012), la détoxification du fongicide par diverses enzymes métaboliques (exemple de la métabolisation de l'edifenphos par *Pyricularia orizae* ; Uesugi *et al.* 1993), l'augmentation de l'efflux de fongicides, lié à la surexpression de transporteurs membranaires (Kretschmer 2012) ou l'activation de voies métaboliques alternatives (par exemple, l'alternative oxidase compensant l'inhibition du complexe respiratoire III ; Wood *et al.* 2003). Enfin, la résistance acquise se distingue de la résistance naturelle, qui préexiste dans les populations à l'introduction du fongicide (exemple de *B. pseudocinerea* naturellement résistant au fenhexamid, ou de *Oculimacula acuformis* naturellement résistant aux triazoles (Leroux *et al.* 1999 ; Leroux *et al.* 2013a).

Botrytis cinerea est un champignon à risque fort de résistance (Tableau 2), (1) parce que de nombreuses caractéristiques de sa biologie rendent plus efficace l'effet de la sélection naturelle (grandes tailles de populations, dissémination des conidies par le vent sur de longues distances, importante variabilité génétique, existence d'une reproduction sexuée et asexuée, large spectre d'hôtes…) et (2) par ce que la sensibilité intrinsèque des fongicides est généralement inférieure chez cette espèce comparativement à d'autres, ce qui oblige à des traitements à doses fortes et limite le recours aux stratégies de mélanges (généralement sous-dosés). En conséquence, la résistance spécifique a été sélectionnée pour toutes les familles d'anti-*Botrytis* homologuées, à l'exception du thirame (multisite avec risque de résistance faible, de par le mode d'action), du fluazinam (découplant de la phosphorylation oxydative) et du fludioxonil (perturbateur de la transduction du signal osmotique ; fitness des mutants de laboratoire altérée *in vitro*) (Tableau 5).

Mode d'action	Famille chimique	Fongicides	Résistance spécifique en France[a]		Articles de référence chez *B. cinerea* (mode d'action et mécanisme de résistance)
			Facteurs de résistance	*Fréquence de résistance*	
Multisites	Dithiocarbamates	Thirame[b]	-	-	
Assemblage de la β-tubuline	Thiophanates et benzimidales	Thiophanate-méthyl, carbendazime[b]	Forts	Généralisée	Yarden *et al.* 1993 ; Davidse *et al.* 1995 ; Kim *et al.* 2001 ; Leroux *et al.* 2002 ; Zhao *et al.* 2010
	N-phenylcarbamates	Diéthofencarbe[b]	Forts	Faible	
Respiration (complexe II)	Pyridine carboxamides	Boscalid	Faibles à forts	Faible	Avenot *et al.* 2010 ; Kim *et al.* 2010b ; Leroux *et al.* 2010b ; Kim *et al.* 2011 ; Veloukas *et al.* 2012
	Pyridinyl-ethyl-benzamides	Fluopyram[c]			
Respiration (complexe III)	Strobilurines	Azoxystrobine[d], pyraclostrobine[d]	Forts	Généralisée	Gisi *et al.* 2002 ; Ishii *et al.* 2007 ; Kim *et al.* 2010a ; Leroux *et al.* 2010b ; Yin *et al.* 2010 ; Vallieres *et al.* 2011 ; Yin *et al.* 2012
Respiration (découplant de la phosphorylation oxidative)	Pyridinamines	Fluazinam	-	-	Guo *et al.* 1991 ; Tamura 2000
Transduction du signal osmotique	Dicarboximides	Iprodione, procymidone[b], vinchlozoline[b]	Modérés	Faible	Fujimura *et al.* 2000 ; Kim *et al.* 2001 ; Leroux *et al.* 2002 ; Cui *et al.* 2004 ; Liu *et al.* 2008 ; Fillinger *et al.* 2012
	Phénylpyrroles	Fludioxonil	-	-	
Synthèse de la méthionine	Anilinopyrimidines	Pyrimethanil, mépanipyrim	Modérés à forts	Modérée	Masner *et al.* 1994 ; Forster *et al.* 1996 ; Fritz *et al.* 1997 ; Sierotzki *et al.* 2001 ; Fritz *et al.* 2003 ; Bardas *et al.* 2005
C4-demethylation des stérols	Hydroxyanilides	Fenhexamid, fenpyrazamine[c]	Forts	Modérée	Debieu *et al.* 2001 ; Leroux *et al.* 2002 ; Albertini *et al.* 2004 ; Fillinger *et al.* 2008 ; Billard *et al.* 2012 ; Grabke *et al.* 2013

Tableau 5 : Modes d'action des fongicides anti-*Botrytis* et phénomènes de résistance. – indique l'absence de résistance en France.

[a] Statut indicatif de la résistance en France, dans les années récentes. [b] Molécule ancienne non autorisée. [c] Homologation prochaine. [d] Autorisé dans d'autres pays que la France

4.5 Mode d'action et résistance aux SDHIs

Les SDHIs (inhibiteurs de la succinate deshydrogénase) ou carboxamides correspondent à une famille de fongicides développés dans les années 70s, de structure cis-croto-anilide (deux sous familles : les oxathiin-carboxamides, représentées par la carboxine et l'oxycarboxine) et les benzamides (représentées par le bénodanil et le flutolanil). Des modifications chimiques de la partie aminée ont permis d'élargir le spectre d'action de ces molécules (Avenot *et al.* 2010), uniquement efficaces sur les Basidiomycètes (White *et al.* 1992 ; Kulka *et al.* 1995). En vigne, le boscalid (pyridine carboxamide) a été homologué pour lutter contre la pourriture grise en 2005, et le fluopyram (benzamide) l'est déjà dans d'autres pays européens que la France. Les N-methylpyrazole carboxamides constituent l'essentiel des nouveautés fongicides actuellement développées sur grandes cultures (exemples : bixafen, fluxapyroxad, isopyrazam, penflufen, penthiopyrad, sedaxane ; Leroux *et al.* 2010a).

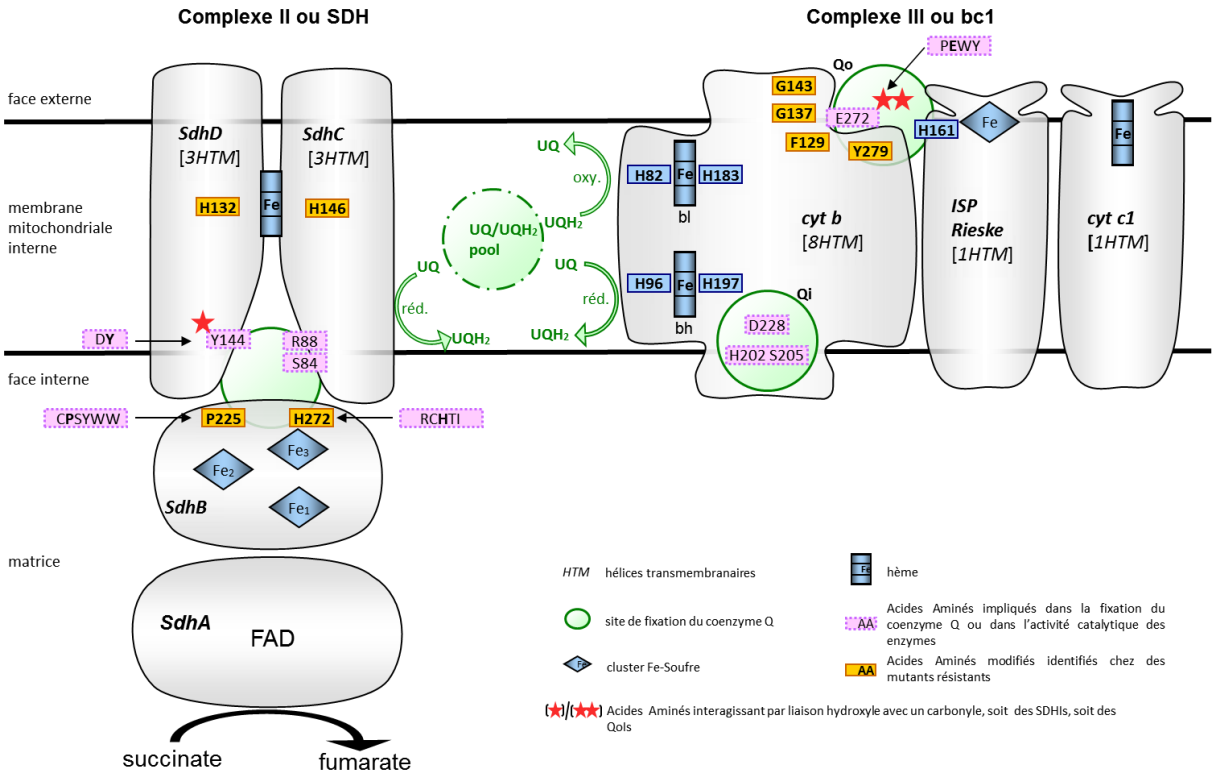

Figure 17 : Fonctionnement de la chaîne respiratoire mitochondriale au niveau des complexes II et III, montrant la structure de la succinate deshydrogénase ainsi que les acides aminés impliqués dans les différentes interactions. Le complexe III est la cible des fongicides QoIs et QiIs alors que le complexe II est la cible des carboxamides. D'après Leroux *et al.* 2010b.

Ces fongicides interagissent avec le complexe II de la chaîne respiratoire mitochondriale (Figure 17) et se substitue au coenzyme Q dans sa poche de fixation, constituée par des acides aminés des sous-unités SdhB (protéine fer-soufre), SdhC et SdhD (protéines d'ancrage dans la membrane), bloquant ainsi le transfert d'électrons du succinate (oxydé en fumarate au sein du cycle de Krebs) à l'ubiquinone (Hagerhall 1997). Le fonctionnement de la SDH, et son interaction avec le coenzyme Q et les fongicides SDHIs font l'objet d'une littérature abondante, notamment grâce à la résolution de la structure de cette protéine Sdh chez *Escherichia coli* et le poulet (voir entre autres (Hagerhall 1997 ; Yankovskaya *et al.* 2003 ; Oyedotun *et al.* 2004 ; Horsefield *et al.* 2006 ; Oyedotun *et al.* 2007 ; Tran *et al.* 2008 ; Glättli *et al.* 2011).

La résistance aux SDHIs a été observée chez des mutants de laboratoire de nombreuses espèces d'ascomycètes et de basidiomycètes (Gunatilleke *et al.* 1975; White 1988; Skinner *et al.* 1998; Irie *et al.* 2001; Ito *et al.* 2004; Shima *et al.* 2009; Scalliet *et al.* 2012). Avec la mise sur le marché de la nouvelle génération de SDHIs, la résistance est observée chez de nombreux champignons pathogènes et sur une grande variété de cultures. Ces résistances sont déterminées principalement par des modifications de la succinate deshydrogénase, en particulier sur la sous-unité B, au niveau des clusters Fer-Soufre S2 et S3 interagissant avec l'ubiquinone (positions équivalentes aux acides aminés 225 et 272 chez *B. cinerea ;* Tableau 6). Ces mutations ne déterminent pas toujours une résistance croisée entre toutes les sous-familles de carboxamides (voir Chapitre 2).

4.6 La résistance multidrogues chez *Botrytis cinerea*

La résistance multidrogues (ou MultiDrug Resistance MDR) correspond à un mécanisme de résistance original, jusqu'alors surtout décrit chez les pathogènes humains comme *Candida albicans* (Morschhäuser 2010) ou chez les cellules cancéreuses résistantes aux drogues (Gottesman *et al.* 2002). Ce phénomène résulte de la surproduction de transporteurs membranaires qui excrètent des molécules toxiques variées. Cet efflux est sous la dépendance de transporteurs ABC (ATP-binding cassette, nécessitant l'énergie de l'ATP) ou MFS (major-facilitator superfamily, utilisant le gradient de protons membranaire), (Stergiopoulos *et al.* 2002). Depuis quelques années, plusieurs cas de MDR ont été détectés chez les champignons phytopathogènes : chez *M. graminicola*, agent de la septoriose du blé (Roohparvar *et al.* 2007 ; Leroux *et al.* 2011), chez *Oculimacula yallundae*, l'un des agents du piétin-verse des céréales (Leroux *et al.* 2013a), chez *Penicillium digitatum*, agent de la pourriture des agrumes (Nakaune *et al.* 1998 ; Hamamoto *et al.* 2001) et également chez *B. cinerea* (Leroux *et al.* 1999 ; Kretschmer *et al.* 2009 ; Leroux *et al.* 2013b). Dans tous les cas, la MDR est caractérisée par des spectres de résistance croisée positive entre modes d'action indépendants et associée à des facteurs de résistance faibles à moyens, sauf si d'autres

Espèce[a]	Culture	Pays	Subsitution d'acide aminé			Reference[b]
			SdhB	SdhC	SdhD	
Alternaria alternata	Pistachier	Californie	H277Y/R	H134R	H133R D123E	Avenot *et al.* 2008 ; Avenot *et al.* 2009
Botrytis cinerea[c]	Vigne Fraisier Pommier	France Allemagne Grèce USA	H272Y/R/L P225L/T/F N230I		H132R	Leroux *et al.* 2010b ; Veloukas *et al.* 2011 ; Yin *et al.* 2011 ; Bardet *et al.* 2012 ; Leroch *et al.* 2013
Botrytis elliptica	Lys	?	H272Y/R			FRAC (www.frac.info)
Corynespora cassiicola	Concombre	Japon	H278Y/R	S73P	S89P G109V	Ishii *et al.* 2008 ; Glättli *et al.* 2009 ; Miyamoto *et al.* 2010
Didymella bryoniae	Courge	?	H277Y/R			Stevenson *et al.* 2008 ; Avenot *et al.* 2010
Mycosphaerella graminicola	Blé tendre	France		T79N		Note nationale « Maladies des céréales » 2013
Podosphaera xanthii	Concombre Melon	Japon	H278Y			Miyamoto *et al.* 2010
Sclerotinia sclerotiorum	Colza	France			H132R	Glättli *et al.* 2009 ; Note nationale « Sclerotinia » 2013
Pyrenophora teres	Orge d'hiver	Allemagne	H277Y			Note nationale « Maladies des céréales » 2013
Puccinia horiana	Chrysanthème	Japon France	?	?	?	Abiko *et al.* 1977; Grouet *et al.* 1981
Stemphylium botryosum	Asperge	?	H272Y/R P225L			FRAC (www.frac.info)
Ustilago nuda	Orge d'hiver	France	?	?	?	Leroux *et al.* 1988

Tableau 6 : Cas de résistance spécifique aux SDHIs détectés chez des souches du champ de différents champignons phytopathogènes et déterminisme moléculaire de cette résistance (changement d'acides aminés modifiant la structure des sous unités de la succinate deshydrogénase).

[a] Toutes ces résistances ont été sélectionnées par le boscalid, à l'exception des mutants de *P. horiana* et *U. nuda* sélectionnés respectivement par l'oxycarboxine et la carboxine. [b] Les notes nationales françaises sont disponibles sur le site de l'AFPP http://www.afpp.net/. [c] Des mutants résistants sans mutation sont également détectés.

mécanismes de résistance sont associés à la MDR (résistance multiple ; par exemple, résistance aux inhibiteurs de la déméthylation des stérols (IDMs) chez *M. graminicola*).

Chez *Botrytis cinerea*, trois catégories de souches MDR (MDR1, MDR2 et MDR3) sont détectées au vignoble, différenciées par leur spectre de résistance croisée (Figure 18). Si les trois phénotypes sont très résistants au tolnaftate (un fongicide inhibiteur de la squalène epoxidase, intervenant dans la biosynthèse des stérols), les souches MDR1 sont plutôt résistantes aux phénylpyrroles et aux anilinopyrimidines, alors que les souches MDR2 sont plutôt résistantes aux SDHIs, aux IDM et au fenhexamid. Les souches MDR3 cumulent les deux spectres de résistance précédents et résultent de l'hybridation entre les deux phénotypes précédents (Leroux *et al.* 1999; Kretschmer *et al.* 2008; Leroux *et al.* 2013b). Récemment, la résistance multidrogues de type MDR1 a été associée à l'ABC-transporteur BcAtrB (Kretschmer *et al.* 2009). Plusieurs mutations différentes ou une délétion chez les souches du groupe *s* dans le facteur de transcription *mrr1* induisent la surexpression de ce transporteur, attestant ainsi de sélections multiples et indépendantes pour ce phénotype (Leroch *et al.* 2013). Le phénotype MDR2 est associé à la surexpression du MFS-transporteur BcmfsM2, générée par une insertion unique dans son promoteur, suggérant ainsi une origine unique en Champagne, suivie d'une diffusion large, pour ce phénotype (Mernke *et al.* 2011). Le phénotype MDR3 comporte logiquement les deux types de réarrangements génétiques précédents (Figure 19).

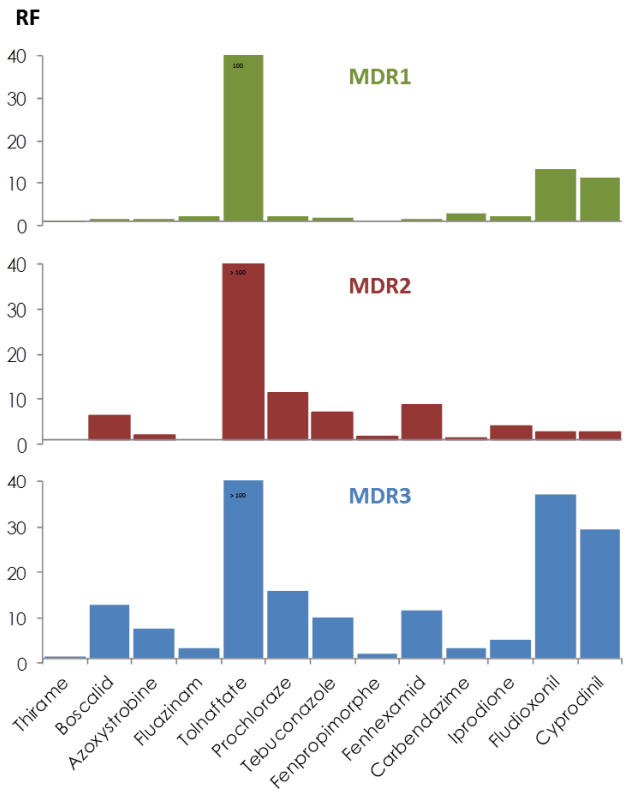

Figure 18 : Spectre de résistance croisée à différents fongicides pour les trois phénotypes multidrogues résistants de *B. cinerea.* D'après (Leroux *et al.* 2013b). RF : facteur de résistance.

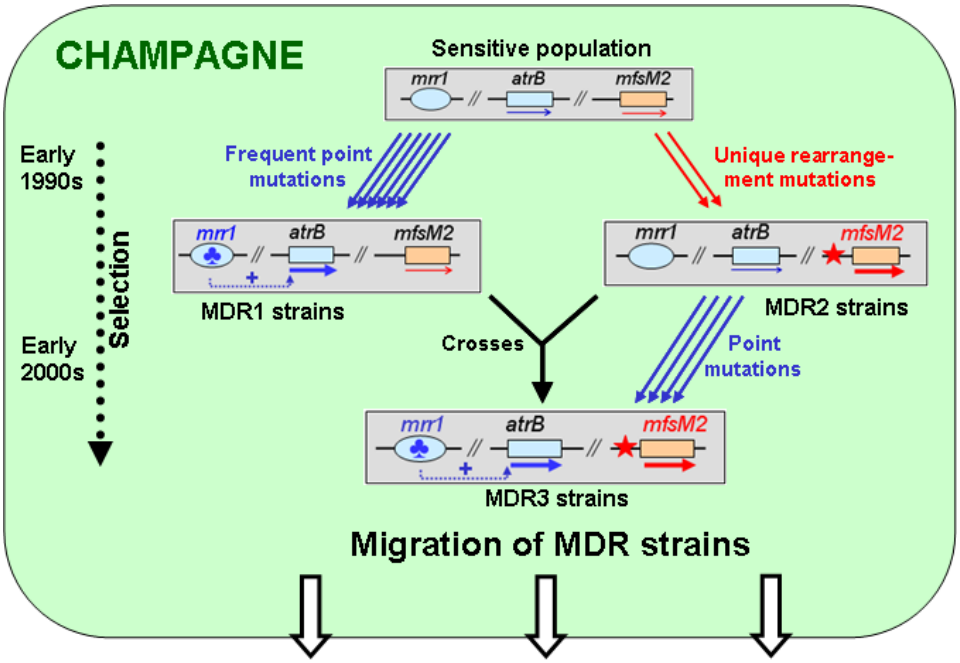

Figure 19 : Mécanisme de résistance et scénarios de sélection chez les trois phénotypes MDR de *B. cinerea*. D'après Kretschmer 2012.

5. Problématique et objectifs de la thèse

Cette introduction a montré que :

- Les populations de *B. cinerea* sont décrites comme très diverses, et potentiellement structurées par l'hôte, la géographie et la tolérance au froid. Cependant, les déterminants de cette diversité sont encore mal explorés ; de plus, les populations de *B. cinerea* subissent d'autres contraintes, dans les paysages agricoles, pouvant participer à complexifier l'image définie par ces études exploratoires ; enfin, le complexe d'espèces cryptiques mérite un établissement définitif.

- *B. cinerea* est une espèce dotée d'un fort pouvoir adaptatif, décrit *in natura* pour la résistance aux fongicides. Cependant, très peu d'éléments sont connus concernant les mécanismes évolutifs de la sélection au niveau populationnel.

Face à ce constat, cette thèse cherche (1) à parfaire la description de la diversité génétique et à expliciter comment les principales forces évolutives et le mode de reproduction structurent les populations, et (2) à comprendre comment les applications fongicides contribuent à cette diversité, dans quelles proportions et par quels mécanismes.

Ces deux questions seront successivement traitées dans les deux chapitres de la thèse :

- Le premier chapitre « Diversité et structure des populations de pourriture grise » valorise des échantillonnages populationnels emboîtés réalisés entre 2005 et 2007 en

France dans différentes niches écologiques, caractérisées par la plante hôte, le système de culture, le type écologique et la géographie. Dans un premier temps, il examine le statut de la nouvelle espèce, *B. pseudocinerea* pour en établir la position taxonomique définitive comparativement à *B. cinerea*, caractériser les abondances relatives et proposer des outils discriminants utilisables en routine pour les études populationnelles (article 1). Dans un second temps, ce chapitre se concentre sur l'espèce *B. cinerea* pour en étudier la structure fine des populations au cours du temps et évaluer l'important de la reproduction sexuée dans la diversité des populations (article 2).

- Le second chapitre « Adaptation aux fongicides chez *B. cinerea »* traite en particulier de la sélection de la résistance aux fongicides chez *B. cinerea*, au niveau individuel, par la caractérisation du mécanisme de résistance aux inhibiteurs respiratoire (article 3), puis au niveau populationnel, par la description de l'évolution des résistances aux anti-*Botrytis* en France pendant les vingt dernières années (article 4). Enfin, la troisième partie de ce chapitre vise à comprendre les mécanismes évolutifs qui sous-tendent l'adaptation des populations aux fongicides, en utilisant l'échantillonnage champenois également valorisé dans le premier chapitre (article 5).

Les différentes parties de ces chapitres sont constituées d'articles publiés, en cours de publication ou soumis dans des revues scientifiques. Ils sont donc présentés en anglais. Les principales conclusions et perspectives directes sont détaillées en français à la fin de chaque article. Enfin, une dernière partie présente les principales conclusions et perspectives de l'ensemble de l'étude.

CHAPITRE I

DIVERSITE ET STRUCTURATION DES POPULATIONS DE POURRITURE GRISE

Moulins, Aisne

« Telle est la loi du ciel dont la sage équité
Sème dans l'univers cette diversité »
Cinna, ou la Clémence d'Auguste (Pierre Corneille)

CHAPITRE I : DIVERSITÉ ET STRUCTURATION DES POPULATIONS DE POURRITURE GRISE

Avant-propos

Deux articles sont présentés dans ce chapitre.

ARTICLE 1 : ETABLISSEMENT DE LA NOUVELLE ESPÈCE *BOTRYTIS PSEUDOCINEREA*

L'objectif de cet article est d'établir définitivement l'entité *B. cinerea* groupe I comme une nouvelle espèce indépendante, en utilisant les critères d'espèces biologique, phylogénétique, écologique et morphologique, et de produire une diagnose décrivant les formes sexuée et asexuée de la nouvelle espèce.

Walker, A-S., A. Gautier, J. Confais, D. Martinho, M. Viaud, P. Le Pecheur, J. Dupont, and E. Fournier. 2011. "*Botrytis pseudocinerea*, a new cryptic species causing gray mold in French vineyards in sympatry with *Botrytis cinerea*." *Phytopathology* no. 101 (12):1433-1445. doi: 10.1094/phyto-04-11-0104.

Ce travail a également produit une version vulgarisée et a donné lieu à une communication orale :

Walker, A. S., and E. Fournier. 2012. *Botrytis pseudocinerea*, une nouvelle espèce distincte de *B. cinerea* responsable de la pourriture grise de la vigne. *Phytoma - La Défense des Végétaux*, 659, 2-10.
Walker, A. S., and E. Fournier. 2012. *Botrytis pseudocinerea*, une nouvelle espèce distincte de *B. cinerea* responsable de la pourriture grise de la vigne. *10ème Conférence Internationale sur les Maladies des Plantes (CIMA)*, AFPP. Tours, France.

ARTICLE 2 : STRUCTURE ET DIVERSITÉ DES POPULATIONS FRANÇAISES DE *BOTRYTIS CINEREA*

Cet article a plusieurs objectifs : (1) identifier les principaux facteurs qui structurent les populations de *B. cinerea*, (2) démontrer l'existence de cette structure dans différentes régions françaises, (3) démontrer la stabilité temporelle de cette structure, (4) estimer les flux de génotypes avec et sans admixture entre les compartiments et (5) détecter les signatures de recombinaison génétique.

Walker, A. S., P. Gladieux, V. Decognet, M. Fermaud, J. Confais, J Roudet, M. Bardin, A Bout, P. C. Nicot, C Poncet, and E. Fournier. 2013. "Population structure in the multihost fungal pathogen *Botrytis cinerea*: causes and implications for disease management." Soumis à *Heredity* le 3 mars 2013.

Ce travail sera également présenté oralement au « 17th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds» en avril 2013 (Friedrichroda, Allemagne) ainsi qu'au « XVI Botrytis Symposium » en juin 2013 (Bari, Italie).

Enfin, ce travail a fait l'objet d'un financement par le département SPE de l'INRA. J'ai animé ce projet pendant trois ans, en collaboration avec des collègues des centres INRA de Bordeaux, Avignon et Sophia-Antipolis.

Botrytis pseudocinerea, a New Cryptic Species Causing Gray Mold in French Vineyards in Sympatry with *Botrytis cinerea*

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ABSTRACT

Walker, A.-S., Gautier, A., Confais, J., Martinho, D., Viaud, M., Le Pêcheur, P., Dupont, J., and Fournier, E. 2011. *Botrytis pseudocinerea*, a new cryptic species causing gray mold in French vineyards in sympatry with *Botrytis cinerea*. Phytopathology 101:1433-1445.

Botrytis cinerea is a major crop pathogen infesting >220 hosts worldwide. A cryptic species has been identified in some French populations but the new species, *B. pseudocinerea*, has not been fully delimited and established. The aim of this study was to distinguish between the two species, using phylogenetic, biological, morphological, and ecological criteria. Multiple gene genealogies confirmed that the two species belonged to different, well-supported phylogenetic clades. None of the morphological criteria tested (spore size, germination rate, or mycelial growth) was able to discriminate between these two species. Sexual crosses between individuals from the same species and different species were carried out. Only crosses between individuals from the same species were successful. Moreover, population genetics analysis revealed a high level of diversity within each species and a lack of gene flow between them. Finally, a population survey over time showed that *B. cinerea* was the predominant species but that *B. pseudocinerea* was more abundant in spring, on floral debris. This observation could not be explained by temperature adaptation in tests carried out in vitro or by aggressiveness on tomato or bean leaves. This study clearly establishes that *B. cinerea* and *B. pseudocinerea* constitute a complex of two cryptic species living in sympatry on several hosts, including grapevine and blackberry. We propose several biological or molecular tools for unambiguous differentiation between the two species. *B. pseudocinerea* probably makes a negligible contribution to gray mold epidemics on grapevine. This new species has been deposited in the MycoBank international database.

Additional keywords: cryptic speciation, fungi, phylogeny.

Species definition has long been a matter of debate in evolutionary biology, leading to various species concepts (35) and, finally, to a consensus that species are segments of evolutionary lineages that evolve independently of each another (5) or subsets of organisms connected to one another through the global genealogical network, existing between two successive speciation events or between a speciation and an extinction (44). Indeed, the issue of species delimitation has long been confused with that of the criteria for species definition (6,36). In operational terms, species criteria are used to recognize and delimit species in experimental studies (6,49). Several such criteria can help to illustrate various species concepts (21,49). The biological species concept (BSC) highlights reproductive isolation (37) and is of particular interest for many fungi, particularly those that can be handled easily and crossed in the laboratory, with the generation of progeny in a reasonable amount of time. The morphological species concept (MSC) is, historically, the criterion most widely used for species delimitation. It is based on morphological similarity. Morphological observations may be challenging in microscopic organisms but the diversity of spores and sexual or asexual structures remains a widely used criterion in the description of fungal entities. The ecological species concept (ESC) focuses on adaptation to particular ecological niches and, thus, is particularly relevant for fungi, which can adapt to a wide range of environments, including extreme ones, due to the variability of their life style (i.e., parasitism, saprophytism, and symbiotism). Finally, the phylogenetic species concept (PSC) distinguishes between species on the basis of nucleotide sequence divergence. Genealogical concordance phylogenetic species recognition (GCPSR) is an extension of the PSC that makes use of the phylogenetic concordance between multiple unlinked genes to indicate a lack of genetic exchange and, thus, the evolutionary independence of lineages. This last criterion is highly discriminating and suitable for use with almost all organisms. Therefore, it has been widely used, particularly for species in which the other criteria have proved not to be relevant. In particular, GCPSR has proved immensely useful for discriminating between cryptic species (i.e., species that are morphologically similar) in fungi (7,15,27).

The mode of speciation is still the subject of active research. Allopatric speciation is thought to occur when groups of organisms are separated by geographic barriers, such as mountains or oceans, preventing gene flow between populations and resulting in reproductive isolation. It has been suggested that this mode of speciation occurs in fungi with no demonstrated substrate specialization (7). Alternatively, isolation may occur within a given geographic area in the absence of an obvious barrier to gene flow, leading to sympatric speciation. Fungi produce large amounts of spores that may easily be dispersed over large distances, reducing the likelihood of allopatric speciation. Ecological speciation (i.e., sympatric speciation promoted by disruptive selection for contrasting ecological niches) is more likely to occur in fungal species reproducing within the habitat in which they grow. For these species, adaptation to a particular niche (i.e., specialization) is a "magic" trait that is sufficient in itself to restrict gene flow in sympatry (20–22). This hypothesis applies

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particularly well to fungi that live as parasite on plants, for which specialization is often strict.

The genus *Botrytis* (Ascomycota) contains 22 highly specialized species and one hybrid. A multiple-gene genealogy study recently showed that this genus could be subdivided into two clades, one consisting of *Botrytis* spp. acting as parasites on both monocots and dicots, and the other containing *Botrytis* spp. acting as parasites on eudicots only (45). Within this second clade, *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) has the widest host range, being able to infect >220 types of eudicot, including grapevine and many fruit and vegetable crops. This species has a highly variable phenotype (i.e., production of sclerotia, density, and color of the mycelium) (34). Genetic diversity, measured either with restriction fragment length polymorphisms (RFLPs) (19) or microsatellite markers (14), is extremely high in vineyards, and linkage disequilibrium is consistent with regular recombination, even if sexual reproduction is cryptic in nature. Two types of strain have been defined, based on the presence or absence of two transposable elements, *boty* and *flipper* (9,18,31): *transposa* strains carry both *boty* and *flipper*, whereas both of these elements are absent from *vacuma* strains. Therefore, *boty* and *flipper* have frequently been used to characterize *Botrytis cinerea* populations worldwide (24,32,38,39,48). However, *B. cinerea* has recently been shown to be a complex of two sibling species living in sympatry and not corresponding to the *transposa/vacuma* subpopulations (15). *B. cinerea sensu stricto* is the predominant species, also referred to as group II; it includes both *vacuma* and *transposa* strains. The other species,

TABLE 1. Origin of strains and their use in this study

Species[a]	Strain	Origin[b]	Location	Year	Host plant	Fl[c]	Bc[d]	Bc6[e]	Analyses[f]					
									Phy	Con	Ge	Gr	T	Ag
Botrytis cinerea	VD230	INRA	Courteron, France	2007	*Vitis vinifera*	+	2	...	X	X	X	X	...	...
	VD231	INRA	Courteron, France	2007	*V. vinifera*	+	2	120	X	X	X	X	...	X
	1754	INRA	Bahlingen, Germany	1994	*V. vinifera*	+	2	120	X	X	X	X	X	X
	1755	INRA	Venningen, Germany	1994	*V. vinifera*	+	2	120	X	X	X	X	...	...
	1767	INRA	Kirweiler, Germany	1995	*V. vinifera*	+	2	124	X	X	X	X	...	...
	1768	INRA	Edenkoben, Germany	1995	*V. vinifera*	+	2	120	X	X	X	X	X	X
	1807	INRA	Cadillac, France	1996	*V. vinifera*	+	2	120	X	X	X	X	X	X
	1810	INRA	Athee, France	1996	*V. vinifera*	+	2	120	X	X	X	X	X	X
	1824	INRA	Tauxières-Mutry, France	1997	*V. vinifera*	+	2	120	X	X	X	X	...	...
	1826	INRA	Mareuil, France	1997	*V. vinifera*	+	2	120	X	X	X	X	...	...
	1905	INRA	Sydney, Australia	2001	*V. vinifera*	+	2	...	X	X	X	X	X	X
	1907	INRA	Sydney, Australia	2001	*V. vinifera*	+	2	120	X	X	X	X	...	...
	1909	INRA	Sydney, Australia	2001	*V. vinifera*	+	2	120	X	X	X	X	...	...
	1911	INRA	Sydney, Australia	2001	*V. vinifera*	+	2	124	X	X	X	X	...	...
	1916	INRA	Sydney, Australia	2001	*V. vinifera*	+	2	124	X	X	X	X	...	...
	SAS405	U. Bari	Italy	<1990	*V. vinifera*	...	2	...	X	X	X	X	...	...
	SAS56	U. Bari	Italy	<1990	*V. vinifera*	+	2	120	X	X	X	X	X	...
	T4	INRA	Eyragues, France	1991	*Lycopersicon esculentum*	...	2	124	X	X	X	X	...	X
	B05.10	U. Münster	Germany	1994	Unknown	...	2	...	...	...	...	...	...	...
B. pseudocinerea	VD233	INRA	Courteron, France	2007	*V. vinifera*	–	1	86	X	X	X	X	...	...
	VD256	INRA	Courteron, France	2007	*V. vinifera*	–	1	86	X	X	X	X	X	X
	VD296	INRA	Courteron, France	2007	*V. vinifera*	–	1	86	X	X	X	X	X	X
	VD312	INRA	Courteron, France	2007	*V. vinifera*	–	1	86	X	X	X	X	...	...
	VD320	INRA	Courteron, France	2007	*V. vinifera*	–	1	86	X	X	X	X	...	...
	VD579	INRA	Hautvillers, France	2007	*V. vinifera*	–	1	86	X	X	X	X	X	X
	VD606	INRA	Hautvillers, France	2007	*V. vinifera*	–	1	86	X	X	X	X	X	...
	VD165	INRA	Vandières, France	2007	*Rubus fruticosus*	–	1	86	X	X	X	X	...	X
	VD184	INRA	Vandières, France	2007	*R. fruticosus*	–	1	86	X	X	X	X	...	...
	VD195	INRA	Vandières, France	2007	*R. fruticosus*	–	1	86	X	X	X	X	...	...
	VD200	INRA	Vandières, France	2007	*R. fruticosus*	–	1	86	X	X	X	X	...	...
	VD110	INRA	Courteron, France	2007	*V. vinifera*	–	1	86	X	X	X	X	...	...
	VD117	INRA	Courteron, France	2007	*V. vinifera*	–	1	86	X	X	X	X	X	...
	VD43	INRA	Courteron, France	2007	*V. vinifera*	–	1	86	X	X	X	X	X	X
B. aclada	MUCL 8415	MUCL	Kitzeberg, Germany	1965	*Allium cepa*	...	...	...	X	...	...	...	...	...
B. calthae	MUCL 1089	MUCL	Lovenjoel, Belgium	1960	*Caltha palustris*	...	...	...	X	...	...	...	...	...
B. croci	MUCL 436	MUCL	Breezand, The Netherlands	1968	*Crocus versicolor*	...	...	...	X	...	...	...	...	...
B. fabae	MUCL98	MUCL	Spain	1965	*Vicia fabae*	...	...	...	X	...	...	...	...	...
B. ficariarum	MUCL 376	MUCL	Heverlee, Belgium	1957	*Ficaria verna*	...	...	...	X	...	...	...	...	...
B. galanthina	MUCL 435	MUCL	Texel Is., The Netherlands	1958	*Galanthus nivalis*	...	...	...	X	...	...	...	...	...
B. globosa	MUCL 444	MUCL	Waterloo, Belgium	1958	*A. ursinum*	...	...	...	X	...	...	...	...	...
B. hyacinthi	MUCL 442	MUCL	Breezand, The Netherlands	1958	*Hyacinthus* sp.	...	...	...	X	...	...	...	...	...
B. narcissicola	MUCL 2120	MUCL	Hull, Canada	1961	*Narcissus poeticus*	...	...	...	X	...	...	...	...	...
B. pelargonii	CBS 497.50	CBS	Frederikstad, Norway	1949	*Pelargonium inquinans*	...	...	...	X	...	...	...	...	...
B. porri	MUCL 3234	MUCL	...	1926	*A. porrum*	...	...	...	X	...	...	...	...	...
B. ranunculi	CBS 178.63	CBS	Apula, U.S.A.	1963	*Ranunculus abortivus*	...	...	...	X	...	...	...	...	...
B. sphaerosperma	MUCL21482	MUCL	...	1963	*A. triquetum*	...	...	...	X	...	...	...	...	...
B. squamosa	MUCL1107	MUCL	California, U.S.A.	1923	*A. cepa*	...	...	...	X	...	...	...	...	...
B. tulipae	BT9901	U. Wageningen	Tollebeet, The Netherlands	2000	*Tulipa* sp.	...	...	...	X	...	...	...	...	...
Sclerotinia sclerotiorum	1980	U. Florida	Nebraska, U.S.A.	...	*Phaseolus vulgaris*	...	...	...	X	...	...	...	...	...

[a] *B. cinerea* is group II and *B. pseudocinerea* is group I.
[b] U. = University of.
[c] Presence (+) or absence (–) of the transposable element *flipper*.
[d] Allele found at the *Bc-hch* locus (17).
[e] Allele size found for the microsatellite marker Bc6 (16).
[f] Symbol X indicates whether the strain was used in the phylogeny (Phy), conidia size (Con), germination rate (Ge), growth rate (Gr), effect of temperature (T), and aggressiveness (Ag) analysis.

also known as group I, has been found at low frequency in French populations and is suppose to include strains of the *vacuma* type only. This species also had a different pattern of fungicide susceptibility (phenotype HydR1), displaying natural resistance to fenhexamid and hypersusceptibility to fenpropidin and edifenphos (28). Although these two species are easily separated on the basis of phenotype and several molecular markers (14,15,17), a relevant description and characteristics for identifying *Botrytis* group I are still lacking, and the phylogenetic position of this species within the genus *Botrytis* remains unknown. Moreover, the low abundance of this species in French vineyards and its absence from many parts of the world suggest that it may have specific but as-yet-undetermined ecological requirements.

The aim of this study was to establish *B. cinerea* group I definitively as a new species, using the species criteria described above, and to complete its description, with the name *B. pseudocinerea*. The PSC was applied in a multiple-gene genealogy approach, to determine the systematic position of *B. pseudocinerea* in the genus *Botrytis*. The MSC was applied, with the measurement of several phenotypic characters in both species. The BSC was applied through inter- and intraspecific crosses. We also carried out population genetics analysis to estimate reproductive isolation between populations. Finally, we calculated the relative frequencies, over time, for each species within populations, and carried out aggressiveness and temperature adaptation experiments, to make use of the ESC. A Latin name-based diagnosis system was also generated, with description of the anamorph *B. pseudocinerea* and the teleomorph *Botryotinia pseudofuckeliana*.

MATERIALS AND METHODS

Fungal strains and populations. The fungal strains used for morphological, biological, and phylogenetic characterization were chosen from INRA collections (Table 1). The group II strains had diverse geographic origins, because this group is found worldwide, whereas the group I strains came from France only. B05.10 and T4 are reference strains, the genomes of which have been fully sequenced and are publicly available (strain T4: http://urgi.versailles.inra.fr/index.php/urgi/Species/Botrytis; strain B0510: http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/). All strains were purified from a single conidium and cultured on malt-yeast-agar (MYA) medium (malt extract at 20 g liter^{-1}, yeast extract at 5 g liter^{-1}, and agar at 12.5 g liter^{-1}), at 19°C, under continuous illumination, to induce sporulation. Reference strains of various *Botrytis* spp. were also included (kindly provided by J. van Kan, WUR Wageningen, or bought from the CBS).

The fungal populations analyzed for the assessment of genetic diversity within the two species (Table 2) were collected from vineyards in Champagne, from two host plants (grapevine and blackberry). The fungi were collected from diseased berries in September 2005 and 2006 and from floral debris in June 2006 and 2007. We generated 1,088 single-conidium strains, which were cultured on MYA medium.

All strains were stored as spore suspensions in 20% glycerol at –80°C and were subcultured on MYA before their use in experiments (Table 1) or genotyping for population genetics analysis.

Nuclear gene sequencing and phylogenetic analysis. DNA was extracted with the DNEasy Plant Mini Kit (Qiagen), adapted for automatic extraction. We assessed the phylogenetic position of the new species within the genus *Botrytis* by sequencing segments of three nuclear genes for the 31 strains analyzed in this study and for 18 representatives of the genus *Botrytis* (Table 1). Consistent with the revision of the phylogeny of the genus *Botrytis* (45), we used the glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*; gene reference in the *Botrytis cinerea* B0510 genome: BC1G_11968.1) and the heat shock protein 60 (*HSP60*; gene reference in the *B. cinerea* B0510 genome: BC1G_09341.1) genes. We added one nuclear gene from the Funybase phylogenomic database (2,33): *MS547* (encoding the ATP-dependent RNA helicase DBP7; reference gene in the *B. cinerea* B0510 genome: BC1G_03202.1). The primers used to amplify *MS547* were 5′-AAGGAGGACGTTGGAAGGAT-3′ and 5′-AAGTCC AGAATCTCGATGTATTTGT-3′. The three gene fragments were amplified by polymerase chain reaction (PCR) with specific primers, in a total volume of 50 μl containing 0.2 μg genomic DNA, 5 μl of reaction buffer, 1 μM $MgCl_2$, 2.5 μl of a 10 μM solution of each primer, and 0.2 U of EUROGENTEC *Taq* polymerase, with 30 cycles of 30 s at 94°C, 90 s at the optimal hybridization temperature for the primers, and 1 min at 72°C. The sequences obtained with the two primers were determined directly from PCR products purified on PEG 8000 (polyethylene glycol), with a CEQ8000 capillary sequencing system (Beckman Coulter). Electropherograms were analyzed and sequences were assembled and aligned with the Muscle algorithm and the CodonCode Aligner (CodonCode Corporation).

For each alignment, we identified the DNA substitution model best fitting the data with ModelTest 3.7 (42), which estimates the likelihood of each model and the parameter values under a maximum likelihood framework. The AIC-c criterion was used to rank the 56 different evolution models tested with ModelTest (41). We used the information for the chosen model and its parameters to infer the corresponding maximum likelihood gene trees with PHYML v3.0 (23). Bootstrap analyses (100 replicates) were performed to assess node support. The majority rule criterion was used to obtain the consensus trees from the bootstrap analysis, with the Consense program in the PHYLIP 3.66 package (12). The three datasets were then concatenated, and the same procedure was used to reconstruct the global tree. The topologies of the four individual trees were compared in pairs, and all the individual topologies were compared with the concatenated topology. Trees were compared by eye and by calculating the Icong (8) index of congruence between pairs of trees.

We used two methods to test the molecular clock hypothesis for the three genes. We first carried out Tajima's relative rate test (46), implemented in the MEGA4 package (47). We then used the baseml program of the PAML4 package (51) to test for constant rates of evolution along trees in a maximum likelihood framework.

Sexual crosses. The mating types of the strains included in Figure 1 were checked by PCR amplification with primers amplifying the *Botrytis* group I or group II MAT locus (A. Gautier, L. Gout, and E. Fournier; *personal communication*). The MAT1

TABLE 2. Origin and characteristics of *Botrytis* populations

Botrytis spp.	Host plant	Collection date				Total
		September 2005	June 2006	September 2006	June 2007	
B. cinerea (group II)	Grapevine	209	22	292	106	800
	Blackberry	40	215	85	63	210
	Total	249	193	377	169	1,010
B. pseudocinerea (group I)	Grapevine	0	19	11	29	60
	Blackberry	0	3	6	9	18
	Total	1	22	17	38	78
Total	...	250	237	394	207	1,088

allele yielded a PCR fragment of ≈930 bp, whereas the MAT2 allele gave a 750-bp amplicon.

Before crosses, each strain was cultured at 17°C in the dark on potato dextrose agar (PDA; ready-to-mix Difco at 39 g liter^{-1}) or malt agar (MA; malt at 20 g liter^{-1} and agar at 12.5 g liter^{-1}) medium for 2 weeks and was then incubated at 4°C for 2 months, to induce the formation of microconidia and sclerotia, respectively. Microconidia from MA petri dishes were scraped into sterile water and sclerotia were collected from PDA cultures by brushing in sterile water to separate them from agar debris and macroconidia. We mixed one to three sclerotia with 3 ml of microconidial spore suspension in six-well microtiter plates, in accordance with the interactions indicated in Figure 1 and including all reciprocal crosses. SAS405 and SAS56 are reference strains from *Botrytis* group II, and are considered good controls for the production of apothecia (11). Cross preparations were stored at 10°C in 16-h photoperiod conditions until apothecia developed. When the apothecia had turned brown, suggesting that they were mature, we dissected a number of apothecia under the microscope, to check for the presence of ascospores.

Morphological and ecological characterization. Suspensions of conidia were obtained by scraping the surface of a 10-day-old sporulated petri dish culture into sterile water. Suspensions were adjusted to a density of 200,000 spores ml^{-1} with a hemocytometer. Aliquots were observed under the microscope (×200) and three pictures were taken for each aliquot. These images were analyzed with ImageJ 1.43 software, to measure the length, width, circumference, and area of at least 100 conidia per strain.

The same spore suspensions were then used to estimate the germination rate of conidia. PDA petri dishes (50 mm in diameter) were inoculated with 300 µl of spore suspension, and the proportion of spores that germinated was evaluated after 12 h of incubation in the dark at 19°C. We also inoculated 100 ml of liquid potato broth medium with the spore suspensions, to a final density of 200,000 spores ml^{-1}. The medium was then incubated in the same conditions, with shaking at 150 rpm on an orbital shaker. The number of spores germinating (germ tube at least as long as the diameter of the spore) was determined under the microscope, with a hemocytometer, for two aliquots from each liquid culture.

Growth rate was estimated by culturing a plug of each strain on solid PDA medium in 90-mm petri dishes, incubated at 19°C in the dark. We performed three replicates per strain. For each dish, two diameters of each growing colony were measured daily, until

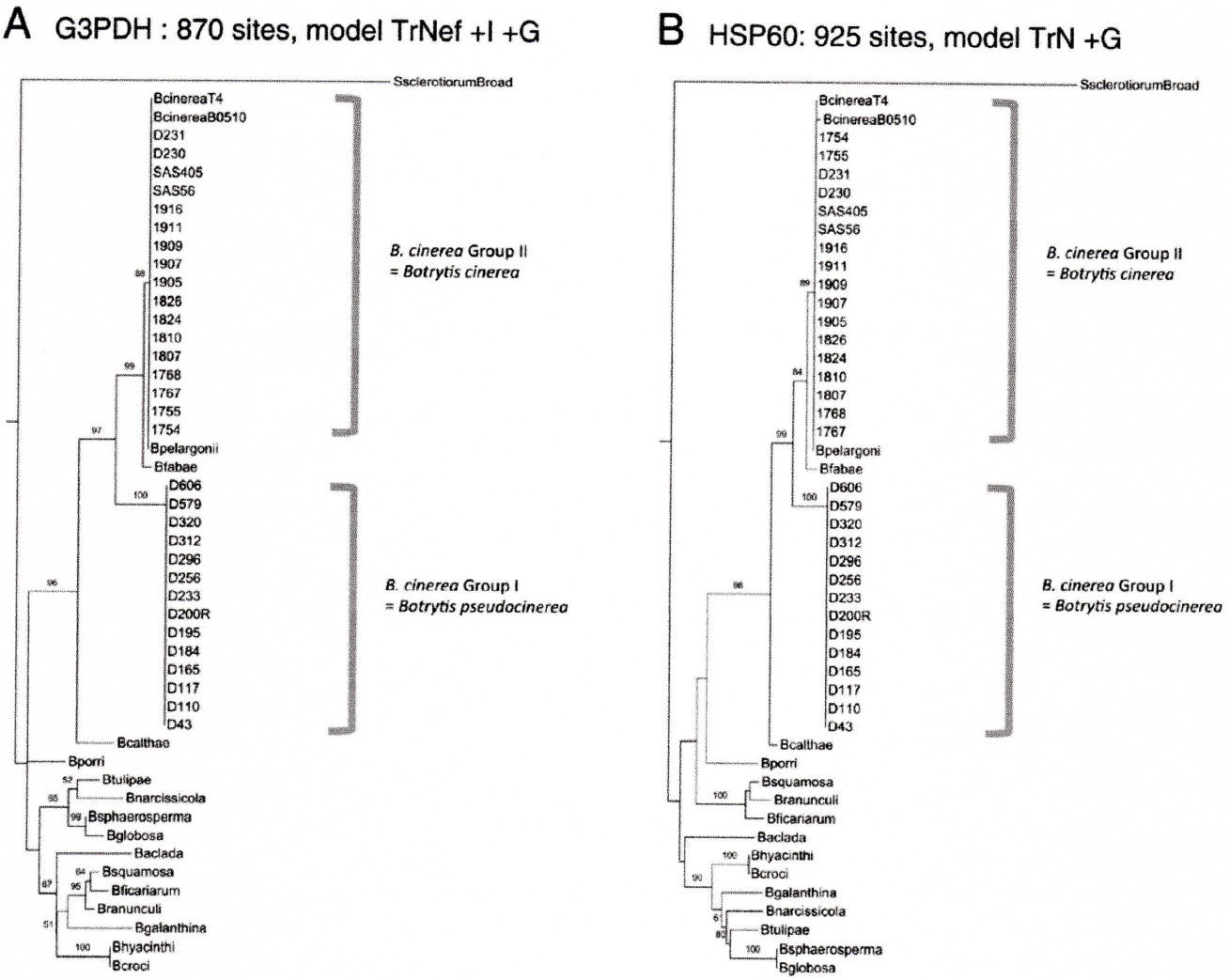

Fig. 1. Phylogenetic position of *Botrytis pseudocinerea* within the *Botrytis* genus. Trees were constructed with maximum likelihood methods for the *G3PDH*, *HSP60*, and *MS547* loci and for the concatenated dataset (the best-fitting substitution model is indicated in parentheses). Bootstrap values >50% (after 100 replications) are indicated above branches. The sequences of G3PDH and HSP60 have already been deposited (45). Those of *B. cinerea* and *B. pseudocinerea* generated in this study have been deposited in GenBank under accession numbers JF421573–74 and JF421575–76, respectively. Sequences of MS547 established for the various *Botrytis* species were deposited under accession numbers JF421577–95.

the culture reached the edge of the dish. The growth rate was then calculated in millimeters per day as the slope of the regression line fitting the curve of mean colony diameter against time.

The diagnosis was established after thorough macroscopic and microscopic examinations of both sexual and asexual stages. The Munsell Book of color (40) was used for color descriptions in the diagnosis of the new species *Botryotinia pseudofuckeliana*.

We measured fungal aggressiveness on plants by picking leaves from fresh green bean (L2 to L3, 'Caruso') and tomato (L4 to L5, 'Moneymaker') plants and placing them on damp paper in plastic boxes. We placed plugs (5 mm in diameter) of 4-day non-sporulating mycelium on the surface of the leaves, such that the mycelium was in contact with the unwounded plant tissue (eight to nine replicates per strain). These humid chambers were then incubated at laboratory temperature. The diameter of necrotic lesions was measured daily on four consecutive days. Growth rates were then calculated as described above for mycelial growth.

Optimal growth temperatures were determined by subculturing strains for 7 days in the dark at 7, 10, 15.5, 20, or 27°C. We used a minimal medium (glucose at 20 g liter^{-1}, $NaNO_3$ at 2 g liter^{-1}, and agar at 12.5 g liter^{-1}) and MYA medium for each strain (three replicates per strain per medium). Plugs of mycelium (5 mm in diameter) were deposited in the middle of 90-mm-diameter petri dishes, such that the mycelium was in contact with the medium. For each dish, we measured two diameters of the growing colony until the culture reached the edge of the dish (21 days for the most slowly growing cultures). Growth rate was calculated as described above. Sclerotia were counted on all replicates for each strain 3 weeks after inoculation, whatever their size and shape.

Statistical analyses were performed with R. We searched for outliers with boxplots and Cleveland boxplots, and we then used conditional boxplots and QQplots to assess the homoscedasticity and normality of the data. We tested for significant differences between groups for the criteria considered, by carrying out nested analysis of variance ("strain label" being nested within "i") with the LME procedure ("strain label" declared as a random effect). The normality of the residuals was checked by plotting residuals against fitted values, the histogram of residuals, and QQplots of the residuals, and by performing the Shapiro-Wilk tests. For germination rate, an arcsin(ln(x:100)) transformation was required to render the data normal.

Genetic differentiation and population phenology. DNA was extracted from fungal populations, as described above (2.2), for phylogenetic studies. Fungal strains (Table 2) were genotyped with eight microsatellites displaying cross-amplification in *Botrytis* group I and group II (16). We carried out multiplex amplifications, as previously described (30). Population structure was assessed by the clustering method implemented in Structure

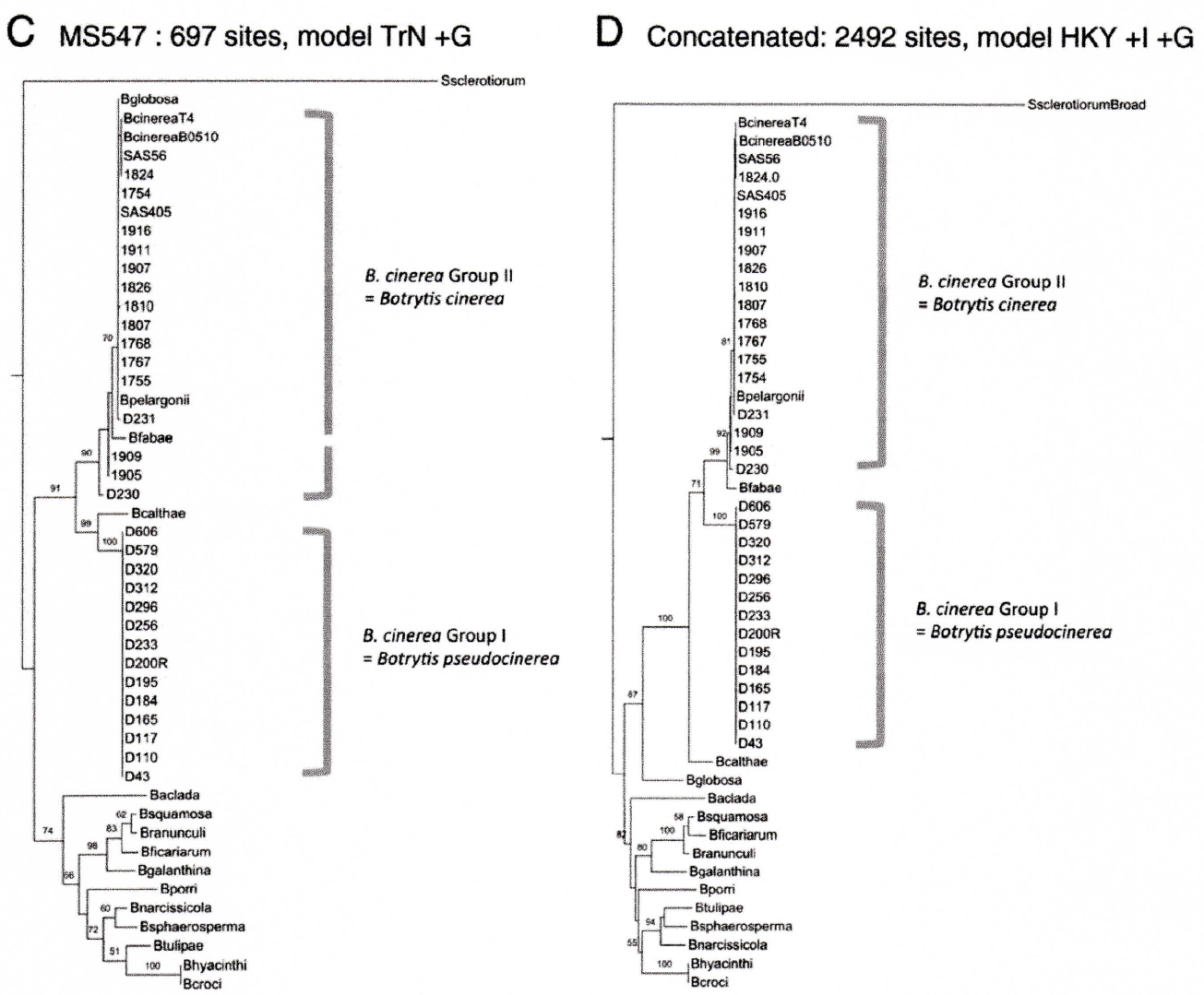

Fig. 1. (*Continued from previous page*)

(10,43), with the admixture model and a 10,000-run burn-in period followed by 100,000 Markov Chain Monte Carlo repetitions. The number of unique multilocus genotypes (G), genotypic diversity, and the r_d index, an estimate of multilocus linkage disequilibrium and its significance after 1,000 randomizations, were calculated with Gimlet (50) and Multilocus (1), within the K clusters detected after the Structure analysis. We assessed the temporal distribution of the clusters by calculating the frequency of the strains belonging to each cluster for the four collection periods. In addition, the transposable element *flipper*, which has been used to distinguish subpopulations of *Botrytis*, was detected in all strains, with primers F300 and F1500 (31). *Flipper* detection was finally carried out for the *Botrytis* group I and II strains from Table 1.

Alleles of the microsatellite Bc6 and of the *Bc-hch* gene (17) were also determined in strains from Table 1, because private alleles are usually recognized for *Botrytis pseudocinerea*.

RESULTS

PSC criterion. We investigated whether *Botrytis* group I and group II corresponded to isolated gene pools (i.e., phylogenetic species) by assessing the genealogical concordance between three independent nuclear genes (49). We sequenced the *G3PDH* and *HSP60* loci, previously used for revision of the *Botrytis* genus (45), and we added a third locus, *MS547*, extracted from the Funybase database (33). Each of the resulting alignments comprised 49 sequences (19 *B. cinerea* group II; 14 *B. cinerea* group I; 15 other *Botrytis* spp., each represented by a single strain; and the *Sclerotinia sclerotiorum* public sequence from the Broad Institute as an outgroup) and were 870, 925, and 697 bp in length for *G3PDH*, *HSP60*, and *MS547*, respectively. In the three genealogies (Fig. 1) estimated by maximum likelihood methods, all group I strains clustered into a fully supported monophyletic lineage (100% bootstrap support). The sister clade of the group I lineage was well supported in all phylogenies (84 to 99% bootstrap support). It encompasses all group II strains, together with the *B. pelargonii* representative, separated from the *B. fabae* representative by a strongly supported node, at least in the *G3PDH* and *HSP60* phylogenies (88 and 89% bootstrap support, respectively). Phylogenetic analysis of the concatenation of the three loci resolved this clade similarly, confirming that *Botrytis* group I forms a well-supported phylogenetic species that is not the sister species of *Botrytis* group II (*B. fabae* branching between them). In all phylogenies, the other *Botrytis* representatives were grouped in a separate clade, the topology of which was consistent with that reported by Staats et al. (45). The only exception was *B. globosa* in the *MS547* phylogeny; its position within the group II lineage may be due to incomplete lineage sorting at this locus, generating unsorted polymorphisms. In addition to comparing topologies by eye, we confirmed the overall congruence between phylogenies by calculating the Icong index (8). The six pairs of topologies tested were more congruent than would be expected by chance alone (Table 3).

TABLE 3. Icong index (8) for all pairs of phylogenetic trees[a]

	HSP60	MS547	Concatenated
G3PDH	2.11**	1.62**	1.78**
HSP60	...	1.46**	1.29**
MS547	...	...	2.11**

[a] A significant *P* value indicates that the compared trees are more congruent than expected by chance alone; ** indicates *P* values < 0.008 (the significance threshold after Bonferroni correction).

TABLE 4. Molecular polymorphism at the three loci sequenced in this study[a]

Nucleotide differences	G3PDH	HSP60	MS547
Fixed (total)	17	11	19
Fixed in group I and not in group II	0	1	11
Fixed in group II and not in group I	0	0	0
Shared between group I and II	0	0	0
d_I	0	0	0
d_{II}	0	1.1×10^{-4}	0.019
d_{I-II}	0.019	0.012	0.036
t_{I-II}	9.8	7	18

[a] Symbols: d_I (d_{II}) = nucleotide sequence diversity within *B. cinerea* group I (II); d_{I-II} = mean nucleotide sequence diversity between groups (number of nucleotide differences per site); t_{I-II} = divergence time between groups (millions of years), assuming a molecular clock and a mutation rate of 10^{-9} substitution/site/year.

Most of the genetic variation at the three loci tested was between groups (Table 4): no mutation was common to two groups, and most were fixed in both species, with the exception of the *MS547* locus, for which 11 of the 19 mutations were polymorphic within *B. cinerea* group II. Tajima's relative rate test for molecular clocks did not reject the hypothesis of a constant rate of evolution along branches in all three topologies (Table 5). This was confirmed by the maximum likelihood test for molecular clocks performed with PAML (data not shown). Because the molecular clock hypothesis was confirmed, we used the mean pairwise nucleotide sequence diversity between groups (d_{I-II}) to estimate the time of divergence between *B. cinerea* groups I and II ($t_{I-II} = d_{I-II}/2r$), assuming a mutation rate (r) of 10^{-9} substitutions/site/generation (25) (Table 4). Divergence times of 7 to 18 million years were obtained.

MSC criterion. Both *Botrytis* group I and group II produced tree-like (branched) conidiophores and elliptic conidia (Fig. 2). The larger of the two conidial diameters (the major diameter) did not differ significantly between the two groups (12.04 ± 1.55 µm for group I and 11.86 ± 1.45 µm for group II) and neither did conidial area (mean = 91.29 ± 21.85 µm^2 for group I and 87.77 ± 18.84 µm^2 for group II) (Table 6). There was no significant difference between the two groups in terms of the sporulation rate measured on solid PDA (mean = 96.58% for group I and 94.60% for group II) or in liquid potato broth (93.56% for group I and 92.42% for group II) (Table 6). The two groups did not differ in growth rate on PDA (mean = 27.10 mm/day for group I and 25.56 mm/day for group II) (Table 6). Therefore, we were unable to identify any morphological criterion that reliably distinguished between the two species.

TABLE 5. Tajima's relative rate test of the molecular clock hypothesis[a]

Third sequence	G3PDH	HSP60	MS547
Botrytis cinerea group II (1,754)	0.25 (0.62)	1.60 (0.20)	2.13 (0.14)
B. fabae	0.06 (0.8)	0.33 (0.56)	2.13 (0.14)
B. calthae	0.43 (0.5)	3 (0.08)	0.06 (0.14)
B. narcissicola	3.67 (0.06)	2.31 (0.13)	0.22 (0.64)
B. galanthina	0.23 (0.63)	0.42 (0.52)	0.02 (0.88)
B. porri	1.96 (0.16)	2 (0.16)	0.08 (0.77)

[a] *Sclerotinia sclerotiorum* was considered as the outgroup, and we used *Botrytis* group I D195 as the reference sequence. The third sequence used for the test is indicated in column 1. Values shown are χ^2 statistics (*P* value in parentheses, 1 df). A nonsignificant *P* value indicates that the molecular clock hypothesis cannot be rejected.

BSC criterion. *Crosses between and within groups.* We carried out 69 intra- and interspecific crosses (8 intragroup II, 50 intragroup I, and 11 intergroup crosses), including 26 control crosses (Fig. 3). As expected, the control cross between SAS56 and SAS405 (11) produced mature apothecia (i.e., containing viable ascospores). The other control crosses of each strain with itself produced no apothecia, as expected. Interspecific crosses of the strains of group I and group II with a strain of the opposite mating type produced viable apothecia in only one case (VD184 × SAS56), in which a single apothecium devoid of ascospores was produced. These experiments confirmed the existence of a barrier to reproduction between the two species.

The eight crosses in addition to the SAS505 to SAS56 control cross that produced mature apothecia all involved the crossing of strains of opposite mating types from the same group (one intragroup II cross and seven intragroup I crosses). The only exception was the cross between the MAT2 strains VD230 and VD231 (group II), which produced two mature apothecia, possibly due to contamination of this cross or one of the strains being a dual-mating type strain. No difference in the production of mature apothecia was observed between species. The size, shape, and color of apothecia from group I, produced here for the first time, were very similar to those of group II, confirming the lack of morphological differences observed at the sexual stage.

Genetic structure between and within B. cinerea groups I and II. We extended the BSC criterion by using population genetics to assess gene flow between the two groups. Single-spore isolates from *Botrytis* groups I and II used for morphological and phylogenetic characterization (Table 1) were genotyped for several molecular markers. As expected from the results of a previous study (17), all *Botrytis* group I strains carried allele 1 for the Bc-*hch* gene, whereas all group II strains carried allele 2. Furthermore, all the group I strains carried a unique allele (encoded as allele 86 in our genotyping) at the microsatellite locus Bc6. This allele size was not found in any of the tested *Botrytis* group II strains. Therefore, these two markers can be used to distinguish between the two genetic entities in routine conditions (Table 1). In the reduced dataset used in Table 1, the *flipper* transposable element was detected in all strains from group II and in none of those from group I (but see below).

We then used the eight microsatellite markers to genotype larger population samples of both species (Table 2). We analyzed the multilocus genotypes of the whole sample (strains from the two cryptic species considered together) or by collection date, using Structure software to determine the optimal number of genetic clusters (K) without the need for prior assumptions. This analysis identified two strongly supported clusters corresponding precisely to groups I and II, referred to hereafter as cluster I and cluster II, respectively. Cluster I contained 78 strains, all from group I, and the mean ancestor probability for this cluster was 0.971. We checked for substructuring within cluster II by performing a second Structure analysis for the individuals of this cluster only. This analysis subdivided cluster II into two clusters: IIa and IIb. Cluster IIa comprised 200 strains, mostly collected from blackberry (55%), with a mean ancestor probability of 0.913, and cluster IIb comprised 810 individuals, mostly collected from grapevine (87%), with a mean ancestor probability of 0.956. The *flipper* transposable element was detected at variable frequency in all clusters and subclusters, including cluster I. The proportion of strains containing *flipper* within populations differed significantly between seasons ($\chi^2 = 38.33$, $P = 0.0005$, df = 8). The frequency of *flipper* was higher in clusters IIa and IIb than in cluster I (Fig. 4A), as expected. The number of unique multilocus genotypes (G) was calculated for each cluster and for each collection date. Clonal richness, measured by determining G/N ratio, was 0.89 to 1 in clusters I and IIa but lower (0.51 to 0.80) in cluster IIb, indicating a higher proportion of clones in this cluster (Fig. 4B). Clonal richness was systematically higher in June than in September for cluster IIb, possibly reflecting more intense clonal multiplication between September and August in any given year. The size and level of G/N variation of clusters I and IIa were too low for any firm conclusions to be drawn. Genotypic diversity

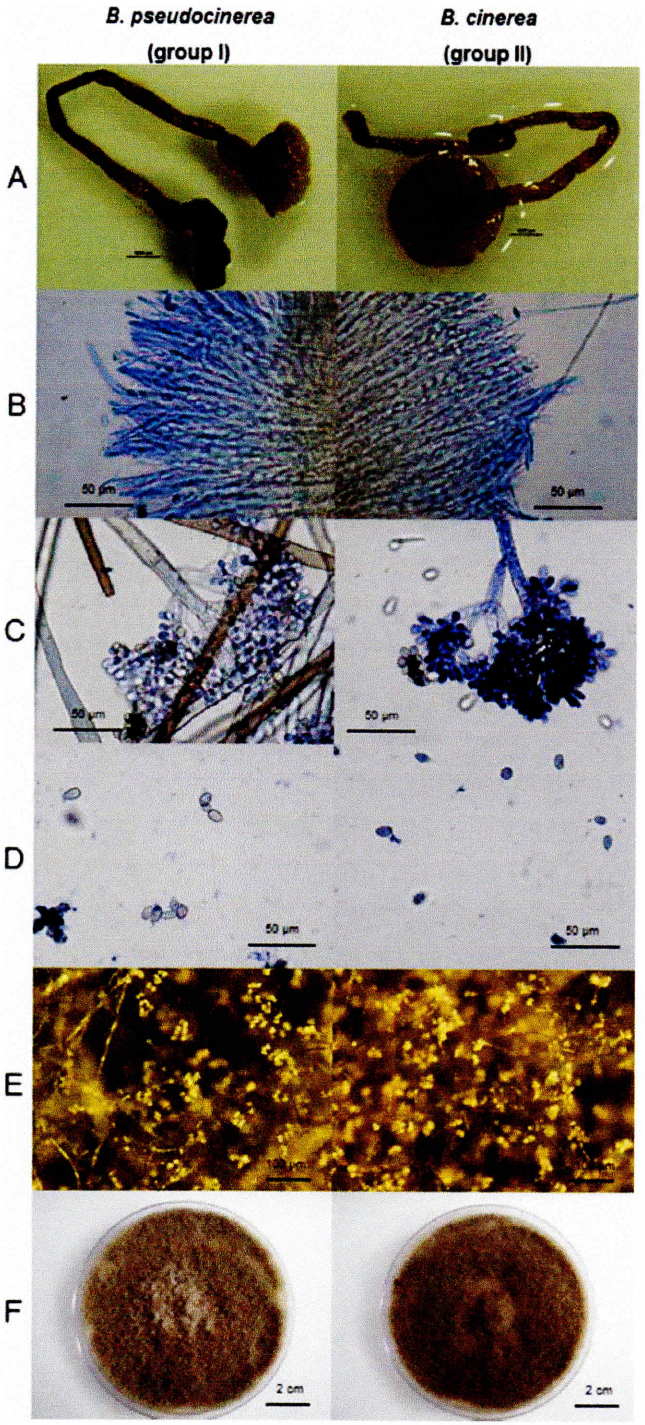

Fig. 2. Morphological characteristics of *Botrytis pseudocinerea* (left) and *Botrytis cinerea* (right). **A,** Apothecia, the sexual structure of *Botrytis* spp. **B,** Asci, containing ascospores (sexual spores), observed after the dissection of apothecia. **C,** Tree-like conidiophores, bearing conidia. **D,** Conidia (asexual spores). **E,** Sporulating mycelium (10 days). **F,** Ten-day sporulating culture on malt-yeast-agar medium. Observation of strains VD256 (*B. pseudocinerea*) and 1810 (*B. cinerea*). Typical apothecia obtained after the crosses described in Figure 3. Apothecia obtained after cross VD110 × VD184 for *B. pseudocinerea* and VD230 × VD231 for *B. cinerea*. Cotton blue staining was used for microscopy.

(Fig. 4C) was >0.99 in all clusters at all collection dates. Multilocus linkage disequilibrium, estimated by calculating the r_D index (Fig. 4D), was <0.25 for all clusters at all collection dates, reflecting very low levels of clonality in all populations ($r_D = 1$ for full clonality). These findings support the hypothesis of regular sexual reproduction in all clusters and, thus, in both species, as previously shown (14,19).

ESC criterion. The proportion of individuals in each cluster at each date was used to compare phenology between the two cryptic species. Groups I and II were found at all collection dates but in very different proportions, indicating phenological differences between the two species, although these differences were not significant over time ($\chi^2 = 3.24$, $P = 0.357$, df = 3) (Fig. 4A). Group II strains were systematically the most frequent but group I strains were more abundant in June (9.3 and 18.4% of the whole population in 2006 and 2007, respectively) than in September (0.4 and 4.3% of the whole population in 2005 and 2006, respectively). It is cooler early in the season and the June strains were collected from dead floral debris, whereas the September strains were collected from living berries. Therefore, we checked for ecological adaptation of the two species by assessing their aggressiveness on plants and their tolerance to extreme temperatures.

In planta aggressiveness was measured on tomato and bean plants from which none of the strains were collected, to limit bias due to host plant adaptation. Both species grew more slowly on tomato leaves (6.08 ± 0.38 mm/day for group I strains and 5.57 ± 0.39 mm/day for group II strains) than on green bean leaves (9.93 ± 0.41 mm/day for group I strains and 12.38 ± 0.34 mm/day for group II strains) but the difference in growth between groups I and II was not significant, regardless of the plant considered (P = 0.26 on tomato and 0.06 on green bean leaves, respectively) (Fig. 5). Similarly, we found no significant difference in optimal growth temperature or tolerance to extreme temperatures between the two species (Table 6). Temperature had a significant effect in both species when colonies were grown on rich medium, with an optimum growth temperature of 20°C (mean growth at this temperature = 21.80 mm/day for group I and 21.27 mm/day for group II) but no such effect was observed on minimal medium. Neither the group effect nor the interaction between temperature and group was significant, indicating that growth rates at the temperatures tested and optimal temperatures were similar for both groups. On minimal medium, the number of sclerotia produced differed significantly between groups I and II ($P = 0.04$ for the group effect) at all tested temperatures ($P = 10^{-4}$ for the temperature effect) but the direction of the difference was changed between temperatures ($P = 10^{-4}$ for the group–temperature interaction). These significant effects were not found on rich medium.

DISCUSSION

Identification of a new *Botrytis* sp. On the basis of several classical descriptors (e.g., phenotypic, ecological, and genetic), *B. cinerea* has long been known to be a highly variable species (18,34). Giraud et al. (19) was the first to suspect that there were two separate genetic entities, on the basis of RFLP and transposable element data, but was unable to delimit these two entities adequately. *B. cinerea* has recently been effectively divided into two hermetic gene pools, groups I and II (15). However, there was no complete description of the new species corresponding to *B. cinerea* group I. In this study, we used several species criteria to develop an unequivocal description of the former *B. cinerea* group I as a new species. We compared the genealogies of three new nuclear loci, applying the PSC, and confirmed that *B. cinerea* group I corresponded to an isolated gene pool (i.e., a phylogenetic species). This was also confirmed by population genetic analysis based upon microsatellite markers. Several combinations of sexual crosses confirmed the status of *B. cinerea* group I as a biological species, in accordance with the BSC. We were unable to distinguish between the two species according to the MSC, because they did not differ significantly for any of the morphological characters measured (major diameter and area of conidia, growth

TABLE 6. Morphological and ecological characteristics of *Botrytis pseudocinerea* and to *B. cinerea*[a]

Measured criterion[b]	*B. cinerea* (group II)	*B. pseudocinerea* (group I)	*P* value[c]
Conidial major diameter (µm)	11.86 ± 1.45	12.04 ± 1.55	0.14
Conidial area (µm²)	87.77 ± 18.84	91.29 ± 21.85	0.08
Germination rate (%) on solid PDA medium	94.60 ± 5.65	96.58 ± 3.72	0.83
Germination rate (%) on liquid PDB medium	92.42 ± 6.77	93.56 ± 4.57	0.52
Growth rate (mm/day) on PDA medium	25.56 ± 4.61	27.10 ± 2.96	0.29
Growth rate (mm/day) on minimum medium at			
7°C	7.85 ± 1.35	5.62 ± 1.56	...
10°C	10.86 ± 1.87	8.83 ± 1.98	...
15.5°C	15.63 ± 2.80	12.28 ± 2.82	G: 0.08
20°C	16.67 ± 1.40	15.63 ± 1.91	T: 0.98
27°C	4.14 ± 0.87	3.92 ± 0.57	G × T: 0.31
Growth rate (cm/day) on MYA medium at			
7°C	6.05 ± 1.20	7.16 ± 0.83	...
10°C	10.19 ± 1.52	11.39 ± 1.57	...
15.5°C	16.66 ± 0.91	17.25 ± 0.17	G: 0.59
20°C	21.27 ± 0.40	21.80 ± 0.29	T: 0.004
27°C	4.52 ± 2.17	10.20 ± 2.48	G × T: 0.17
Number of sclerotia on minimum medium at			
7°C	73.33 ± 72.43	34.05 ± 12.02	...
10°C	79.22 ± 41.72	72.22 ± 17.93	...
15.5°C	78.83 ± 32.11	103.50 ± 15.64	G: 0.004
20°C	76.05 ± 31.62	117.39 ± 20.26	T: 10^{-4}
27°C	31.22 ± 37.15	105.72 ± 138.23	G × T: 10^{-4}
Number of sclerotia on MYA medium at			
7°C	106.33 ± 105.08	20.28 ± 16.61	...
10°C	55.78 ± 45.78	47.72 ± 14.63	...
15.5°C	77.33 ± 43.38	85.06 ± 18.34	G: 0.86
20°C	45.22 ± 20.53	84.56 ± 15.15	T: 0.24
27°C	196.17 ± 173.13	43.22 ± 38.41	G × T: 0.07

[a] Measurements are mean value for each species, followed by standard deviation.
[b] PDA = potato dextrose agar, PDB = potato dextrose broth, and MYA = malt-yeast-agar.
[b] *P* value of the group (G) effect, unless another factor or interaction is specified; T = temperature effect.

rates, and conidial germination rates on different media). Nevertheless, conidial area was higher in group I than in group II and this difference was close to the threshold for statistical significance ($P = 0.08$), and this is consistent with previous observations (15). Finally, according to the ESC, the only element confirming that *B. cinerea* group I was a new species was the difference in phenology, this species being found predominantly in spring, at least on grapevine and blackberry. However, this finding was not supported by differences in optimal temperature in tests in vitro. We suggest that this species should be named *Botrytis pseudocinerea* (anamorph)/*Botryotinia pseudofuckeliana* (teleomorph). The name *Botrytis cinerea* (anamorph)/*Botryotinia fuckeliana* should, hereafter, refer to *Botrytis cinerea* group II only. We provide below a diagnosis of this new species.

Botryotinia pseudofuckeliana A.-S. Walker, A. Gautier, J. Confais, D. Martinho, M. Viaud, P. Lepêcheur, J. Dupont, E. Fournier, sp. nov. MycoBank no.: MB 561818

Fungus heterothallicus. Apothecia cupulata et longo stipite praedita, infundibuliformia, extus intusque brunnea, usque ad 3.5 mm diam lata. Stipes concolor, flexuosus, usque ad 15 mm longus, oriens ex nigris sclerotiis. Asci cylindracei, octaspori, 150 µm longi, mixti paraphysibus filiformibus, hyalinis. Ascosporae unicellulares, hyalinae, ovoideae, 12 to 16 × 4.5 to 5.5 µm.

Status anamorphus: *Botrytis pseudocinerea.*

Botrytis pseudocinerea A.-S. Walker, A. Gautier, J. Confais, D. Martinho, M. Viaud, P. Lepêcheur, J. Dupont, E. Fournier, sp. nov.

Coloniae in PDA ad 19°C 27 mm diam d^{-1}, effusae, griseae. Sclerotia grisea vel nigra, solitaria vel gregaria. Conidiophori simplices, erecti, septati, brunnei, cellulis conidiiferis leviter inflatis ad apicem praediti. Conidia racemosa, unicellularia, hyalina vel brunnea, ellipsoidea vel ovoidea, 8 to 13 × 5 to 6.5 µm. Spermatia unicellularia, hyalina, globosa, 2.5 to 3 µm diam, orientia ex phialidibus aggregatis in spermodochiis.

Heterothallic. Apothecia cupulate and stalked, dark reddish brown (5YR/3/2 to 5YR/3/3 after (40). Cup infundibuliform up to 3.5 mm diam. Stipes flexuous, same color, up to 15mm long, arising from black (2.5Y/2/0) sclerotia. Asci cylindrical, 8-spored, 150 µm long, interspersed with filiform, hyaline, paraphyses. Ascospores ovoid, hyaline, 12 to 16 × 4.5 to 5.5 µm. Anamorph: *Botrytis pseudocinerea*.

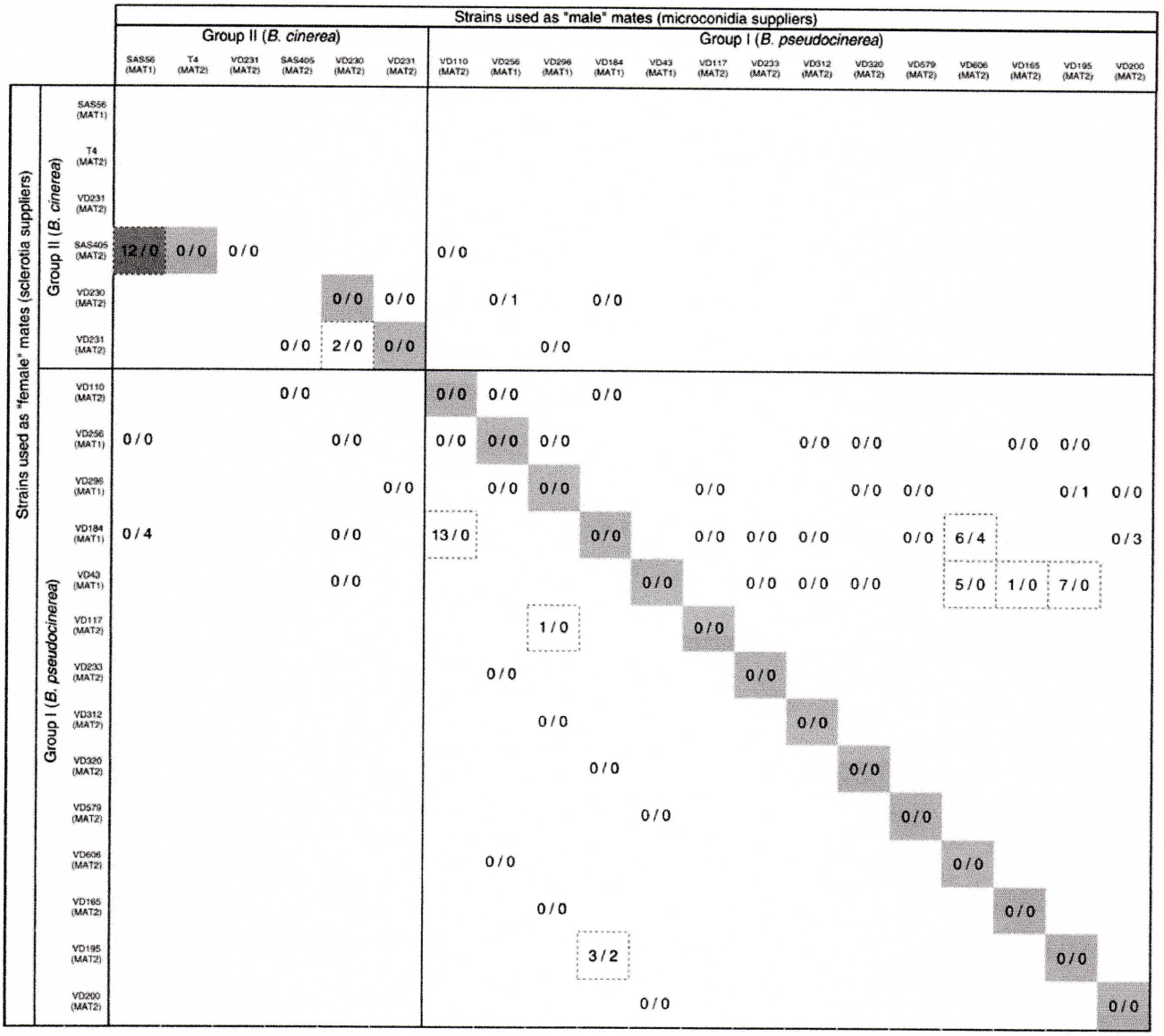

Fig. 3. Sexual crosses between *Botrytis* strains. Pale gray boxes indicate control crosses. The dark gray box corresponds to the SAS405 × SAS56 cross, considered as the reference cross in literature. Left and right numbers indicate the number of mature and aborted apothecia produced, respectively. Boxed cells indicate crosses that produced at least one viable apothecia.

On PDA at 19°C in the dark, radial mycelial growth rate 27 mm d^{-1}, colony surface gray (10YR/6/1). Sclerotia gray (2.5Y/3/0) to black (2.5Y/2/0), solitary or aggregated. Conidiophores simple, erect, septate, and brown, bearing alternate conidiogenous cells slightly inflated at the apex. Conidia in botryose clusters, one-celled, hyaline to pale brown, elliptical to ovoid, 8 to 13 × 5 to 6.5 µm. Spermatia one-celled, hyaline, globose, 2.5 to 3 µm in diam produced from phialides aggregated into clusters forming spermodochia.

Isolation: ex petal cap of *Vitis vinifera* from the Champagne vineyard, Courteron, France, 2007, collector A.-S. Walker.

Holotypus: The type specimen PC0655988, deposited in Paris herbarium (PC) is a dried culture from a pair of the isolates VD110 × VD184. Ex type: live cultures of isolates VD110 and VD184 deposited at the Fungal Culture Collection of the national Museum of natural history, Paris, with accession number LCP 05896 and LCP 05897, respectively).

Divergence between *B. cinerea* and *B. pseudocinerea*. Our study revealed that *B. cinerea* and *B. pseudocinerea* are not sister species, the species most closely related to *B. cinerea* being *B. fabae*. Considering the three genes used for our phylogeny to have evolved clockwise, and assuming a mutation rate of 10^{-9} substitution/site/year, a reasonable value for filamentous fungi (25), we estimated a divergence time between *B. cinerea* and *B. pseudocinerea* of 7 to 18 million years. These values are similar to the 7 to 15.5 million years reported in a previous study with four different nuclear genes (15) and confirm that the speciation between *B. pseudocinerea* and the common ancestor of *B. cinerea* and *B. fabae* is ancient. Moreover, because most crosses between groups I and II produced no viable apothecia, the reproductive barrier may be due to prezygotic isolation, as is often reported for ancient or sympatric divergences (26).

Further investigations are required to disentangle the speciation mechanisms and dynamics responsible for the divergence between *B. pseudocinerea, B. cinerea*, and *B. fabae*. *B. cinerea* and *B. pseudocinerea* have similar large host ranges and are found in sympatry but clearly differ in their phenology, demonstrating differences in ecological niche. If these species diverged in sympatry through ecological speciation (20), the adaptive traits targeted by disruptive selection might be related to climate, plant phenology, or trophic resources. We found no difference in cold tolerance or aggressiveness on living plants but many other param-

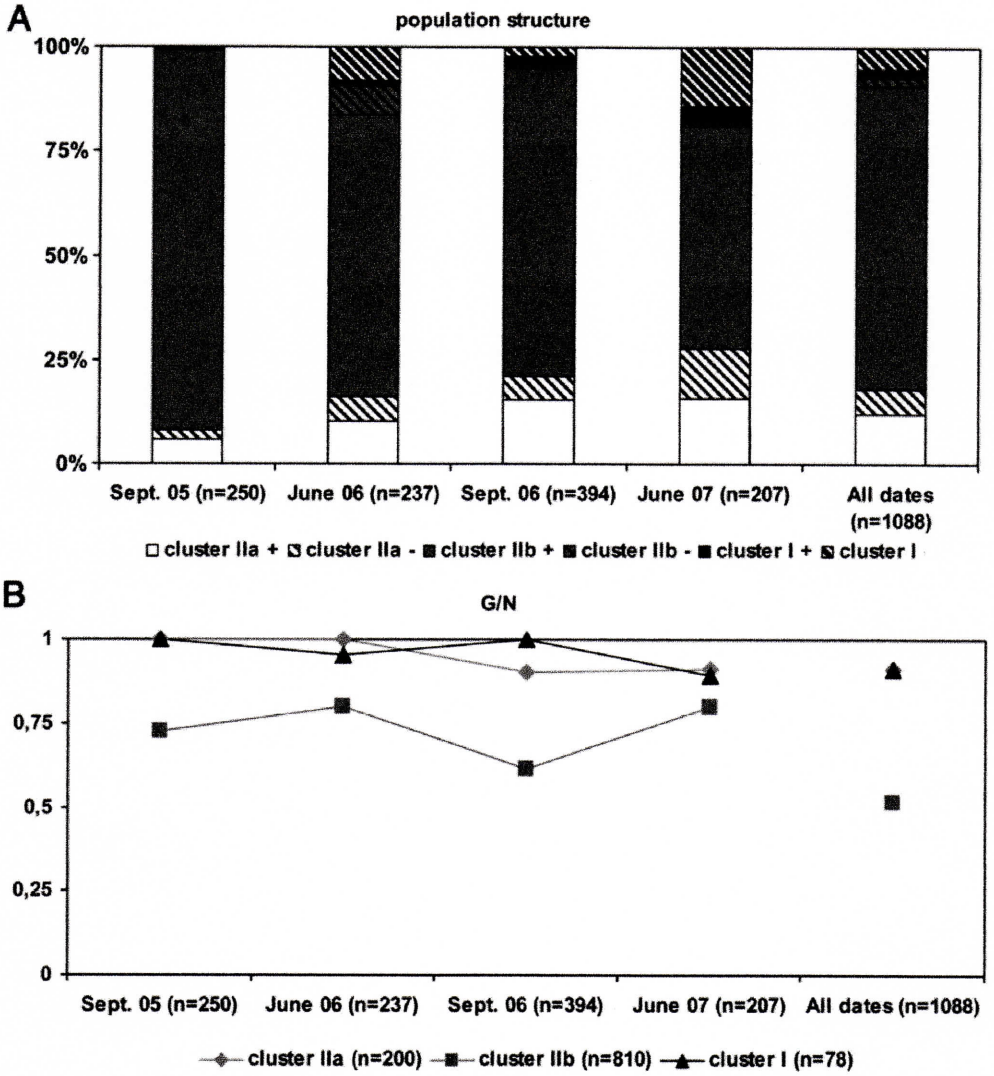

Fig. 4. Changes in the genetic structure of *Botrytis* over time. **A,** Frequencies, for each sampling date, of each cluster detected in Structure analysis. For each cluster, we indicate (hatches) the frequency of individuals for which the *flipper* transposable element was detected (+) or not detected (–). Cluster IIa and cluster IIb are *Botrytis cinerea* (group II) strains, whereas cluster I contains only *B. pseudocinerea* (group I) strains. **B,** Proportion of unique genotypes in each cluster at each sampling date. **C,** Genotypic diversity in each cluster at each sampling date. **D,** Multilocus linkage disequilibrium in each cluster at each sampling date. Values marked with an asterisk (*) are significant at the 5% level.

eters and conditions should be tested. However, the possibility of allopatric speciation followed by secondary contact cannot be ruled out. Phylogeographic approaches should help to determine which of these two scenarios is the correct one. Surprisingly, *B. pseudocinerea* has rarely been reported outside France. This may be due to poor monitoring in the spring or in other areas and to the lack of discriminatory tools, a problem that this work has rectified (see below). The observation of *B. pseudocinerea* in a limited number of European regions suggests that it may be a "relict species", the survival of which depends on undetermined local adaptation conditions.

Impact of *B. pseudocinerea* in populations and consequences. Our data suggest that *B. pseudocinerea* is present at low frequencies in populations collected from grapevine in France (0 to 15%; monitoring data; not shown). Therefore, it is unlikely to have a major effect on gray mold epidemics or to make a substantial contribution to the damage observed on grape berries at harvesting. The management of gray mold may be simpler if we need to consider only one species. The rarity of this species is all the more surprising because *B. pseudocinerea* is naturally resistant (phenotype HydR1) to the hydroxyanilide fungicide fenhexamid (28,29). Because this sterol biosynthesis inhibitor is widely used and because it is highly effective against *Botrytis* on grapevine and, particularly, as the first treatment (flowering time) of the spraying program, we would expect this species to be selected in populations, which does not seem to be the case according to population survey data. This is consistent with *B. pseudocinerea* being less fit on ripe grapevine berries than *B. cinerea* and with significant differences in the ecological niches of the two species. More generally, fungicide resistance spectrum is one of the only phenotypic criteria of any use for distinguishing between the two species. In routine monitoring, *B. pseudocinerea* strains can be easily recognized, due to their resistance to fenhexamid, particularly in the mycelial growth test, and their hypersusceptibility to fenpropidin, fenpropimorph, and, to a lesser extent, sterol demethylation inhibitors. Analysis of the full spectrum of resistance or susceptibility in an appropriate test is required for clear differentiation of HydR1 strains from those of *B. cinerea* with acquired resistance to fenhexamid (HydR2, $HydR3^+$, and $HydR3^-$ phenotypes) (13,28).

In addition to this phenotypic test, many molecular markers are now available for discriminating between the two species. Diagnostic sequence polymorphisms exist in several nuclear genes, such as the vegetative incompatibility locus *Bc-hch*, the sterol 14-α demethylase gene *cyp51*, the β-tubulin gene, the noncoding region 63R, the 3 ketoreductase gene (3,4,17), and the three genes used in our study (*G3PDH*, *HSP60*, and *MS547*). Therefore, PCR-RFLP tests, like that for *Bc-hch* (17), could be developed. The population genetics analysis conducted in this study also confirmed a unique allele at the microsatellite locus Bc6 (16) for *B. pseudocinerea*. Conversely, the *vacuma/transposa* genotyping method, based on the presence or the absence of the transposable elements *boty* and *flipper*, is clearly irrelevant for distinguishing between the two species. Indeed, although our study showed that

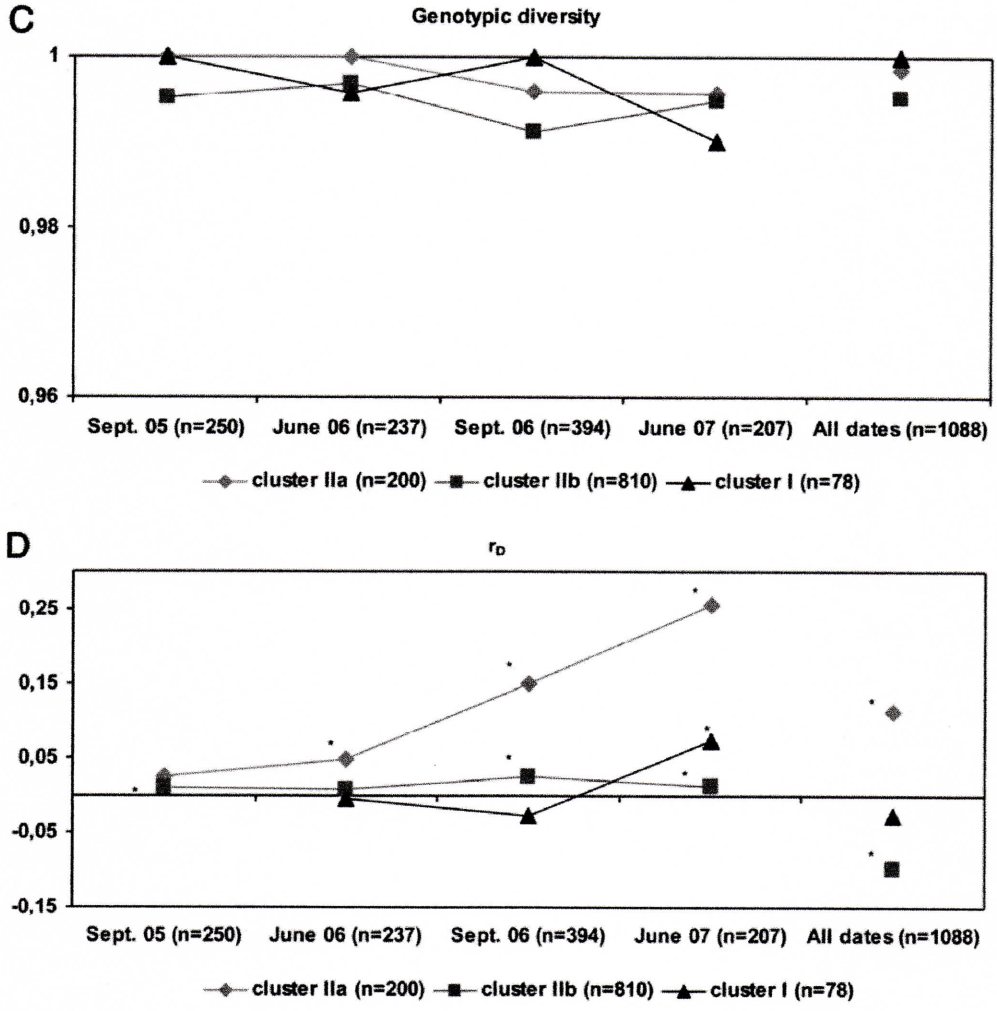

Fig. 4. (*Continued from previous page*)

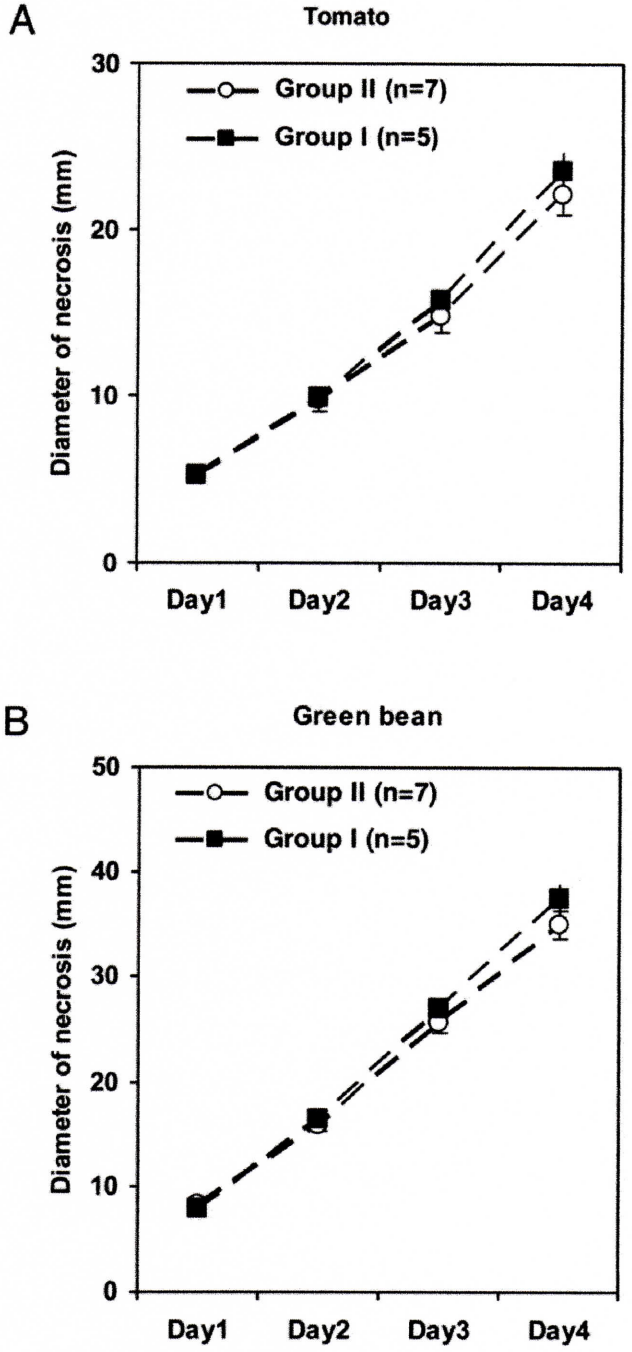

Fig. 5. Comparative aggressiveness of *Botrytis cinerea* and *B. pseudocinerea* on **A,** tomato and **B,** green bean leaves.

most of the *B. cinerea* strains harbored the *flipper* element whereas most of the *B. pseudocinerea* strains did not, many exceptions were recorded (Fig. 4A) and, therefore, this marker cannot be used for reliable species diagnosis.

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Conclusions

- *B. pseudocinerea* est décrite définitivement comme une nouvelle espèce distincte de *B. cinerea*, après l'établissement de sa diagnose et le dépôt d'une souche type dans une collection internationale (Mycobank).

- Les deux espèces sont différenciables par les critères biologique (croisements interspécifiques non fertiles ; structuration totale des populations), phylogénétique (topologie congruente pour 3 gènes) et écologique (temporalités différentes d'abondance). Le critère morphologique (taille des spores asexuées) n'est pas pertinent.

- La nomenclature *vacuma vs transposa* largement utilisée dans la littérature est obsolète. Les deux éléments transposables sont rencontrés chez les deux espèces, mais avec des fréquences contrastées.

- Les deux espèces cryptiques vivent en sympatrie sur les mêmes hôtes.

- De nombreux marqueurs phénotypiques et moléculaires sont désormais disponibles pour différencier les deux espèces.

Perspectives

- La spéciation sympatrique et les différences de temporalité suggèrent des spécificités écologiques distinctes pour ces deux espèces, que nous n'avons pas pu mettre en évidence. Une perspective serait d'affiner ce travail par des mesures de fitness plus fines, *in vitro, in planta* et *in natura.*

- L'espèce *Botrytis pseudocinerea* est résistante naturellement au fenhexamid mais n'est pas sélectionnée par ce fongicide principalement utilisé à la floraison, moment où cette espèce est la plus abondante. La caractérisation de ce mécanisme de résistance, en complément des études citées plus haut pourrait permettre d'expliciter cette observation (pour partie, thèse d'Alexis Billard, 2008-2011 et de Saad Azzedine, 2010-2013).

POPULATION STRUCTURE IN THE MULTIHOST FUNGAL PATHOGEN *BOTRYTIS CINEREA*: CAUSES AND IMPLICATIONS FOR DISEASE MANAGEMENT

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ABSTRACT

An understanding of the causes of genetic subdivision in pathogens is essential for forecasting disease emergence and implementing sound disease management strategies. We investigated population subdivision in the multi-host fungus *Botrytis cinerea,* by comprehensive multiyear sampling on different hosts (*Solanum, Vitis, Rubus*) in different cropping systems (indoor, outdoor) in three French regions. Population structure analyses revealed a clear differentiation between populations from indoor and outdoor environments, indicating that cropping system is a potentially powerful isolating factor in pathogens. Indoor and outdoor populations were significantly differentiated across sampling dates, with a quite higher magnitude than subdivision due to host plant, and to a lesser extent, to geography, in outdoor populations. The significant association between genetic differentiation and host of origin is consistent with host adaptation, but the distribution of inferred genetic clusters and the frequency of admixed individuals indicated a lack of strict host-specificity. Three genetic clusters coexisted on the same plant, *Vitis*. Linkage disequilibrium analysis indicated that recombination was not synchronous in the different clusters, suggesting a possible role of temporal isolation in genetic differentiation. Our findings open up new perspectives for disease control by managing plant debris in outdoor conditions, and prophylactic measures in greenhouses.

Keywords: population structure, temporal isolation, crop management, reproduction, migration, host plant, geography, *Botrytis cinerea*

INTRODUCTION

"Everything is not everywhere", and most eukaryotic microbial pathogens are subdivided into distinct populations (Taylor *et al.* 2006). An accurate understanding of the structure of pathogen populations and of the historical and contemporary relationships between the inferred demes is important for several reasons. First, the quantification of population subdivision and its changes over space and time provides information about the existence of reservoirs, and the transmissibility or longevity of populations; it also makes it possible to design effective identification tools targeting particular genotypes (Milgroom *et al.* 2003; Taylor *et al.* 2003; Gladieux *et al.* 2011a; Simwami *et al.* 2011). Second, on the basis of inferences concerning the processes that shaped population structure in the past, we can forecast and prevent the emergence of genotypes, populations or species with negative effects on ecosystem health and human welfare (McDonald *et al.* 2002; Giraud *et al.* 2010; Williams 2010). Third, it is also important to delimit the barriers to gene flow and to assess their permeability in the context of emerging diseases, given the recently recognized relationship between disease emergence on new hosts and reproductive isolation (Giraud *et al.* 2010; Gladieux *et al.* 2011a).

Population differentiation may be adaptive or non adaptive, and may be caused by limited dispersal, limited mating preferences and/or limited adaptation, the consequences being a divergence in gene frequencies between demes. Host-specific or geographic differentiation has been extensively investigated in fungal plant pathogens, and subdivision into multiple populations associated with different hosts or regions has been demonstrated for many species (Peever *et al.* 2000; Giraud *et al.* 2006; Gladieux *et al.* 2008; Gladieux *et al.* 2011b; Dutech *et al.* 2012; Robert *et al.* 2012). The role of other structuring factors, such as i) ecosystem features, *e.g.* wild *vs*. agricultural ecosystems (Stukenbrock *et al.* 2008; Gladieux *et al.* 2010); ii) abiotic factors, *e.g.* temperature; (Enjalbert *et al.* 2005; Zhan *et al.* 2011; Mboup *et al.* 2012); iii) agrosystem subunits or cropping systems, *e.g.* nursery *vs* commercial fields (Peever *et al.* 2000), has been much less thoroughly investigated. Temporal changes in the amount and distribution of neutral genetic variation have also seldom been investigated. However, multiyear sampling can potentially provide new insight into features of pathogen population dynamics, such as variation in migration intensity over time and space, disease spillover, hybridization, or barriers to gene flow (Gladieux *et al*. 2011a).

Botrytis cinerea (teleomorph *Botryotinia fuckeliana;* the name of the anamorph, *B. cinerea*, was chosen for this study, according to the recommendation for "One fungus, one name" nomenclature; (Wingfield *et al.*

2012)) is a filamentous, heterothallic ascomycete fungus causing gray mold on more than 220 host plants, including high-value crops, such as grapevine and tomato, and wild species, such as bramble (Elad *et al.* 2004). This fungal pathogen can also develop saprophytically, leading to the suggestion that it may recently have been derived from strict saprobes (Martinez *et al.* 2005; Gordon *et al.* 2010). *Botrytis cinerea* was long thought to be a single, but morphologically variable and generalist species. However, several recent studies have shown *B. cinerea* to be a species complex, the cryptic species *Botrytis pseudocinerea* (teleomorph *Botryotinia pseudofuckeliana*) being found in sympatry with *B. cinerea,* but at low frequency (Albertini *et al.* 2003; Fournier *et al.* 2003; Fournier *et al.* 2005; Martinez *et al.* 2008; Walker *et al.* 2011). Population genetic surveys have reported contrasting patterns of host-specific differentiation within *B. cinerea*. No significant genetic differentiation was found between isolates collected from grape, kiwifruit, pea, and squash in the Californian Central Valley (Ma *et al.* 2005), but other studies revealed significant differentiation between isolates collected from grape, tomato, kiwifruit and bramble in Chile (Munoz *et al.* 2002), from grape and bramble in France (Fournier *et al.* 2008), and from grape, tomato, faba bean and strawberry in Tunisia (Karchani-Balma *et al.* 2008). The existence of host-specific differentiation raises the question of the role of adaptation to the host in the establishment of barriers to gene flow between sympatric *B. cinerea* populations. The life cycle of *B. cinerea* is not fully understood, but dispersal between selection on the host and mating may be limited, as in many ascomycete pathogens that reproduce on the plant on which their spores initially landed. In life cycles of this type, adaptation to the host can be seen as a “magic trait”, assortative with respect to host use and leading to host-specific differentiation (Giraud *et al.* 2010). Simulations have shown that adaptation to the host in pathogens that do not disperse between development on the host and mating can even totally prevent gene flow between sympatric populations adapted to different hosts, leading to ecological speciation (Giraud *et al.* 2006). In addition to the divergent selection pressures exerted by hosts, other factors may shape population structure. These factors merit further investigation, and the role of the saprotrophic phase in the pathogen life cycle is of particular interest because it might allow mating between populations adapting to different hosts, thereby preventing host-specific differentiation. Another major factor that should be considered is geographic distance. This factor has been shown to affect the differentiation of populations of *B. cinerea* from different continents (South Asia and Australia)(Isenegger *et al.* 2008), but its effects seem to be weak at the scale of a single country (France) (Fournier *et al.* 2008). Extensive multiyear sampling nationwide in France is a useful approach for investigating the contribution of geographic distance to the subdivision of the

French *B. cinerea* population

Here, we investigated the population structure of *B. cinerea* by comprehensive hierarchical sampling over a two-year period, with four sampling dates, and several host plants, regions and cropping systems in France. We addressed the following questions: (i) What are the main factors structuring genetic variation in *B. cinerea*? (ii) Is the same population structure observed in different regions? (iii) Is population structure stable over time? (iv) What is the level of admixture and genotype flow between populations? (v) Is there evidence for recombination?

MATERIALS AND METHODS

BIOLOGY OF THE SYSTEM

Botrytis cinerea has a haplontic life cycle. The somatic mycelial system is haploid and produces asexual macroconidia and sclerotia (a melanized mycelium enabling the fungus to survive unfavorable environmental conditions). Sclerotia can germinate to produce a somatic mycelium. The fungus can propagate and disperse through several asexual cycles in spring and summer (Giraud *et al.* 1997; Elmer *et al.* 2004). In laboratory conditions, after appropriate preconditioning, a sexual stage is observed, which includes a transient diploid phase restricted to karyogamy. During this sexual stage, the mycelium produces microconidia (spermatia) that mediate the fertilization of female sexual cells within the sclerotia. The sclerotia then develop apothecia containing haploid ascospores generated by meiosis. By germinating and developing a new vegetative mycelium, the ascospores re-initiate the asexual haploid stage. Apothecia, the only macroscopic evidence of the sexual stage, are rarely found in the field (Beever *et al.* 2004). However, genetic signatures of recombination in field populations indicate that the sexual stage does indeed occur *in natura* (Giraud *et al.* 1997; Fournier *et al.* 2008), probably during winter.

SAMPLE COLLECTION

Samples were collected on four dates: September 2005, June 2006, September 2006 and June 2007. June corresponds to the start of the cropping season and the samples collected in this month were presumed to have undergone sexual reproduction during the winter. By contrast, September corresponds to the end of the cropping season, after the occurrence of asexual multiplication on the host (Table 1). Samples were collected in Champagne, the South West (Aquitaine) and South East (Provence and Côte d'Azur), at two to six

Table 1: Populations of *Botrytis cinerea* collected in three French regions, on various host plants and in different cropping systems, between 2005 and 2007

Region	Location	Host plant[a]	Cropping system[b]	Sampling date				
				September 2005	June 2006	September 2006	June 2007	**Total**
South West	Couhins	Grapevine	Open-field	54	24	63	59	200
	Fauguerolles	Tomato	Greenhouse	26	16	32	28	102
	Fauillet	Tomato	Greenhouse	32	18	24	-	74
	Grande-Ferrade	Bramble	Open-field	26	33	25	27	111
		Grapevine	Open-field	50	41	65	55	211
		Litter	Open-field	30	42	73	46	191
	Léoville-Lascaze	Bramble	Open-field	28	31	28	27	114
		Grapevine	Open-field	55	44	56	57	212
	Marmande	Tomato	Greenhouse	-	19	11	30	60
Champagne	Courceroy	Tomato	Greenhouse	-	6	55	48	109
	Courteron	Bramble	Open-field	18	4	46	25	93
		Grapevine	Open-field	53	50	99	30	232
		Litter	Open-field	19	1	-	-	20
	Foissy-sur-Vanne	Tomato	Greenhouse	24	50	-	-	74
		Bramble	Open-field	-	-	22	10	32
	Hautvillers	Grapevine	Open-field	93	59	108	47	307
	Vandières	Bramble	Open-field	23	18	17	28	86
		Grapevine	Open-field	63	84	85	29	261

South East	Carnoules	Grapevine	Open-field	8	-	-	-	8
	La Farlède	Grapevine	Open-field	20	7	14	7	48
		Litter	Open-field	22	32	-	1	55
	Berre	Bramble	Open-field	16	-	31	18	65
		Grapevine	Open-field	30	29	30	30	119
		Litter	Open-field	30	13	24	30	97
		Tomato	Greenhouse	30	30	30	30	120
	Pierrelatte	Tomato	Greenhouse	30	30	30	30	120
	Sarrians	Bramble	Open-field	31	-	30	31	92
		Grapevine	Open-field	30	25	30	29	114
		Litter	Open-field	39	-	30	30	99
		Tomato	Greenhouse	30	30	30	30	120
Total				910	736	1088	812	3546

[a] On grapevine (*Vitis vinifera)* and bramble (*Rubus fruticosus*), samples were collected from diseased berries in the fall, and from flower caps or decaying flower parts in spring. On litter, samples were collected from asymptomatic wild- or crop-plant debris on the ground in all seasons. On tomato (*Solanum lycopersicum*), samples were collected from diseased fruits in all seasons.

[b] *Solanum* samples were collected from glass greenhouses. *Vitis* samples were collected from open-field cultivated plots. *Rubus* samples were collected from plants surrounding the grapevine plots. Litter samples were collected within or very close to the grapevine plots.

separate sites per region (Fig. 1). In each region, samples were collected in two cropping conditions: greenhouses (indoors) and open-field environments (outdoors); and from four different hosts/substrates: tomato (*Solanum lycopersicum)* in greenhouses, grapevine (*Vitis vinifera*) in open-field conditions, bramble (*Rubus fruticosus*) in open-field conditions, from bushes surrounding vineyards or greenhouses, and litter in open-field conditions, on various dead wild plants and/or grapevine debris, on the soil within and/or outside the grapevine plots (Table 1). Strains were collected on cotton swabs, from sporulating lesions for greenhouse crops, grapevine and bramble in September, and from asymptomatic grapevine flower caps and dried blackberry flowers in June. For June and for litter samples, the fungus was collected following the incubation of the plant material in a moist chamber at room temperature until conspicuous sporulation was observed.

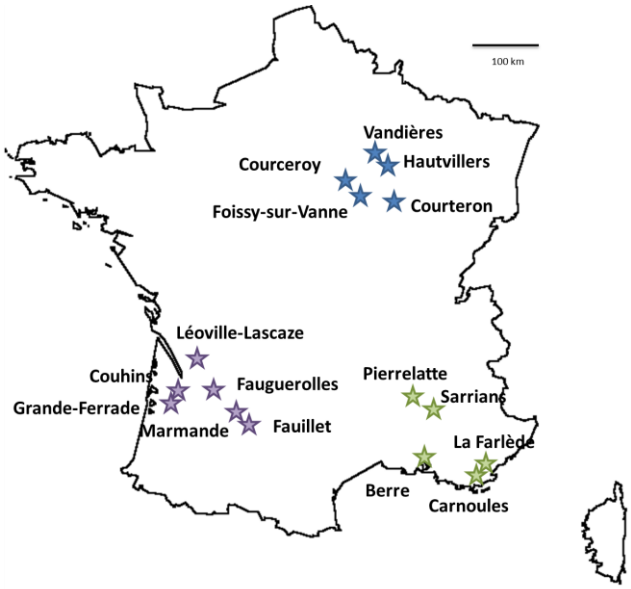

Figure 1: Map of *Botrytis cinerea* populations collected from three French regions on various host plants and in various cropping conditions, on four dates between 2005 and 2007. Locations from Champagne, the South West and South East are shown in blue, purple, and green, respectively.

Strains were grown on malt-yeast-agar (MYA) medium (20 $g.l^{-1}$malt extract, 5 $g.l^{-1}$ yeast extract and 12.5 $g.l^{-1}$ agar) or potato dextrose agar (PDA; 39 $g.l^{-1}$ ready-to-mix DIFCO potato dextrose agar), at 19°C to 21°C, under continuous illumination, to induce sporulation. Single-spore cultures, referred to hereafter as “isolates”, were obtained for all strains. Stocks of spore suspensions were for each isolate were stored in 20% glycerol, at -80°C, until required.

MICROSATELLITE GENOTYPING

For each isolate, DNA was extracted after seven days of culture on MYA or PDA medium at 21°C. DNA was extracted in an automated system, with the

DNeasy adapted kit (Qiagen), or manually (Martinez *et al.* 2008; Decognet *et al.* 2009). All samples were genotyped for eight microsatellite markers — Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7 and Bc10 (Fournier *et al.* 2002) — either in multiplex PCR, as previously described (Leroux *et al.* 2010), or in simplex PCR. Automatic allele recognition and annotation (binning analysis, Beckmann Coulter CEQ 8000 software) was carried out for the microsatellites for the multiplex analyses. In addition, as genotyping was achieved at several laboratories, a panel of 21 reference isolates was distributed to all the laboratories and used to cross-validate allele assignment. We excluded isolates of the cryptic species *B. pseudofuckeliana*, which is morphologically undistinguishable from *B. cinerea*, on the basis of a previously described diagnostic allele at locus Bc6 (Walker *et al.* 2011). Only isolates genotyped at all microsatellite markers were included in the analyses.

ANALYSES OF POPULATION SUBDIVISION

The relative contributions of sampling date, region of origin, cropping system and host plant species to the partitioning of genetic variance were estimated by hierarchical analyses of molecular variance (AMOVA) implemented in ARLEQUIN V3.5 (Excoffier *et al.* 2010).

Population subdivision was investigated by the Bayesian clustering method implemented in STRUCTURE (Pritchard *et al.* 2000), with the admixture model and correlated allele frequencies. Burn-in length was set at 100,000 Markov Chain Monte Carlo iterations. The burn-in period was followed by a run phase of 500,000 iterations, with the number of clusters *K* ranging from 1 to 10 and 10 independent replicates for each value of *K*. STRUCTURE outputs were processed with CLUMPP (Jakobsson *et al.* 2007); a *G'* statistic greater than 80% was used to assign groups of runs to a common mode (*i.e.* clustering solution). The amount of additional information explained by increasing *K* was determined by calculating the Δ*K* statistic (Evanno *et al.* 2005) in STRUCTURE HARVESTER (Evanno *et al.* 2005; Earl *et al.* 2011).

We also investigated population subdivision by a non parametric clustering method, the discriminant analysis of principal components (DAPC, (Jombart *et al.* 2010)). This multivariate method involves a discriminant analysis on genetic data transformed after principal component analysis. The DAPC was carried out with the *adegenet* 1.3-1 package in the R 2.13.1 environment. We used the K-means procedure implemented in the function *find.clusters* to determine *K,* the optimal number of clusters, by letting *K* vary between 1 and 30. We used the Bayesian information criterion (BIC) to determine the 'optimal'

value of *K,* defined as the value for which BIC was minimal, or at which the rate of change of BIC changed abruptly.

GENETIC VARIABILITY AND DIFFERENTIATION

Calculations were performed on the clusters obtained in STRUCTURE clustering analyses. GENETIX (http://www.genetix.univ-montp2.fr/genetix/intro.htm) was used to estimate within-cluster variability on the basis of genic diversity (*He*) and allele richness (*Ar*). GENEPOP V4.1 (Raymond *et al.* 1995) was used to calculate pairwise Weir & Cockerham's F_{ST} between pairs of clusters (Weir & Cockerham 1984).

ANALYSES OF THE MODE OF REPRODUCTION

The number of unique multilocus genotypes (G) and the clonal fraction (1-G/N) were calculated with MULTILOCUS V1.3b (Agapow *et al.* 2001). We also used this software to calculate the r_D index, an estimate of multilocus linkage disequilibrium. Unlike the I_A index, r_D is corrected for the number of loci considered, and ranges from 0 (complete panmixia) to 1 (strict clonality). Significance was established by comparing the observed values with the distributions obtained by 1000 randomizations (Agapow *et al.* 2001). We also evaluated the significance of pairwise linkage disequilibrium by contingency tests (with default parameters) implemented in GENEPOP V4.1 (Raymond *et al.* 1995).

RESULTS

POPULATION SUBDIVISION

We first examined the partitioning of genetic variation between the various sources of population subdivision considered in our study (sampling date, cropping system, host plant and geographic location), by hierarchical analyses of molecular variance (AMOVA) (Supporting Information Table 1). In the first AMOVA, the 52 populations of the complete dataset were organized by sampling date, nested into cropping system (indoors/outdoors). In this analysis, variation within populations accounted for most of the molecular variance (67.43 %; $P < 0.0001$; $F_{ST} = 0.32$). Variation among cropping systems was the second most important source of variation (31.72%; $P < 0.03$; $F_{CT} = 0.32$). Variation among dates within cropping systems was also significant (0.85 %; $P < 0.001$; $F_{SC} = 0.32$). The genetic variability of *B. cinerea* populations therefore seemed to be strongly structured by cropping system. We performed a second set of hierarchical

AMOVAs to explore, for each sampling date, the distribution of genetic variation among outdoor populations, by host of origin (*Vitis, Rubus* or litter) nested into the three possible geographic origins (Champagne, South East and South West). Again, variation within populations accounted for most of the molecular variance (85-92 %; $P < 0.001$; $0.06<F_{ST}<0.14$). Variation among hosts within regions accounted for 5 to 17% of molecular variance ($P<0.001$), whereas variation among regions was not significant. These results suggest that the three factors significantly affecting the genetic variance were sampling date, cropping system, and host of origin for outdoor populations. The cropping system was the main structuring factor, whereas geographic origin made little contribution, at least for outdoor populations.

We also investigated population subdivision with the admixture and correlated allele frequency model implemented in STRUCTURE. Analyses were performed independently for each sampling date, without predefining populations. The mode of the ΔK statistic was observed at K=5 for the June 2006, September 2006 and June 2007 datasets, and at K=2 for the September 2005 dataset (Supporting Information Table 2). Non parametric clustering by DAPC yielded the same pattern as STRUCTURE analyses, with five distinct groups inferred whatever the sampling date considered (Supporting Information Fig. 1). In the rest of the analyses, we consider the clustering patterns inferred with STRUCTURE. These were highly similar across sampling dates (Figure 2).

Consistent with the results of AMOVA, clustering analyses indicated that whatever the sampling date, genetic variation in *B. cinerea* populations was structured principally according to the cropping system (indoors/outdoors) and the host plant, with geographic location having a much weaker effect (Supporting Information Fig. 2). Indeed, whatever the sampling date considered, one cluster consisted mostly of isolates collected in greenhouses on *Solanum* (94.3 - 98.7% of individuals from this habitat were assigned to the same cluster); this cluster was named *INDOOR*. Another cluster contained mostly isolates collected on *Rubus* (57.1 - 95.0% of the individuals assigned to this cluster), and was named *RUBUS*. The proportion of isolates collected on *Rubus* grouped in this cluster was higher in spring (June 2006 and 2007), than in the fall (September 2005 and 2006). The last three clusters, the sizes of which remained stable over time, grouped together isolates collected mostly from *Vitis* and were named *VITIS1, VITIS2* and *VITIS3*. These clusters included a mean of 69.2% of the isolates collected on grapevine (43.1% to 84.6% according to sampling date and clusters). No cluster specifically grouped together the isolates collected on litter, which were distributed between the five clusters. Litter was thus considered to be essentially the same as *Vitis* in subsequent analyses. The four clusters

characterized for outdoor populations (*RUBUS*, *VITIS1*, *VITIS2* and *VITIS3*) are referred to as *OUTDOOR* clusters.

We calculated pairwise F_{ST}, to measure the degree of population differentiation between clusters (Supporting Information Table 3). All F_{ST} values were significant (P <0.001), ranging between 0.12 and 0.65 (mean of 0.40). The genetic differentiation between clusters corresponding to isolates from different hosts was generally greater (range: 0.20 – 0.65, mean 0.43) than that between the clusters coexisting on *Vitis* (range 0.12 – 0.30; mean 0.23). More specifically, differentiation between indoor and outdoor clusters (range 0.32 – 0.65; mean 0.51) was nearly twice than that between outdoor hosts (range 0.17 – 0.57: mean 0.28).

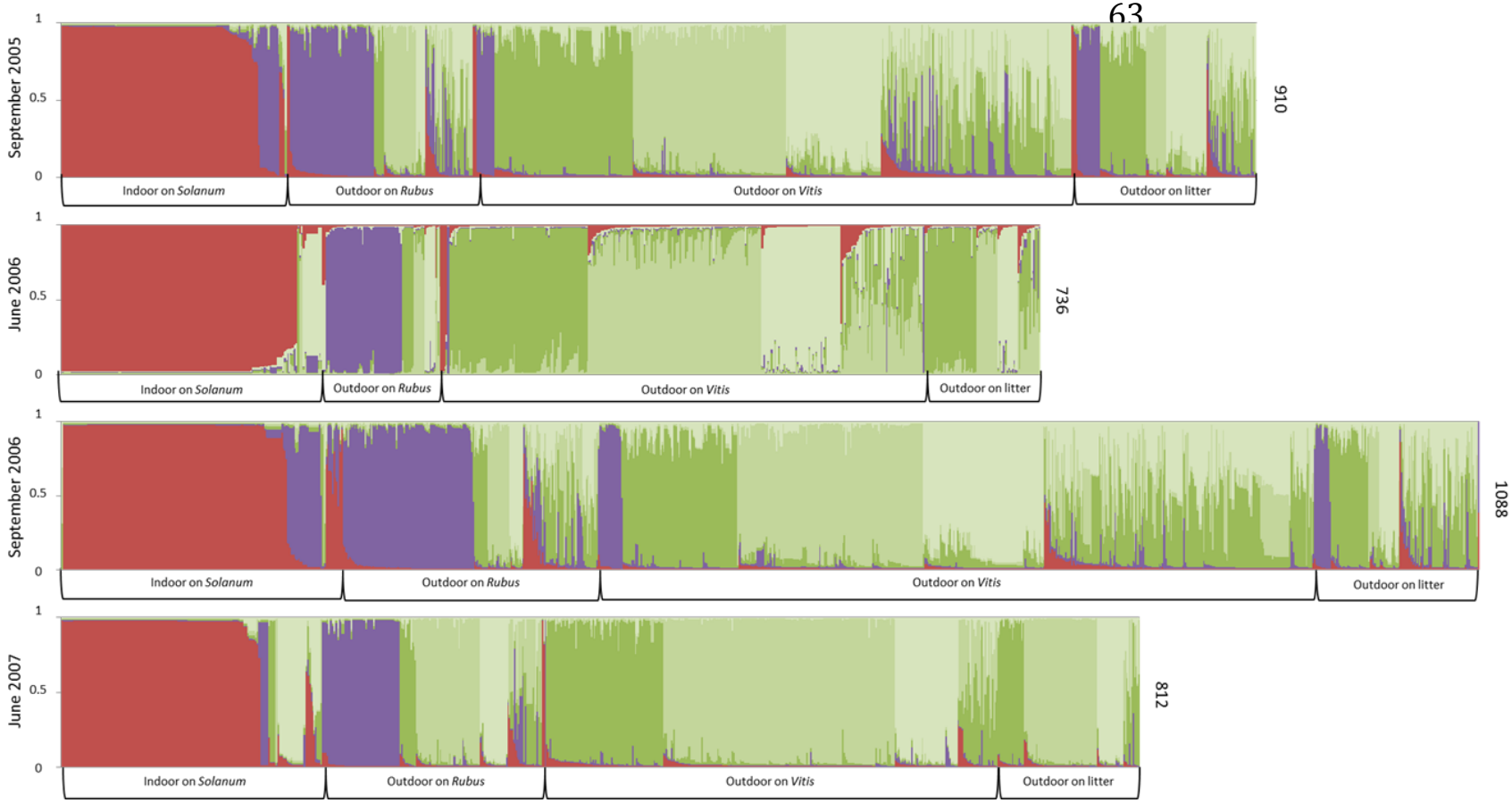

Figure 2: Barplots of the STRUCTURE analysis showing the assignment of the 3546 *B. cinerea* isolates collected between 2005 and 2007 to five genetic clusters. The numbers on the right indicate the sample size at each date. The origin of isolates (cropping system and host plant) is indicated below each horizontal axis.

PATTERNS OF GENETIC VARIABILITY

We assessed genetic variability within each clusters at each sampling date, by estimating the mean number of alleles per locus, A_r, and the genic diversity, H_e (Fig. 3). The *INDOOR* cluster was the least diverse, with A_r values ranging between 2.6 and 3.8 over time, and an H_e not exceeding 0.32. Genetic variability was higher in all outdoor clusters, but with different patterns of variability over time, depending on the cluster considered. The inter-date standard deviations of A_r were 1.7 and 0.7 in *VITIS1* and *VITIS2*, respectively (A_r range: 4.1 - 8.4), whereas they reached 5.2 and 5.6 in *RUBUS* and *VITIS3*, respectively (A_r range: 3.8 - 16.0). Similarly, the inter-date standard deviations of H_e were 0.7 and 0.06 in *VITIS1* and *VITIS2*, respectively (H_e range: 0.43 - 0.69), whereas they reached 0.21 and 0.23 in *RUBUS* and *VITIS3*, respectively (H_e range: 0.33 - 0.81). Moreover, the fluctuations in the *RUBUS* and *VITIS3* clusters were asynchronous: genetic variability was highest in the fall for *RUBUS*, whereas it peaked in spring in *VITIS3*. Thus, at least in the *RUBUS* and *VITIS3* outdoor clusters, inter-date demographic fluctuations were accompanied by substantial, asynchronous variations of genetic composition.

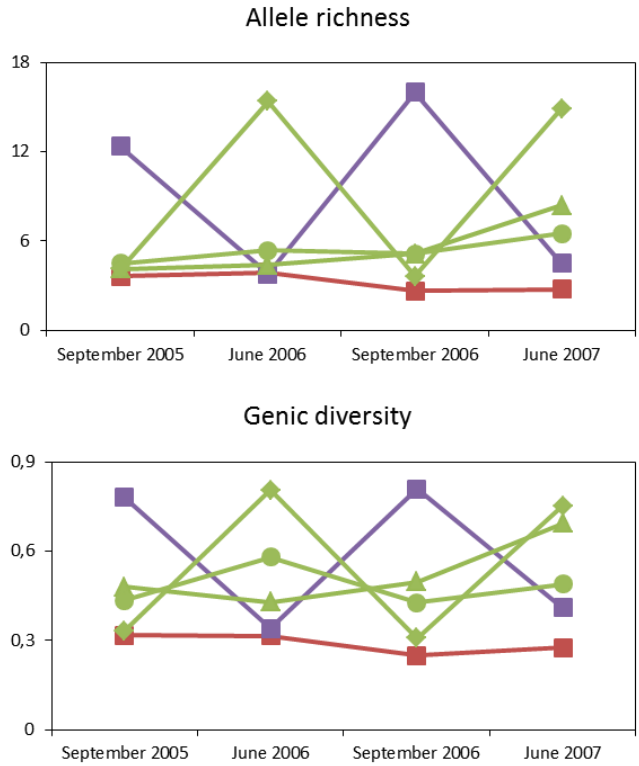

Figure 3: Diversity measured in the five *B. cinerea* clusters and for the four sampling dates. Colors and marks indicate clusters: *INDOOR* (red), *RUBUS* (purple), *VITIS1* (green, triangles), *VITIS2* (green, circles) and *VITIS3* (green, diamonds). Allele richness is calculated as the mean number of alleles per locus. Genic diversity is measured as the mean expected heterozygosity (H_e) over the eight loci. All the values were significant at the 5% confidence level.

ADMIXTURE AND GENOTYPE FLOW BETWEEN CLUSTERS

Each of the four *OUTDOOR* clusters was clearly associated with a particular host plant species, but possible spillover genotypes (i.e. genotypes assigned to a cluster associated with a host plant but sampled on another plant) were systematically observed, suggesting significant genotype flow between clusters (Fig. 2, Supplementary Information Fig. 2). Whatever the sampling date, the *RUBUS* cluster encompassed a mean of 76 % (57-95%) of the individuals sampled on *Rubus*, but it also included a mean of 13.8% (1.5-28.6%) possible spillover genotypes sampled on *Vitis*, and 10% (0-16.9%) possible spillover genotypes sampled on *Solanum* in greenhouses. Similarly, the *VITIS1, VITIS2* and *VITIS3* clusters consisted of a mean of 90%, 88.5% and 80.2%, respectively, of individuals sampled on *Vitis* (ranges: 82-94.6%, 84.6-92.8%, and 61.5-93.6%, respectively). However, the three *VITIS* clusters also included a mean of 9%, 9.8% and 11%, respectively, of possible spillover genotypes sampled on *Rubus* (corresponding ranges: 5-17%, 5.8-15.4% and 6.3-10%), and a mean of 0.5%, 1.7% and 8.8%, respectively, of possible spillover genotypes sampled on *Solanum* in greenhouses (corresponding ranges 0-1.3%, 0-4% and 0-19.2%).

We also determined the proportion of admixed individuals by inference with STRUCTURE and their parental clusters. Admixed individuals accounted for between 9.9 and 30.7% of total isolates, and most were collected on *Vitis* (37.5% to 72.1% of admixed individuals). The clusters for which the membership coefficients of a given admixed genotype were the highest and second highest were considered to be the clusters of origin of the genotype (Supplementary Information Fig. 3B). Admixture between the *INDOOR* cluster and the other clusters was limited (maximum of 6.2% of the admixed genotypes), consistent with the strong isolation between indoor and outdoor *B. cinerea* populations. Admixture between the *RUBUS* cluster and any of the three *VITIS* clusters accounted for less than 12.4% of the admixed individuals, with *VITIS2* the cluster most frequently admixing with the *RUBUS* cluster. Most admixture events were actually detected in pathogen clusters from the same host plant: between 3% and 60.5% of the admixed individuals had their highest membership coefficients for two of the three *VITIS* clusters. Admixture between clusters from different hosts remained stable over time, whereas seasonal variation was observed for admixture between *VITIS* clusters: admixture between *VITIS1* and *VITIS2* predominated in spring, whereas admixture between *VITIS1* and *VITIS3* predominated in the fall.

MODE OF REPRODUCTION

We inferred the existence of recombination within clusters from two different estimates of linkage disequilibrium (Fig. 4). In the *INDOOR* cluster, we found the highest clonal fraction, which ranged from 0.75 to 0.91. In this cluster, multilocus linkage disequilibrium, estimated with the r_D index, varied from 0.18 to 0.34 over time and was lower for samplings in June than for those in the previous September. The proportion of significantly linked pairs of loci ranged between 57% and 76%, and followed the same pattern of seasonal variation. For each season, we determined whether the loci in each pair were significantly linked or not, using Fisher's exact tests, and we then estimated the proportion of pairs for which linkage status changed over time. In the *INDOOR* cluster, most pairs of loci (57 to 76%) remained linked over time (Supporting Information Fig. 4). Together, these results suggest that asexual reproduction is probably the main mode of reproduction in the *INDOOR* cluster.

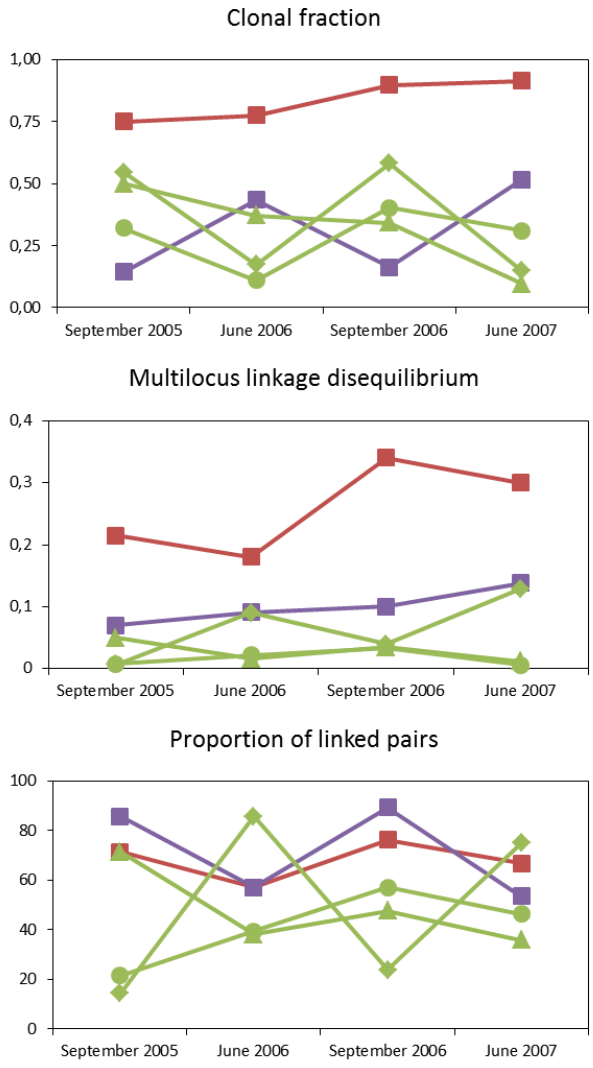

Figure 4: Clonal fraction, multilocus linkage disequilibrium, and numbers of significantly linked pairs of loci in the five clusters identified by clustering analyses. Clusters: *INDOOR* (red square), *RUBUS* (purple square), *VITIS1* (green triangles), *VITIS2* (green circles) and *VITIS3* (green diamonds). The clonal fraction represents the proportion of different multilocus genotypes. Multilocus linkage disequilibrium was estimated with the r_D index.

Contrasting with the *INDOOR* cluster, r_D values were low and varied little between seasons (range 0.07-0.14) in the *RUBUS* cluster (Fig. 4). The clonal fraction was below 0.50 and peaked in the fall (0.14 and 0.16 in September 2005 and September 2006, respectively). The proportion of linked pairs of loci varied between 53.6% and 89.3% and was higher in the fall than in the spring. Transition graphs (Supporting Information Fig. 4) confirmed that 36% to 39% of the statistical associations between pairs of loci disappeared between the fall and the following spring. This suggests that regular recombination events occurred within the *RUBUS* cluster, and that reproduction probably took place during the winter.

The three *VITIS* clusters displayed contrasting modes of reproduction (Fig. 4). In *VITIS1* and *VITIS2,* r_D values were close to 0 (maximum 0.05) and the clonal fraction was between 0.10 and 0.50 and was lower in spring than in the fall. Except in September 2005, between 38% and 57% of the pairs of loci were significantly linked in these clusters, and 11% to 47% of the significant associations disappeared during the winter. Thus, as for the *RUBUS* cluster, recombination probably occurred regularly within the *VITIS1* and *VITIS2* clusters, particularly during the winter. The *VITIS3* cluster also had a very low clonal fraction (ranging from 0.18 to 0.58) and low levels of multilocus linkage disequilibrium (r_D ranging from 0.01 to 0.13), with higher values of r_D in spring than in the fall. The percentage of linked pairs (Supporting Information Fig. 4) varied from 14.3% to 23.8% in the fall, but reached 75% and 85.7% in spring. Associations between loci were mostly broken between June 2006 and September 2006, with 71% of the linked pairs becoming unlinked. Conversely, such associations were mostly generated between the fall and the following spring. Thus, recombination predominates in *VITIS3* but appears to occur between spring and fall, indicating asynchronous reproduction between *VITIS3* on the one hand and *RUBUS, VITIS1* and *VITIS2* on the other.

DISCUSSION

We analyzed the associations between the population structure of the multihost plant pathogen *B. cinerea* and cropping system, host plant and geographic origin, based on the genotyping of samples collected hierarchically across multiple years at multiple sites in France. We found a stable pattern of subdivision into five genetic clusters. For all sites and all years, populations from different cropping systems (indoor *vs.* outdoor) displayed strong differentiation. Genetic variation was also significantly structured according to sampling date, and, in outdoor populations, according to host plant. A higher frequency of

asexual reproduction in greenhouses may account for some of the differentiation observed between indoor and outdoor populations, and asynchronous reproduction may contribute to temporal isolation between populations coexisting on *Vitis*.

ISOLATION-BY-CROPPING SYSTEM

In our sampling, effect of cropping system and of host plant may be confused because samples from the same host were not collected in the two cropping systems. This could not be achieved from a technical point of view, because such cultural situations occur only in a few restricted French areas, which would have not allowed a comprehensive exploration of *B. cinerea* ecological niches. Nevertheless, at all dates, we observed higher differentiation (AMOVA results, pairwise F_{ST}) between *OUTDOOR* and *INDOOR* clusters compared to differentiation between *OUTDOOR* clusters collected on distinct hosts. Moreover, genotypes from the *INDOOR* and the three *OUTDOOR* clusters were almost strictly habitat-specific, with only a few genotypes from the *INDOOR* cluster or the *OUTDOOR* clusters found in the 'wrong' environment (Fig. 2). The level of admixture between *INDOOR* and *OUTDOOR* clusters was also low, and did not increase with time (Fig. 2). This pattern of isolation-by-cropping system may result from a combination of habitat isolation (the greenhouse acts as physical barrier), immigrant inviability (effective prophylaxis indoors and adaptation of populations to outdoor/indoor environments), and differences in the mode of reproduction (higher relative contribution of asexual reproduction indoors (Karchani-Balma *et al*. 2008; Decognet *et al*. 2009), with probably little effect of the host. We also observed differences in diversity between indoor populations from different regions (Supplementary Information Fig. 5), possibly reflecting differences in the prophylactic measures implemented, the greenhouse structure or climatic conditions. Altogether, these results may suggest that cropping system was the most important structuring factor in *B. cinerea* populations.

These findings suggest that strong prophylactic measures should be practiced indoors, such as disinfection between crops and seedlings, quarantine, filtering of the incoming air, weed management in the areas surrounding greenhouses and the use of techniques limiting the introduction of diversity. The confinement of greenhouse populations may also limit the introgression of fungicide resistance selected on outdoor crops. Indeed, analyses of the distribution of resistant genotypes in greenhouse populations have revealed isolates resistant to the restricted number of modes of action used indoors, whereas greater phenotypic diversity is encountered in the surrounding

populations collected on grapevine, for which different modes of action are authorized (AS Walker, unpublished data).

HOST PLANT ADAPTATION AND GEOGRAPHIC ISOLATION

The host plant of origin significantly accounted for the subdivision of the outdoor *B. cinerea* populations. Such cryptic population differentiation has already been described in *B. cinerea* (Munoz *et al.* 2002; Fournier *et al.* 2008; Karchani-Balma *et al.* 2008; Samuel *et al.* 2012), and in many other fungal plant pathogens (Giraud *et al.* 2006). The divergent selection pressures exerted by hosts may thus explain the stable pattern of differentiation we observed over time. The coexistence of populations on different hosts might be accounted for by differences in host phenology or periods of receptivity (temporal isolation, see below). An alternative explanation would involve the large arsenal of genes involved in necrotrophic processes (Choquer *et al*. 2007; Williamson *et al*. 2007), including a large number of secondary metabolites, including fungal toxins in particular, as confirmed by genome analysis (Amselem *et al.* 2011). These genomic features might favor ecological specialization on different hosts in *B. cinerea* (Giraud *et al*. 2010). We also observed different genetic clusters on the same host, the coexistence of which may also be due to temporal isolation or ecological specialization acting at a very fine scale (see below).

Population structure was only weakly associated with geographic location at the (national) scale used in our study. This is consistent with previous findings that geographic structuring of populations is observed only at the continental scale (Isenegger *et al.* 2008). The lack of geographic structure may reflect long-distance migration or very large effective population sizes. The nature of the substrate of origin (living plant organs or litter) had no significant effect on the genetic structure of outdoor populations. Litter isolates were predominantly associated with the *Vitis* populations. The regions sampled in this study have large areas under grapevine, which is probably the principal initial source of *B. cinerea* populations each year. Large inocula released from grapevine may readily colonize dead material present in the immediate neighborhood, which is not systematically sprayed with fungicides and may therefore serve as a reservoir of inoculum. This opens up new possibilities for gray mold management.

MODE OF REPRODUCTION AND POSSIBLE TEMPORAL ISOLATION

The low clonal fraction, low r_D values, and the regular breakdown of linkage between pairs of neutral loci between consecutive sampling dates are signatures of regular recombination events. Our findings are consistent with those of previous studies (Giraud *et al.* 1997; Fournier *et al.* 2008). They suggest

regular cryptic sexual reproduction in *B. cinerea* populations, despite an absence of evidence of sexual structures in natural conditions (Beever *et al.* 2004).

Our results also revealed complex spatio-temporal dynamics in the timing of recombination events and in the intensity and direction of genotype flow between clusters (Fig. 4, Supplementary Information Fig. 4). For the *RUBUS, VITIS1* and *VITIS2* clusters, the proportion of linked marker pairs was higher in the fall than in the spring. This indicates that sexual reproduction occurs during the cold season and is consistent with published findings concerning the epidemiology of gray mold (Elmer *et al.* 2004). However, analyses of the *VITIS3* cluster revealed a different pattern, with more linkage disequilibrium in spring than in the fall. This suggests that the factors triggering the sexual phase may be different for this cluster. It is possible that parasexuality, rather than sexual reproduction, occurs in *VITIS3*. Indeed, it has been hypothesized that parasexuality due to anastomosis occurs in *B. fuckeliana* (Beever *et al.* 2003; Roca *et al.* 2012). A second hypothesis that could be tested relates to the presence of hyperparasites (such as mycoviruses, Wu *et al.* 2007) in the various clusters. Hyperparasites are known to affect the mode of reproduction and virulence levels of their hosts (van Putten *et al.* 2007, Montarry *et al.* 2009, Kiss *et al.* 2011). Whatever the factors at work, the asynchronous recombination in *VITIS3* may contribute to temporal isolation between this cluster and the other two *VITIS* clusters, potentially accounting for their coexistence on the same host.

Temporal differences between clusters were also observed in terms of possible disease spillover. We considered that, for each isolate, the host plant associated with membership cluster of the isolate (i.e. *Solanum* for *INDOOR, Rubus* for *RUBUS, Vitis* for *VITIS1-2-3*) was the "source" plant (the plant from which the isolate originated), and that the plant on which it was actually sampled was the "sink" plant (the plant on which it developed) (Supplementary Information Fig. 3A). This approach revealed that genotype flows from *Vitis* were the most intense, accounting for up to 68% and 37% of the possible spillover genotypes observed on *Rubus* and *Solanum*, respectively. Genotype flows from *Rubus* were less frequent, accounting for 33% and 26% of the possible spillover genotypes observed on *Vitis* and *Solanum*, respectively. Genotype flows to *Solanum* were more frequent (maximum of 37% of the migrants) than genotype flows from this crop (less than 10% of the migrants). In the greenhouse, little temporal variation was observed for migrants coming from or arriving on *Solanum*. Conversely, we observed temporal variation in genotype flows between *OUTDOOR* clusters, with *VITIS* clusters being more prevalent on non *Vitis* hosts in spring and the *RUBUS* cluster emitting a larger number of possible spillover genotypes in the fall. This pattern reflects the phenologies of the hosts and their periods of receptivity to *B. cinerea*, the phenological synchrony between host and parasite optimizing the

contact leading to infections (Desprez-Loustau *et al.* 2010). In France, *Vitis* and *Rubus* flower and produce berries at similar times (June to September). Grapevine berries first become susceptible to gray mold at veraison (mid-July to the end of August, depending on the variety considered and the region) and this susceptibility is maximal at vintage. However, *Vitis* also harbors sclerotia on the vine wood or attacked flower parts in spring. Our results show that most possible spillover genotypes from *Vitis* probably result from these early attacks. Symptoms on *Rubus* are observed principally in the fall, but sporulation on flower parts may also occur in the spring. The observed pattern can also be interpreted as resulting from the action of selective filters with different timings on the two hosts. In this model, *Vitis* would be less receptive to migrants derived from other plants during spring and/or summer (fall and/or winter for *Rubus*).

Another interesting finding is the existence of three distinct clusters on the same host, *Vitis*. Although substantial admixture was observed between the three *VITIS* clusters, their persistence over time and space suggests that *B. cinerea* isolates assigned to a particular cluster may exploit a particular ecological niche on *Vitis*. Temporal isolation may occur, although the mechanisms are unknown and would require further investigation. As highlighted above, the enrichment of the *B. cinerea* genome involved in the production of the secondary metabolites involved in necrotrophy might also enhance the possibility of niche exploration and ecological differentiation at a very fine scale on a given host plant.

CONCLUDING REMARKS

Our comprehensive analysis of *B. cinerea* population structure and dynamics has direct applications for disease management. As our understanding of the various populations and of the source and sink patterns of migration between them improves, it will become increasingly feasible to restrict exchanges between populations. For example, limiting the density of potential host plants in the vicinity of greenhouses or improving litter management in vineyards (as we observed intense genotype flow between *Vitis* and litter) might significantly decrease the intensity of epidemics.

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DATA ARCHIVING STATEMENT

Data for this study are available from: to be completed after manuscript is accepted for publication.

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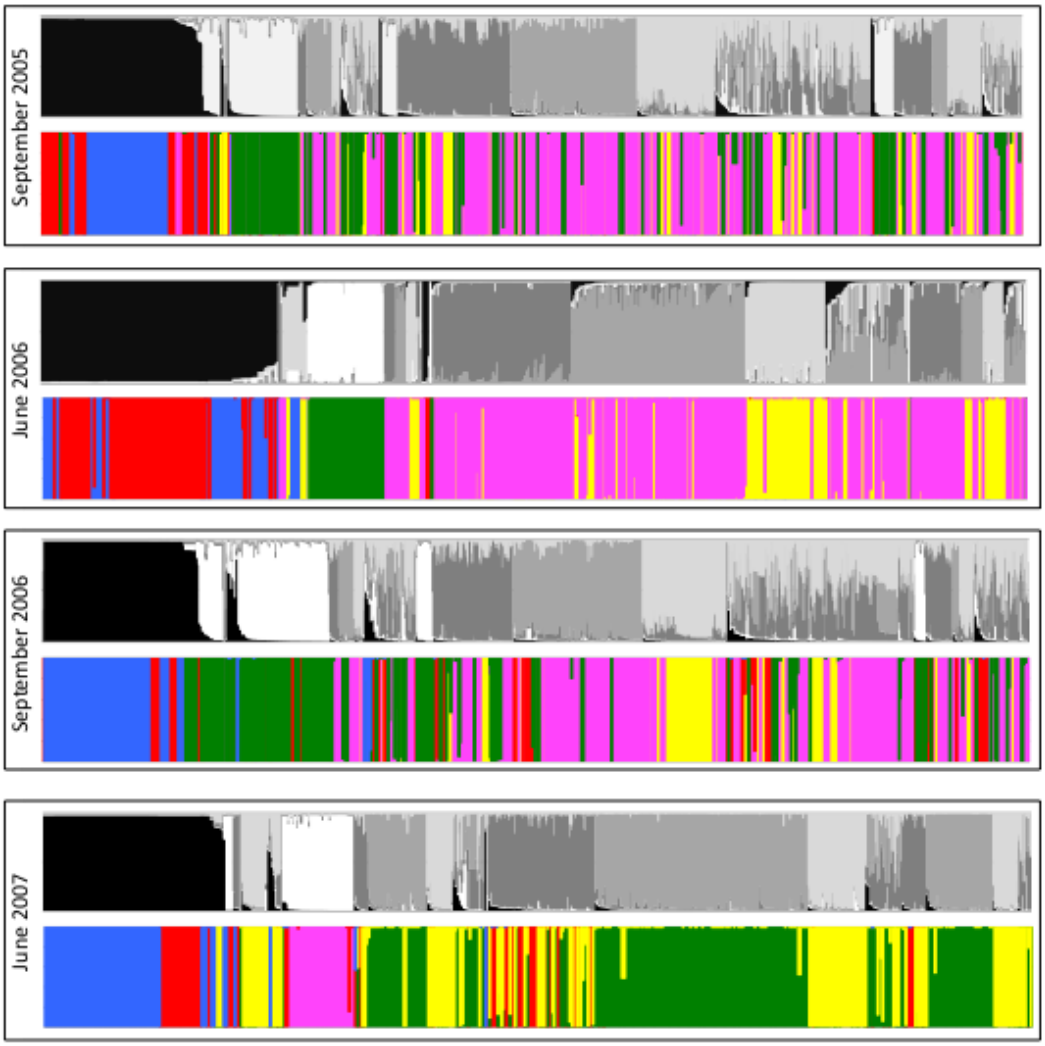
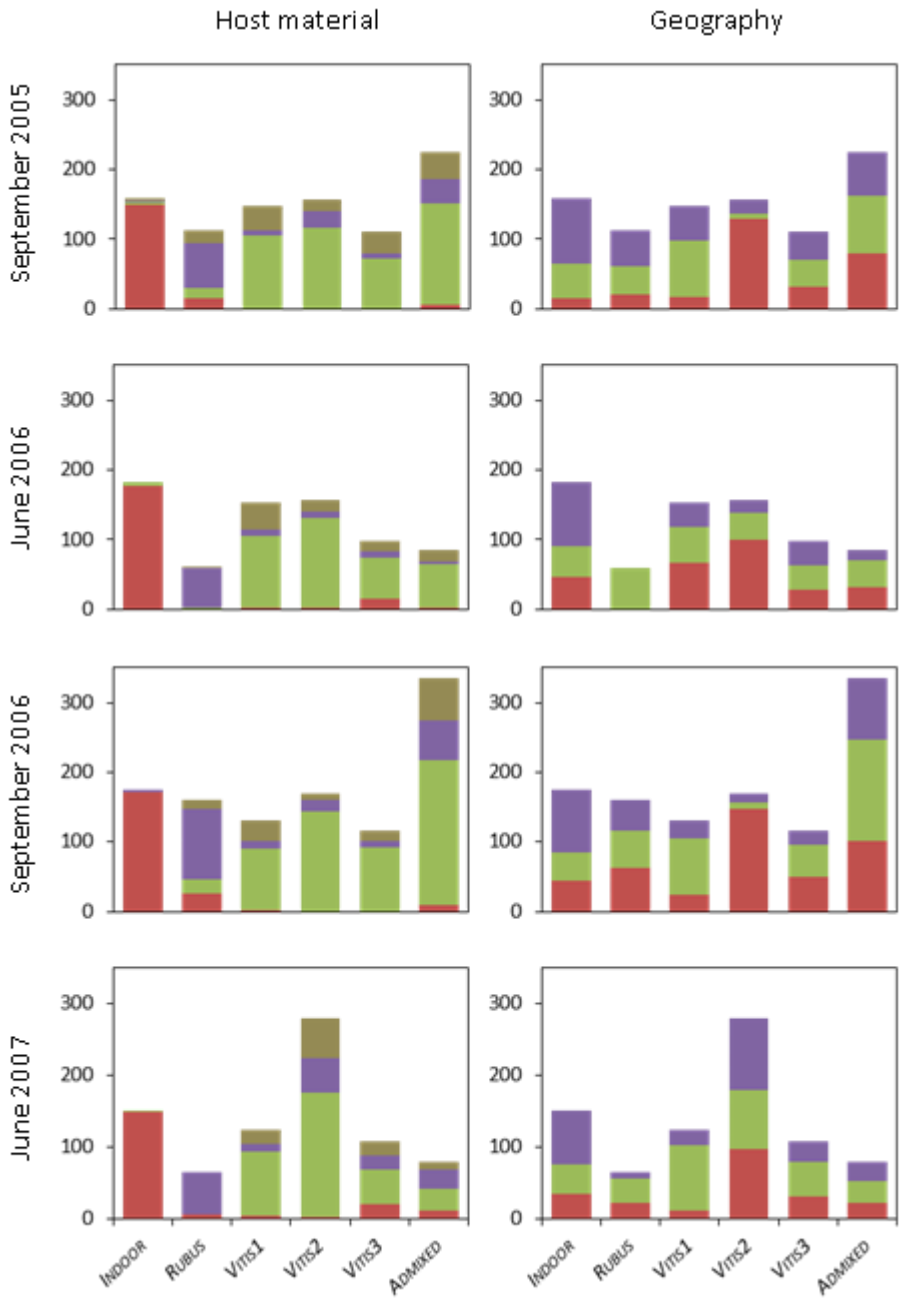


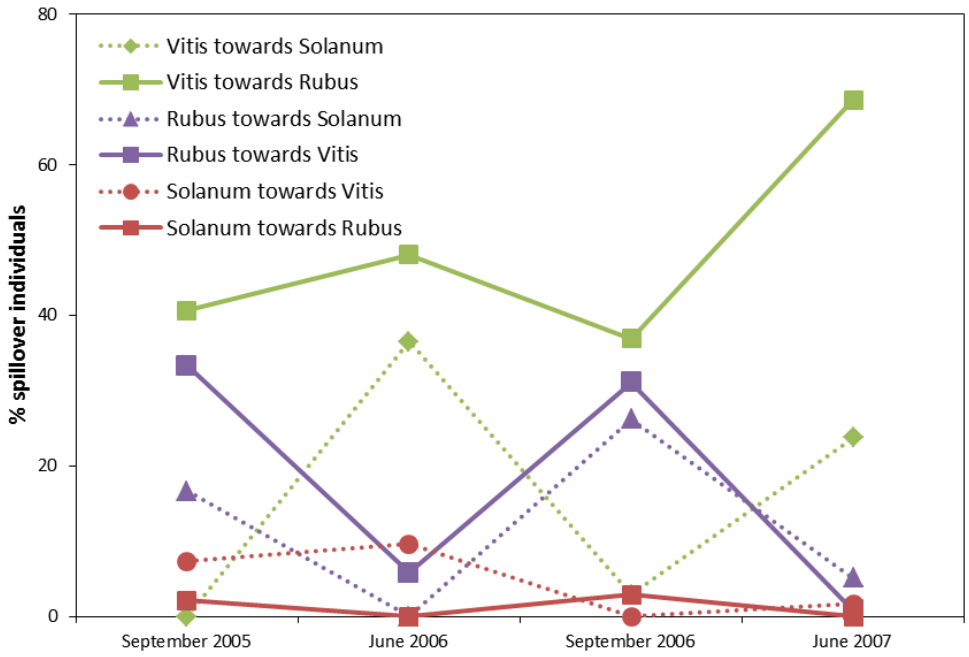
Supporting Information Figure 1: Barplots showing, for each sampling date, the comparison between assignments of individuals to five genetic clusters inferred by the STRUCTURE method (upper black and white panel) or the DPAC method (lower colored panel).



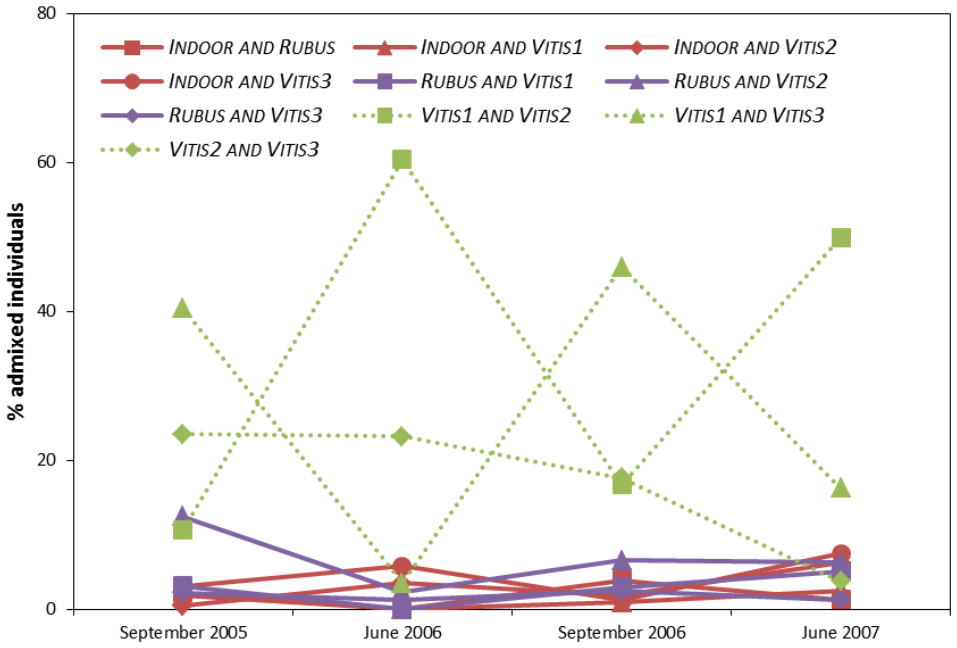
Supporting Information Figure 2: Composition of the five clusters inferred by the STRUCTURE program, as a function of the host plant of origin and geographic origin of the collected *B. cinerea* isolates.

Each barplot shows the number of isolates assigned to the corresponding cluster. Colors indicate the host from which the isolate was collected (*Solanum*: red; *Rubus*: purple; *Vitis*: green; litter: brown) or the region in which the isolate was collected (Champagne: red; South East: purple; South West: green).

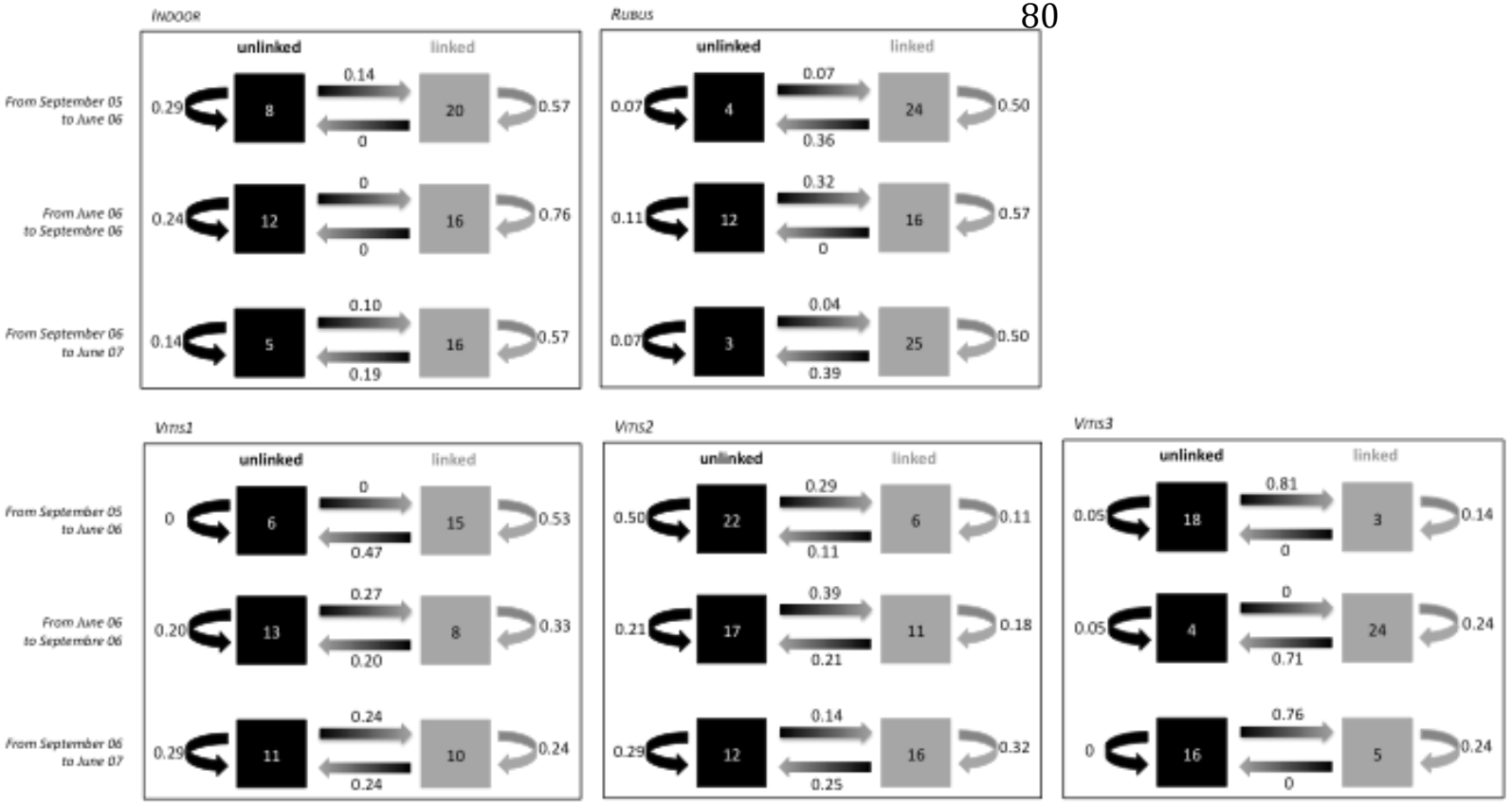
A



B

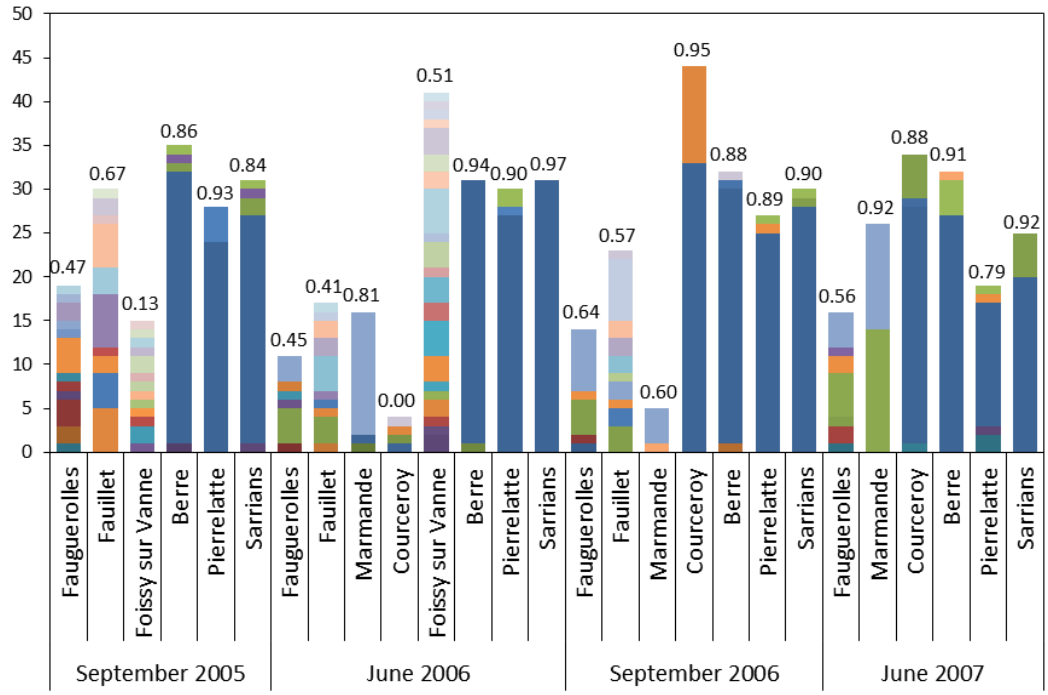


Supporting Information Figure 3. Proportion of spillover isolates (A) and admixed isolates (B) detected as migrating between genetic clusters.



Supporting Information Figure 4: Changes between unlinked/linked status for pairs of loci within the five clusters, over consecutive dates. Each panel corresponds to one of the five genetic clusters inferred with STRUCTURE.

In each panel, each line corresponds to a transition between two consecutive sampling dates (indicated on the left of the figure). On each line, the numbers in the squares indicate the numbers of pairs of loci for each status (unlinked in black squares, linked in gray squares) at the starting date. The possible linkage status transitions between two consecutive dates are indicated by arrows; the numbers on the arrows indicate the proportions of pairs of loci for each transition.



Supporting Information Figure 5: Haplotypic diversity measured in *B. cinerea* greenhouse populations (cluster *INDOOR*).

Barplots indicate the numbers of the various haplotypes (one color = one multilocus haplotype) detected in the *INDOOR* cluster, in the various greenhouses sampled. Figures above the barplot indicate the clonal fraction, i.e. 1 - the proportion of unique haplotypes over the total number of isolates collected in a given greenhouse. South West and Champagne (with the exception of Courceroy) greenhouse populations were more diverse than populations from the South East, although their clonal fraction was nevertheless below that of outdoor populations. South East and Couceroy greenhouses were dominated by the same clone. This clone may have propagated in different regions due to the transfer of infected plant materials. Moreover, these greenhouses had high-technology equipment, with strict prophylactic measures, which may have favored the isolation of the dominant clone, possibly through maintenance on alternative crops (such as lettuce in the South East). The other greenhouses were older and probably more permeable to contaminations from outdoors.

Supporting Information Table 1: Hierarchical analyses of molecular variance (AMOVA).

Upper table: cropping system and sampling dates within cropping systems as grouping factors. Lower table: for outdoor populations, geographic origin and host of origin nested within geographic origin as grouping factors. *P*-values in bold are significant at the 5% confidence level.

	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value	Fixation indices
Among cropping systems	1	1389	1.13	31.72	**0.03**	0.32
Among dates within cropping system	6	94	0.03	0.85	**<0.001**	0.01
Within dates	3538	8518	2.41	67.43	**<0.001**	0.32
Total	3545	10001	3.57			

	d.f.	Sum of squares	Variance components	Percentage of variation	P-value	Fixation indices
September 2005						
Among regions	2	51	0.036	1.46	0.14	0.01
Among hosts within regions	6	58	0.119	4.89	**<0.001**	0.05
Within hosts	729	1666	2.286	93.65	**<0.001**	0.06
Total	737	1775	2.441			
June 2006						
Among regions	2	74	-0.08	-2.82	0.65	-0.03
Among hosts within regions	5	122	0.49	17.00	**<0.001**	0.16
Within hosts	529	1305	2.46	85.82	**<0.001**	0.14
Total	536	1501	2.87			

September 2006						
Among regions	2	52	-0.02	-0.70	0.68	-0.01
Among hosts within regions	5	101	0.21	8.23	**<0.001**	0.08
Within hosts	868	2014	2.32	92.48	**<0.001**	0.07
Total	875	2167	2.50			
June 2007						
Among regions	2	43	-0.02	-0.85	0.42	-0.008
Among hosts within regions	5	104	0.27	9.09	**<0.001**	0.090
Within hosts	608	1638	2.69	91.75	**<0.001**	0.082
Total	615	1785	2.94			

Supporting Information Table 2: Results of the clustering analysis performed with STRUCTURE

	September 2005		**June 2006**		**September 2006**		**June 2007**	
K	|Ln''(K)|	Delta K	|Ln''(K)|	Delta K	|Ln''(K)|	Delta K	|Ln''(K)|	Delta K
1	—	—	—	—	—	—	—	—
2	**1365.750000**	**1893.145619**	745.320000	8.385463	913.340000	12.040038	640.770000	10.552405
3	60.830000	1.499674	470.850000	18.769794	437.440000	31.691119	377.380000	6.662756
4	213.930000	30.145109	215.680000	5.197015	147.060000	2.340117	77.240000	0.966265
5	60.540000	4.806976	**330.130000**	**30.454356**	**384.390000**	**36.771135**	**327.890000**	**100.072767**
6	54.940000	1.816565	544.980000	0.841056	583.310000	0.806773	55.910000	0.720437
7	39.480000	2.869771	831.860000	0.612736	1316.180000	1.290742	817.330000	10.032193
8	83.160000	3.155784	834.790000	0.635917	338.520000	0.244093	1465.200000	1.314896
9	69.980000	1.023889	932.930000	3.085561	244.407500	0.156458	850.150000	11.661261
10	—	—	—	—	—	—	—	—

All analyses were run 10 times. Optimal K values (as determined by the Evanno method) are shown in bold.

Supporting Information Table 3: Pairwise F_{ST} between the five genetic clusters inferred with STRUCTURE

	C_R	C_{V1}	C_{V2}	C_{V3}
September 2005				
C_{IN}	0.322	0.550	0.554	0.612
C_R		0.224	0.256	0.310
C_{V1}			0.229	0.266
C_{V2}				0.226
June 2006				
C_{IN}	0.607	0.562	0.458	0.355
C_R		0.567	0.457	0.317
C_{V1}			0.125	0.264
C_{V2}				0.178
September 2006				
C_{IN}	0.359	0.591	0.595	0.653
C_R		0.198	0.259	0.309
C_{V1}			0.235	0.297
C_{V2}				0.269
June 2007				
C_{IN}	0.582	0.353	0.520	0.429
C_R		0.320	0.497	0.333
C_{V1}			0.228	0.174
C_{V2}				0.258

All pairwise F_{ST} are significant at the 5% confidence level.

ANNEX ON GENE FLOW BETWEEN DEMES

In this manuscript, we studied flows of genotypes between demes without or with admixture, while analyzing spillover and admixed individuals, respectively, *i.e.* with a direct method. As a complement, we propose here to estimate gene flow between the same compartments, using an indirect method.

MATERIAL AND METHODS

Alternatively to the direct counting method, we used the software GENECLASS2 (Piry et al., 2004). For each sampling date, we proceeded in two steps. First, to test the null hypothesis that a given individual sampled on a particular host plant was indeed born on this plant (*i.e.* was a resident on this plant), GENECLASS2 was used to detect first-generation migrants (FGM), the starting-point populations considered being the host plant priors. Since all potential source populations have not been sampled, we used the L_h likelihood-based statistics*, i.e.* the likelihood of a given individual to be a resident in the population in which it was sampled (Paetkau *et al*., 2004). L_h was estimated using the Bayesian method of Rannala and Mountain (1997). The value of L_h beyond which individuals were assumed to be migrants was determined using the resampling method of Paetkau *et al*. (2004) (10000 simulated individuals, type I error = 0.05). The less polymorphic loci (with allelic richness lower than 10) were removed (September 2005: Bc3, Bc4 and Bc7; June 2006: Bc4 and Bc7; September 2006 and June 2007: Bc4). In a second step, GENECLASS2 was then used to assign all FGM, detected either with GENECLASS2 or with the direct method, to one of the STRUCTURE clusters previously described. The reference populations considered here were the 5 clusters determined by the STRUCTURE analysis (see Results section), from which putative migrants and admixed individuals were removed. Again, the Bayesian criteria of Rannala and Mountain (1997) was used for computation, and probability were calculated using the resampling method of Paetkau *et al* (2004) (10000 simulated individuals, type I error = 0.05). For each assigned individual, the “source” population was the reference population with the highest assignment probability, and the “sink” population was the plant on which the individual was sampled. When “source” and “sink” populations were identical, we considered this individual as a migrant from (or towards) this plant with undetermined arrival (starting) point.

Alternatively, we also used GENECLASS2 to assign admixed individuals to one of the five STRUCTURE clusters. The procedure was the same as described for FGM assignment. For each individual, we recorded its most probable parental clusters as the clusters with the highest and sub-highest assignment probabilities.

RESULTS

GENECLASS2 always detected less FGM than the direct (spillovers counting) approach (Figure S6). Altogether, these two methods detected a total of 107, 81, 117 and 146 putative FGM for September 2005, June 2006, September 2006 and June 2007, respectively (that is, 15.6%, 12.5%, 15.5% and 19.9% of the assigned individuals, respectively). GENECLASS2 allowed assigning 48.6% to 81.3% of these FGM to their most probable source cluster, according to the date. The *INDOOR* cluster was detected as the source cluster for 0 to 8 putative FGM, confirming that indoor crops are likely to emit very few migrants. In spring, 89% (June 2006) to 97% (June 2007) of the assigned putative FGM had one of the three *VITIS* clusters as probable source cluster. In autumn, the *RUBUS* cluster was inferred as the major source cluster (56% and 60% of the assigned FGM in September 2005 and September 2006, respectively). This confirms that most migration flows occurred between *Vitis* and *Rubus*, with an inversion of the direction of the flows between spring and autumn.

In comparison, GENECLASS2 allowed assigning 87.5%, 89.5%, 89.2% and 63.7% of admixed individuals in September 2005, June 2006, September 2006 and June 2007, respectively (Figure S7). Using this approach, we confirmed that most of the admixture occurred between *VITIS* clusters in spring: for all admixed individuals in June 2006 and June 2007, the most probable parental cluster was one of the three *VITIS* clusters, and admixture flows involving two *VITIS* clusters could be inferred in 48% of cases. On the other side, significant admixture flows between *RUBUS* and *VITIS* clusters were detected in Autumn: in September 2005 and September 2006, both *RUBUS* and *VITIS* clusters were identified as probable sources in balanced proportions (*RUBUS* is the most probable source for 52.3% and 45.4% of admixed individuals in September 2005 and September 2006, respectively). Admixture flows involving both *RUBUS* and *VITIS* as putative parental clusters were indeed inferred in 30% of the cases in September 2005 and 51% of the cases in September 2006.

DISCUSSION

Gene flow was measured according to two distinct methods. The first one simply counted the proportion of spillover and admixed individuals between demes and over time; individuals were distinguished according to their genotypes. The second inferred Bayesian assignment for these individuals, according to gene frequencies. More generally, the two methods did not detect exactly the same individuals as FGM and the second method was more stringent. It detected less FGM than the first one and their assignment was not complete. Nevertheless, both methods led to similar conclusions, *i.e.* temporal variation of between-deme flows. *Rubus* seem to be a better source of FGM in Autumn, whereas *Vitis* emitted more migrants in Autumn. Nevertheless, between-host admixture flows are

estimated as more intense with the second method compared to the first one and also exhibit contrasted temporal evolution, without any clear explanation.

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A	September 2005	June 2006	September 2006	June 2007
FGM detection				
Nb of direct FGM	96	52	103	118
Nb of GeneClass FGM	41	44	48	46
Nb of "true" GeneClass FGM	32	18	29	22
Nb of "true" GeneClass FGM AND direct FGM	29	8	29	9
Total nb of GeneClass OR direct FGM	107	81	117	146
FGM assignement (GeneClass)				
Nb of total FGM (GeneClass OR direct) assigned	87	57	73	71
% total FGM (GeneClass OR direct) assigned	81.3%	70.4%	62.4%	48.6%
Most probable source cluster: *INDOOR*	8	3	0	1
RUBUS	49	3	44	1
VITIS	30	51	29	69

B

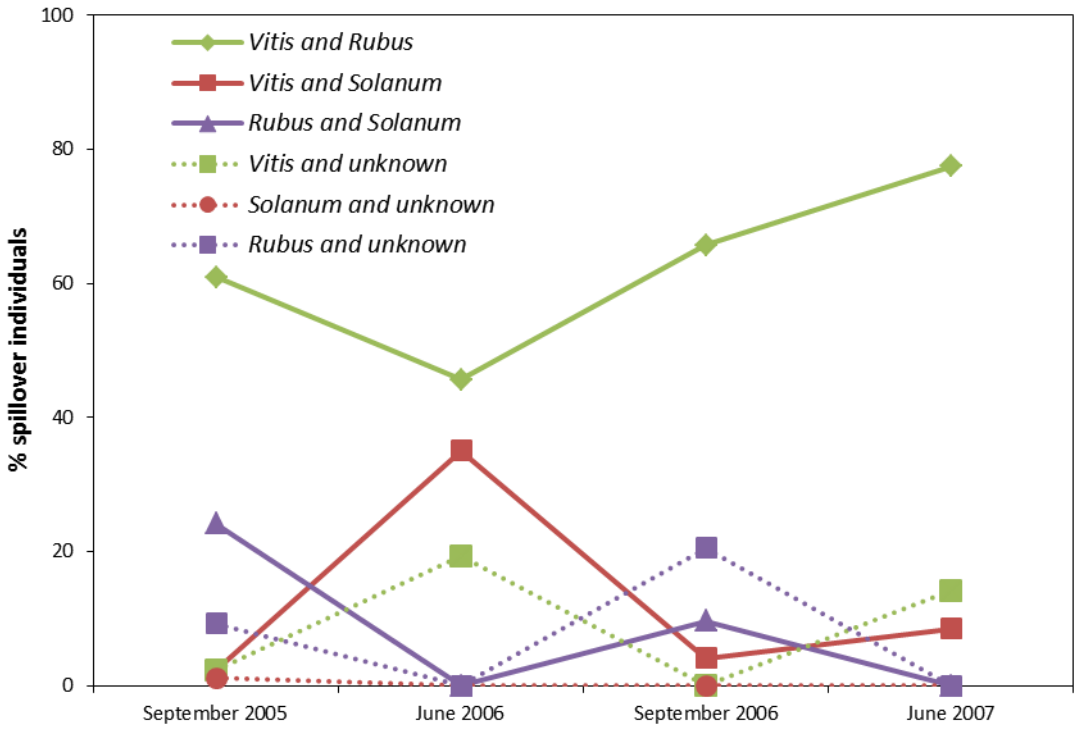

Figure S6: Gene flows without admixture detected over time in *B. cinerea* populations by GENECLASS2.

A: Numbers of migrating individuals and their assignment

B: Gene flow between host plants (not oriented), as the proportion of individuals involved in this flux compared to the total number of FGM.

A	September 2005	June 2006	September 2006	June 2007
Admixed individuals (NA) assignment (GeneClass)				
Nb of NA	225	86	335	80
Nb of NA assigned	197	77	299	51
% of NA assigned	87.5%	89.5%	89.2%	63.7%
Most probable parental cluster: *INDOOR*	0	0	0	0
RUBUS	104	0	136	0
VITIS1	51	0	102	41
VITIS2	40	30	56	0
VITIS3	2	47	5	10

B

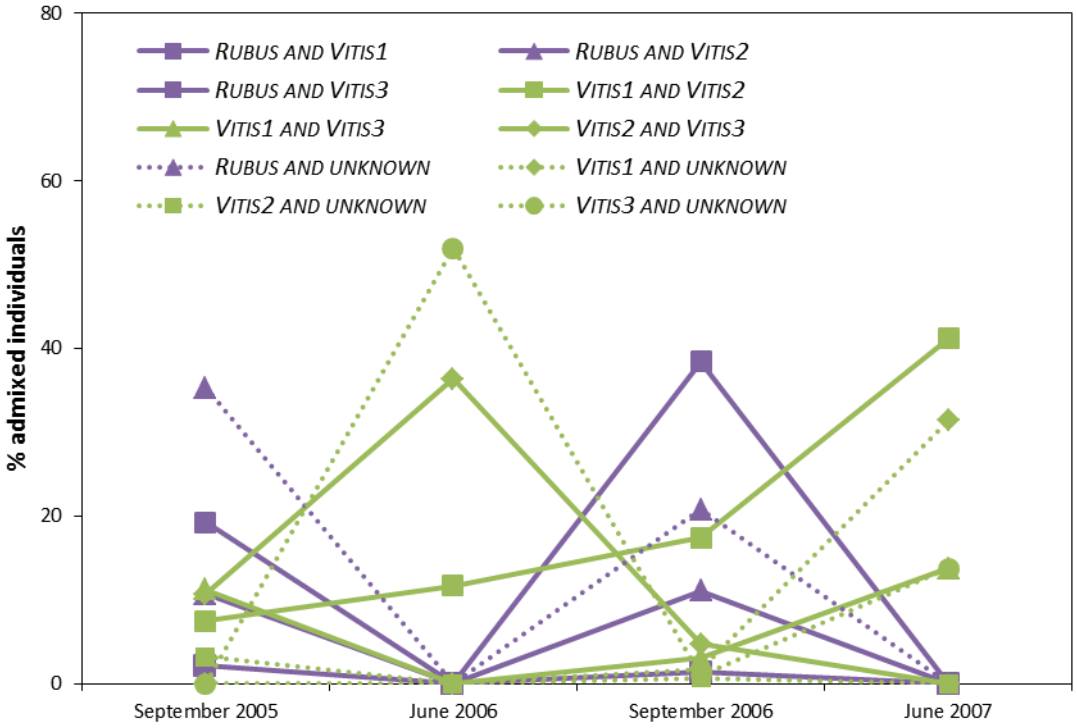

Figure S7: Gene flows with admixture detected over time in *B. cinerea* populations by GENECLASS2.

A: Assignment of admixed individuals

B: Gene flow between host plants (not oriented), as the proportion of individuals involved in this flux compared to the total number of admixed individuals. Some flows were not detected (those especially involving the *INDOOR* cluster).

Conclusions

- Les populations de *B. cinerea* sont très diverses mais néanmoins structurées par (1) le système de culture, (2) la plante-hôte, (3) dans une faible mesure à l'échelle nationale, par la géographie. Le type écologique ne structure pas les populations.
- Le cluster *INDOOR* comprend majoritairement des souches collectées en serre. Les populations appartenant à ce groupe génétique sont essentiellement clonales et les échanges avec les populations extérieures sont limités.
- Les clusters *OUTDOOR* sur ronce (*RUBUS*) et sur vigne (*VITIS1*, *VITIS2* et *VITIS3*) sont significativement différenciés, et sont relativement bien expliqués par l'hôte d'origine. La différenciation observée, qui se maintient dans le temps et dans l'espace, pourrait donc s'expliquer par une adaptation à l'hôte. Ils sont très divers, pratiquent la reproduction sexuée et échangent des génotypes selon des temporalités contrastées, ce qui résulte probablement de la mise en œuvre de filtres sélectifs distincts sur ces deux hôtes.
- Sur vigne, le cluster *VITIS3* subit un isolement temporel accompagné d'une reproduction sexuée non synchrone des autres clusters *OUTDOOR*. Cet isolement permettrait une adaptation écologique, à caractériser pour ces isolats.
- Ces travaux ouvrent de nouvelles perspectives pour la lutte contre la pourriture grise, notamment pas la manipulation des flux de gènes.

Perspectives

- La coexistence des trois clusters génétiques distincts (bien que connectés) sur un même hôte (la vigne) est surprenante. Cette co-existence peut s'expliquer par de l'isolement temporel (les souches des trois clusters exploitent des ressources similaires mais à des moments différents du cycle de développement de la plante), ou écologique à des échelles extrêmement fines.
- Dans ces trois groupes qui coexistent sur la vigne, deux ont des dynamiques identiques, notamment en termes de période de reproduction, le troisième (*VITIS3*) est totalement asynchrone. La cause de cette asynchronie pourrait aussi être recherchée dans l'existence de mycoparasites dans certains clusters et pas dans d'autres. Chez d'autres organismes, il a été montré que la présence d'endoparasites comme les mycoparasites pouvait effectivement perturber et décaler les périodes de reproduction sexuée. Il serait également intéressant de vérifier *in vitro* leur

compatibilité végétative avec des souches des autres clusters. Ces travaux sont prévus fin 2013. L'adaptation des souches du cluster *VITIS3* à des conditions estivales pourrait également être testée *in vitro.*

- L'adaptation à l'hôte pourrait être démontrée par des mesures d'agressivité *in planta* après inoculations croisées entre souches de différents clusters et plantes-hôtes (travaux prévus en juin 2013).

CHAPITRE II

ADAPTATION AUX FONGICIDES CHEZ *BOTRYTIS CINEREA*

Napa Valley, Californie

« A mutable and treacherous tribe. »
Description des champignons par Albrecht von Haller
dans une lettre à Carolus Linnaeous (1745)

CHAPITRE II : ADAPTATION AUX FONGICIDES CHEZ *BOTRYTIS CINEREA*

Avant-propos

Trois articles sont présentés dans ce chapitre.

ARTICLE 3: CARACTÉRISATION DES MÉCANISMES DE RÉSISTANCE AUX INHIBITEURS RESPIRATOIRES CHEZ *BOTRYTIS CINEREA*

Cet article a plusieurs objectifs : (1) caractériser la sensibilité de *B. cinerea* aux strobilurines et SDHI, fongicides unisites « à risque fort de résistance », (2) identifier les mécanismes potentiellement impliqués dans le déterminisme de la résistance à ces inhibiteurs respiratoires et (3) à partir des résultats obtenus, réfléchir aux conséquences possibles pour la gestion de la résistance en pratique.

Leroux, P., M. Gredt, M. Leroch, and A-S. Walker. 2010. "Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold." *Applied and Environmental Microbiology* no. 76 (19):6615-6630. doi: 10.1128/aem.00931-10.

Ce travail a également produit une version vulgarisée et une présentation orale:

Leroux, P, and A. S. Walker. 2010a. "Les fongicides affectant les processus respiratoires. Episode 1: Modes d'action et phénomènes de résistance chez les anciennes substances (multisites et unisites affectant la biodisponibilité de l'ATP) et les nouvelles de type SDHI." *Phytoma - La Défense des Végétaux* no. 631:8-11.
Leroux, P, and A. S. Walker. 2010b. "Les fongicides affectant les processus respiratoires. Episode 2: Modes d'action de résistance chez les fongicides unisites inhibiteurs du complexe III (QoI et QiI), et phénomènes de type MDR (multidrug resistance)." *Phytoma - La Défense des Végétaux* no. 632:46-51.
Walker, A. S., M. Gredt, and P Leroux. 2010. Resistance to QoIs and SDHIs in populations of *Botrytis cinerea*. 16th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds, April 25-29 2010, at Friedrichroda, Germany.

ARTICLE 4: STATUT DE LA RÉSISTANCE AUX ANTI-*BOTRYTIS* AU VIGNOBLE OU COMMENT L'EXPÉRIENCE FRANÇAISE PEUT CONTRIBUER À AMÉLIORER LES STRATÉGIES DE LUTTE CONTRE LA POURRITURE GRISE

Cet article est une revue des mécanismes de résistance aux fongicides anti-*Botrytis,* étayée par le suivi de l'évolution des résistances en France et en particulier dans le vignoble champenois (jusqu'à 20 ans), *via* deux observatoires de résistance. Cet article propose également une réflexion sur l'efficacité relative des stratégies anti-résistance chez *B. cinerea.*

Walker, A. S., A Micoud, F Rémuson, J Grosman, M. Gredt, and P Leroux. 2013. "French vineyards provide information which opens ways for effective resistance management of *Botrytis cinerea* (grey mould)" *Pest Management Science* 69 (6): 667-678.

Ce travail a également fait l'objet d'une intervention orale invitée lors de la conférence « Patholux » (22-23 octobre 2013, Mondorf-les-Bains, Luxembourg).

ARTICLE 5 : SIGNATURE DE SÉLECTION ET STRUCTURE DES POPULATIONS DE *BOTRYTIS CINEREA* RECEVANT DES APPLICATIONS DE FONGICIDES

Cet article a pour objectif (1) de détecter une éventuelle barrière aux flux de gènes, consécutive à l'application de fongicides, entre parcelles traitées et non traitées, (2) de rechercher des pertes éventuelles de diversité génétique dans les parcelles traitées, (3) de détecter les patrons de clines entre zones traitées et non traitées et de déterminer les conditions de l'équilibre migration-sélection qui les sous-tendent et (4) d'inférer les effets relatifs de la migration et de la sélection par le calcul d'estimateurs

Walker, A. S., A Rieux, V Ravigné, and E Fournier. 2013. "Contemporary fungicide applications sign for selection in *Botrytis cinerea* populations collected in the Champagne vineyard (France)" A soumettre dans une revue d'évolution, après estimation des derniers paramètres de sélection.

Ce travail sera également présenté oralement au « 17th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds » en avril 2013 (Friedrichroda, Allemagne) ainsi qu'au « XVI Botrytis Symposium » en juin 2013 (Bari, Italie).

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0099-2240/10/$12.00 doi:10.1128/AEM.00931-10

Exploring Mechanisms of Resistance to Respiratory Inhibitors in Field Strains of *Botrytis cinerea*, the Causal Agent of Gray Mold▽†

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Respiratory inhibitors are among the fungicides most widely used for disease control on crops. Most are strobilurins and carboxamides, inhibiting the cytochrome *b* of mitochondrial complex III and the succinate dehydrogenase of mitochondrial complex II, respectively. A few years after the approval of these inhibitors for use on grapevines, field isolates of *Botrytis cinerea*, the causal agent of gray mold, resistant to one or both of these classes of fungicide were recovered in France and Germany. However, little was known about the mechanisms underlying this resistance in field populations of this fungus. Such knowledge could facilitate resistance risk assessment. The aim of this study was to investigate the mechanisms of resistance occurring in *B. cinerea* populations. Highly specific resistance to strobilurins was correlated with a single mutation of the *cytb* target gene. Changes in its intronic structure may also have occurred due to an evolutionary process controlling selection for resistance. Specific resistance to carboxamides was identified for six phenotypes, with various patterns of resistance levels and cross-resistance. Several mutations specific to *B. cinerea* were identified within the *sdhB* and *sdhD* genes encoding the iron-sulfur protein and an anchor protein of the succinate dehydrogenase complex. Another as-yet-uncharacterized mechanism of resistance was also recorded. In addition to target site resistance mechanisms, multidrug resistance, linked to the overexpression of membrane transporters, was identified in strains with low to moderate resistance to several respiratory inhibitors. This diversity of resistance mechanisms makes resistance management difficult and must be taken into account when developing strategies for *Botrytis* control.

Chemical control methods are widely used to combat fungal plant pathogens in agriculture. Many synthetic fungicides have been used intensively since the 1950s, resulting in the selection of resistant mutants in natural populations of phytopathogenic fungi (17). The evolution of fungicide resistance has become a major problem worldwide, particularly in cases in which high-resistance factors have been reported and the frequencies of mutant phenotypes in the population are high. This phenomenon may greatly decrease the efficacy of the active ingredients concerned, increasing the cost of chemical control and potentially resulting in damage to the environment if repeated treatment is required (11).

Several resistance mechanisms have been identified, but changes in the target site proteins, which remain functional but are less susceptible to fungicides, are the most common resistance mechanism in plant pathogenic fungi. Target site changes may result from point or insertion/deletion mutations in the corresponding gene. However, other mechanisms have been reported at lower frequencies and include (i) target overproduction, (ii) activation of an alternative pathway compensating for inhibition of the major pathway, (iii) greater fungicide detoxification or lower levels of profungicide activation, and (iv) decreases in fungicide influx or increases in fungicide efflux, resulting in lower fungicide content within the cell (11). The first three mechanisms often lead to specific resistance, i.e., resistance concerning only one molecule or a class of fungicides. The last mechanism generally involves the constitutive overexpression of membrane drug efflux proteins, i.e., either ATP-binding cassette (ABC) or major facilitator superfamily (MFS) transporters. Due to their low substrate specificity, both types of transporters can mediate multidrug resistance (MDR) to various unrelated classes of fungicides, also considered multiple resistance even if driven by alteration of a single gene. MDR is an important phenomenon in human pathogenic microbes and cancer cells and is beginning to emerge in plant pathogenic fungi, even though resistance levels against individual fungicides are generally much lower than those achieved by target site mutations (16, 42).

Many of the fungicides used in agriculture inhibit respiration, and many are effective against a wide range of plant-pathogenic fungi. The oldest such fungicides are still in use and include dithiocarbamates (e.g., mancozeb, thiram) or trihalomethylthio derivatives (e.g., captafol, captan, folpet, dichlofluanid, tolylfluanid). These multisite inhibitors block several thiol-containing enzymes involved in respiration (50). A second group of these fungicides includes uncouplers of oxidative phosphorylation; the protonophore may be either a phenol group (i.e., dinocap) or a secondary amine group (i.e., fluazinam) (32, 50). A third group comprises inhibitors of mitochondrial complex III (the cytochrome bc_1 complex), which bind to cytochrome *b* at either an outer quinol-oxidizing pocket (Qo site) or an inner quinone-reducing pocket (Qi site). Most Qo inhibitors (QoIs) are synthetic analogs of natural strobilurins (e.g., azoxystrobin, kresoxim-methyl) introduced in

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the mid-1990s to control a wide spectrum of fungal diseases (8). The only QiIs used in agriculture are sulfonamides (e.g., cyazofamid, amisulbrom), which are active only against oomycetes. The antibiotic antimycin A is also a Qi inhibitor (24). Inhibitors of succinate dehydrogenase (mitochondrial complex II), known as SDHIs, are the last major group of respiratory inhibitors used as agricultural fungicides (72). Chemically, many of these fungicides are carboxamides with a common "*cis*-crotonanilide" structure, as shown in Fig. SA1 in the supplemental material. They are derived from an α,β-unsaturated carboxylic acid, the double bond of which is incorporated into a benzene ring or conjugated to an electron-releasing atom, such as O, N, or S, giving rise to several subgroups. In addition to these "*cis*-crotonanilides," several *N*-methylpyrazole carboxamides with a single double bond between the significant methyl and carboxanilide groups have been designed (see Fig. SA1 in the supplemental material). The first generation of carboxamide fungicides, including carboxin, was discovered in the mid-1960s, and these molecules were effective only against basidiomycetes (72). New carboxamides with a much wider spectrum of activity have recently been discovered (6). Many harbor a lipophilic substituent in the phenyl ring of the anilide (e.g., boscalid, bixafen). Such an extension of the spectrum of activity was described in 1967 for the experimental 2′-phenyl analog of carboxin (F427) (see Fig. SA1 in the supplemental material). All these carboxamides interfere with ubiquinone binding and affect three subunits of succinate dehydrogenase (33, 35, 36, 59, 72, 75).

Botrytis cinerea Pers. ex Fr., the anamorph of *Botryotinia fuckeliana* (de Barry) Whetzel, is a ubiquitous fungus causing gray mold on many crops, including vegetables, ornamental plants, and fruits, such as grapevine in particular (13). The losses for commercial wine production are especially high, being estimated up to 2 billion U.S. dollars per year worldwide (69), in addition to qualitative defaults observed in grapevine musts. Gray mold has recently been found to be caused by a complex of two related fungal species living in sympatry: *Botrytis* group II (*B. cinerea* sensu stricto, the most abundant species in the complex) and *Botrytis* group I (*B. pseudocinerea*), which represents less than 10% in French vineyards (25). It is not possible to surely distinguish between these two species on the basis of morphological criteria (25). Instead, these species can be separated on the basis of polymorphism in many genes (3, 4, 25) or microsatellite private alleles (26). In addition, *Botrytis* group I is naturally more tolerant to fenhexamid and more susceptible to fenpropidin than *Botrytis* group II (4, 47).

Despite the availability of a large number of botryticides, the chemical control of gray mold has been hindered by the emergence of resistant strains. This phenomenon is well documented for the use of benzimidazoles (e.g., carbendazim), dicarboximides (e.g., iprodione, procymidone), and to a lesser extent anilinopyrimidines (e.g., cyprodinil, pyrimethanil) and the hydroxyanilide fenhexamid in vineyards (47). Some shifts in susceptibility to multisite fungicides and fluazinam have also been recorded in ornamental plants and vegetables (44). Moreover, field strains of *B. cinerea* resistant to inhibitors of mitochondrial complexes II and/or III have recently been detected (7, 38, 40, 54, 65). Finally, strains displaying MDR have been isolated from French and German vineyards (42, 44).

In this study, our goal was to assess the susceptibility of *B. cinerea* strains from French and German vineyards to "risky" single-site respiratory inhibitors, including strobilurins and carboxamides, which are representative of the more recently introduced modes of action. We also aimed to identify the various possible mechanisms underlying resistance to these two classes of respiratory inhibitors and to investigate possible consequences for resistance management.

MATERIALS AND METHODS

Fungal strains. Fifty *B. cinerea* field strains were isolated from infected berries collected from French and German vineyards (Table 1). They were plated on MYA medium (20 g malt, 5 g yeast extract, 12.5 g agar in 1 liter of deionized water). After one round of culture, highly diluted spore suspensions were obtained and poured onto GA medium (10 g glucose and 12.5 g agar in 1 liter of deionized water), and after 24 h, pieces of medium bearing single germinated conidia were cut off under the microscope in sterile conditions and isolated in a new petri dish. The single-spore isolates were maintained on the MYA medium, and large numbers of conidia were produced after 1 week of culture under white light at 19°C.

For assessment of the frequency of strobilurin resistance in representative French populations of *B. cinerea*, 169 isolates were collected from Champagne vineyards, where they were growing on grapevine (*Vitis vinifera*) or blackberry (*Rubus fruticosus*), at two sites—Courteron and Vandières—in September 2006 and June 2007. These strains were isolated, maintained, grown, and analyzed in the same conditions as described above. The characteristics of these samples are presented in Table SA1 in the supplemental material.

In these data sets, *Botrytis* group I strains were differentiated from those of *Botrytis* group II by culture on medium containing discriminating doses of fenhexamid (0.4 mg liter^{-1}) and fenpropidin (0.08 mg liter^{-1}), making use of the "natural" differences in susceptibility to these sterol biosynthesis inhibitors (SBIs) of these two groups (46). The results obtained were confirmed in molecular tests (i.e., polymorphism in genes encoding cytochrome *b* or subunits of SDHIs and microsatellite private alleles; see below).

Fungicides. We tested principally respiratory inhibitors but also fungicides with other biochemical modes of action (i.e., carbendazim, fenhexamid, fludioxonil, iprodione, prochloraz, pyrimethanil, tebuconazole, and tolnaftate), which were used to differentiate between strains displaying specific and multidrug resistance (46). All the fungicides tested were of technical grade and most of them were kindly donated by manufacturers. They included boscalid, benodanil, furcarbanil, dimoxystrobin, pyraclostrobin, carbendazim, iprodione, prochloraz, pyrimethanil (BASF, Germany), methfuroxam, carboxin and its derivatives oxa-3a (3′ phenylcarboxin), oxa-3b (3′ benzylcarboxin), oxa-3c (3′ butoxycarboxin), oxa-3d (3′ hexyloxycarboxin), oxa-4a (4′ phenylcarboxin), oxa-4b (4′ butoxycarboxin) (Uniroyal), flutolanil (Nihon Nohyaku, Japan), thifluzamide (Monsanto), pyracarbolid (Hoechst, Germany), azoxystrobin, trifloxystrobin, fenpropidin, fludioxonil (Syngenta, Switzerland), tolylfluanid, fenhexamid, tebuconazole (Bayer, Germany), and fluazinam (ISK, Japan). Antimycin A, salicylhydroxamic acid (SHAM) and 2-thenoyltrifluoroacetone (TTFA) were purchased from Sigma-Aldrich. The structures of the tested carboxamides are given in Fig. SA1 in the supplemental material. These compounds were dissolved in ethanol, with the exception of iprodione and tolnaftate, which were solubilized in acetone, and then added to 50°C molten agar medium after autoclaving. The concentration of solvents in all media, including controls lacking fungicides, was 0.5% (vol/vol). At this solvent concentration in controls, assays revealed no significant change in strain growth in comparison to controls lacking ethanol or acetone (data not shown). For each respiratory inhibitor, we tested 5 to 10 concentrations, following a geometric progression with dose increments of ×2, ×2.5, or ×3, according to the fungicide and susceptibility range (46). Stock solutions, obtained after solubilizing 100 to 500 mg of inhibitor in 100 ml solvent, were stored at 4°C. Fresh dilution series were prepared for each experiment. The other fungicides were tested only at one or two discriminatory concentrations, previously chosen according to the response of known resistant phenotypes (23, 46, 61).

Fungicide susceptibility test. The effect of fungicides was assessed on the spore germination and germ tube elongation of *B. cinerea* as previously described (46). For all inhibitors except carboxamides and TTFA, the medium contained 10 g glucose, 2 g K_2HPO_4, 2 g KH_2PO_4, and 12.5 g agar in 1 liter of deionized water. Previous studies (62, 66) and preliminary tests indicated that the *in vitro* toxicity of the SDHIs tends to be higher if the carbon source in the medium is acetate or succinate rather than glucose. We therefore replaced glucose with 4 g of sodium

TABLE 1. Origin and fungicide resistance phenotype of the tested strains

Strain no.	Origin[a]	Year	Location	Country	*Botrytis* group[b]	Specific resistance to[c]:						MDR[d]
						Benzimidazoles	Dicarboximides	Anilinopyrimidines	Hydroxyanilides	Strobilurins	Carboxamides	
1–3	INRA	2007	Champagne	France	I	−	−	−	[+]	−	−	−
4	INRA	2007	Versailles	France	II	−	−	−	−	−	−	−
5	INRA	2007	Champagne	France	II	−	−	−	−	−	−	−
6	INRA	1994	Champagne	France	II	+	−	−	−	−	−	−
7	INRA	1994	Armagnac	France	II	−	+	+	−	−	−	−
8	INRA	<1980	Bordeaux	France	II	+	−	−	−	−	−	−
9	INRA	2006	Champagne	France	II	−	−	−	+	+	−	−
10	INRA	2007	Champagne	France	II	−	−	−	+	+	−	−
11	INRA	2007	Champagne	France	II	−	−	−	−	−	−	+ (MDR1)
12	INRA	2007	Champagne	France	II	+	−	−	−	−	−	+ (MDR1)
13–14	INRA	2007	Champagne	France	II	−	−	+	−	+	−	+ (MDR1)
15	INRA	2007	Champagne	France	II	−	−	−	−	−	−	+ (MDR2)
16–17	INRA	2007	Champagne	France	II	+	−	−	−	−	−	+ (MDR2)
18	INRA	2007	Champagne	France	II	+	+	−	−	−	−	+ (MDR2)
19–20	INRA	2007	Champagne	France	II	+	−	−	−	−	−	+ (MDR3)
21–22	INRA	2007	Champagne	France	II	−	−	−	−	−	−	+ (MDR3)
23–24	BASF	2006		Germany	II	−	−	−	−	−	+ (CarR1)	−
25–26	INRA	2007	Champagne	France	II	−	−	−	+	+	+ (CarR1)	−
27	INRA	2008	Champagne	France	II	−	−	−	−	+	+ (CarR1)	−
28	INRA	2008	Champagne	France	II	−	−	−	+	+	+ (CarR1)	−
29	Uni. Kai	2007	Palatine	Germany	II	−	−	−	−	+	+ (CarR1)	−
30	Uni. Kai	2007	Palatine	Germany	II	−	−	−	+	+	+ (CarR1)	−
31	Uni. Kai	2007	Palatine	Germany	II	−	−	−	−	+	+ (CarR1)	−
32	Uni. Kai	2007	Palatine	Germany	II	−	−	−	+	+	+ (CarR1)	−
33–35	Uni. Kai	2007	Palatine	Germany	II	−	−	−	−	+	+ (CarR2)	−
36–44	Uni. Kai	2008	Palatine	Germany	II	−	−	−	−	+	+ (CarR2)	−
45	Uni. Kai	2008	Palatine	Germany	II	−	−	−	−	+	+ (CarR3)	−
46	Uni. Kai	2008	Palatine	Germany	II	−	−	−	−	+	+ (CarR4)	−
47–48	INRA	2008	Champagne	France	II	−	−	−	−	+	+ (CarR5)	−
49	BASF	2006		Germany	II	−	−	−	−	−	+ (CarR6)	−
50	Uni. Kai	2008	Palatine	Germany	II	−	−	−	−	+	+ (CarR6)	−

[a] INRA, strains collected by our group; BASF, strains provided by BASF, Limburgerhof, Germany; Uni. Kai, gift from Kaiserslautern University, Germany.
[b] *Botrytis* group I and II strains were differentiated on the basis of genotype and their response to certain sterol biosynthesis inhibitors, including fenhexamid and fenpropidin (25, 46).
[c] Specific resistance (+) is determined by an alteration in the genes encoding the target protein (see the first section of Results). In *Botrytis* group I, there is natural resistance ([+]) to fenhexamid (hydroxyanilides), which is probably determined by an increase in the detoxification of this compound (47). −, lack of specific resistance for a given mode of action.
[d] Multidrug resistance (+) corresponds to positive cross-resistance between unrelated fungicides. It is determined by the overexpression of drug transporters (42). −, lack of MDR in a given isolate.

succinate dibasic hexahydrate (Sigma-Aldrich) in experiments involving carboxamides and TTFA. Moreover, the existence of an active alternative oxidase (68, 74) in *B. cinerea*, as in other fungi, made it necessary to add SHAM to *in vitro* tests involving inhibitors of complex III (i.e., strobilurins, antimycin A). We used a SHAM concentration of 0.5 mM. However, in our experimental conditions, this compound did not enhance carboxamide activity (data not shown).

Fungicides were added to the medium described above, which was then poured into 5.5-cm-diameter plastic petri dishes. We then dispensed 0.3 ml of a conidial suspension (200,000 conidia ml^{-1}) onto the surface of agar plates with a pipette. The plates were incubated for 24 h in the dark at 19°C, and the percentage of spores germinating and the lengths of germ tubes (50 to 100 germinated conidia for each treatment) were estimated under a microscope, using a micrometer. Each test was replicated at least three times. Standard error was calculated and indicated an acceptable level of repeatability between replicates (data not shown).

Fungicides affecting respiration are powerful inhibitors of both germination and germ tube elongation. However, the effect of some of them, especially carboxamides, appears often transient toward germination. We therefore determined for each strain/fungicide couple an EC_{50} (i.e., a concentration causing a 50% decrease of fungicide *in vitro* efficacy) only for the germ tube elongation criterion. EC_{50}s were determined after linear regression of the germ tube length (as percentage of control) plotted against the $\log_{10}$ of fungicide concentration. For a given fungicide, according to the response of susceptible (wild-type) strains, a mean EC_{50} could then be calculated. For each putative mutant strain, a resistance factor (RF) could be estimated as the ratio of the EC_{50} of the mutant to the mean EC_{50} of the wild type. In our experimental condition, a strain with an RF greater than or equal to 0.5 and less than 2 was considered susceptible, whereas RF values greater than 2 or less than 0.5, respectively, suggested resistance and hypersusceptibility (46).

DNA isolation. Conidia from 1-week-old cultures on MYA medium (as described above) were used to inoculate 125 ml of the same liquid medium. The resulting suspension was cultured in an orbital shaker for 36 h at 20°C, and the fungus was then filtered through sterile muslin and rinsed in sterile water. Fungal material was frozen at −20°C for later use. DNA was extracted from this frozen material by a sarcosyl-based protocol (18).

Cytochrome *b* gene sequencing and molecular diagnosis of strobilurin resistance. Primers Qo1 to Qo22 (see Table SA2 in the supplemental material) were designed based on the *B. cinerea* cytochrome *b* (*cytb*) gene sequence (accession number AB262969; 6,072 bp) and provided complete coverage of the entire gene for sequencing purposes. PCR was carried out with 25 ng genomic DNA, 200 μM each deoxynucleoside triphosphate (dNTP), 0.5 μM each primer, 10× titanium buffer, and 50× titanium *Taq* polymerase (Clontech) in a reaction volume of 50 μl. Amplifications were carried out as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 60°C for 30 s, and 68°C for 1 min and a final extension at 68°C for 4 min. Fragments of the appropriate size were sequenced on both strands with a CEQ8000 Beckman Coulter sequencer. Sequences were assembled, translated, and aligned with CodonCode Aligner software (CodonCode Corporation), using the Muscle algorithm.

The cleavable amplified polymorphic sequences (CAPS) test was developed for quick and easy characterization of the G143A mutation, which is known to be the principal cause of resistance to strobilurins in many phytopathogenic fungi (21) in field populations. We digested 5 μl of unpurified PCR fragment, obtained with primers Qo13ext and Qo14ext, with the restriction enzyme SatI (isoschizomer Fnu4HI; Fermentas) at 37°C for 2 h, according to the manufacturer's recommendations. Digested fragments were visualized by electrophoresis in 2% agarose gels.

Population genetics analyses. Only the population samples (169 isolates) collected from the Courteron and Vandières vineyards were analyzed with the Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7, and Bc10 microsatellites developed by Fournier et al. (26). We assessed the allele range variability of all loci and developed a multiplex analysis procedure, with appropriate fluorochrome assignment. Multiplex PCR was first carried out as follows: multiplex PCR1 amplified Bc1 plus Bc9, multiplex PCR2 amplified Bc3 plus Bc6, multiplex PCR3 amplified Bc4 plus Bc10, and multiplex PCR4 amplified Bc2 plus Bc5 plus Bc7. We then carried out a fragment analysis of the PCR products on a CEQ8000 Beckman Coulter sequencer, as follows: one run for multiplex PCR4 products and one run for multiplex PCR1, -2, and -3 products pooled together. Diversity parameters were calculated with GenClone (http://ccmar.ualg.pt/maree/software.php?soft=genclon), Multilocus (2), and Genetix (9). We used Populations (http://bioinformatics.org/~tryphon/populations/#ancre_fonctionnalites) software to reconstruct a tree showing the genetic relationships between the 169 individuals, using Nei's Da genetic distance (56) and a neighbor-joining clustering algorithm with 1,000 bootstrap replicates.

Sequencing of succinate dehydrogenase subunits. Mitochondrial complex II comprises a flavoprotein (SdhA), an iron-sulfur protein (SdhB) with three iron-sulfur clusters (S1, S2, and S3), and two membrane-anchored proteins (SdhC and SdhD) (33). The SdhA and SdhB subunits form the soluble part of the complex, which has succinate dehydrogenase activity, whereas the SdhC and SdhD subunits anchor the SdhA and SdhB proteins in the mitochondrial membrane and display ubiquinone reduction activity, as previously described in *Escherichia coli* (75) and *Saccharomyces cerevisiae* (59).

The *sdhB* primers IpBcBeg and IpBcEnd2 (see Table SA2 in the supplemental material) were designed in a previous study (unpublished data) for NCBI accession numbers AY726618 (*Botrytis* group II) and AY726619 (*Botrytis* group I).

Other subunit sequences were obtained after tBLASTn analysis of NCBI accession numbers for *Saccharomyces cerevisiae* with the *B. cinerea* genome release from the Broad Institute (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html). This made it possible to isolate *B. cinerea* sequences displaying a high level of identity to the published *S. cerevisiae* sequences M86746, X73884, and L26333 for *sdhA*, *sdhC*, and *sdhD*, respectively. Sequences were retrieved from *B. cinerea* supercontigs 44, 143, and 4, respectively, based on Broad Institute genome release numbering. *B. cinerea*-specific primers were designed to bind to *sdhA* (primers SdhA1 to SdhA8), *sdhC* (primers SdhC1 to SdhC5) and *sdhD* (primers SdhD1 and SdhD2) (see Table SA2 in the supplemental material). PCR and sequencing protocols were as described for the cytochrome *b* gene, except that primers IpBcBeg and IpBcEnd2 were used with a hybridization temperature of 68°C.

RESULTS

Phenotypic characterization of fungal strains. Among the 50 strains collected in French and German vineyards, three belonged to *Botrytis* group I (1 to 3) and the others to *Botrytis* group II (4 to 50). Resistance to botryticides was mainly found within group II (Table 1).

The three tested strains of *Botrytis* group I were susceptible to all fungicides tested except fenhexamid. It has been previously hypothesized that the natural tolerance of sterol C4 demethylation to this inhibitor is probably due to an increase in detoxification (47).

Some *Botrytis* group II strains were moderately to highly resistant to benzimidazoles, dicarboximides, anilinopyrimides, and/or fenhexamid. As previously shown, these specific resistances are determined by changes in the respective target genes of benzimidazoles (i.e., β-tubulin, a component of microtubules), dicarboximides (i.e., a histidine kinase involved in osmoregulation), and fenhexamid (i.e., a 3-keto reductase involved in sterol C4 demethylation). A similar mechanism has been proposed for anilinopyrimides, which have an unknown biochemical mode of action (23, 47). No specific resistance to fludioxonil was recorded.

In addition, several *Botrytis* group II strains displayed simultaneous decreases in susceptibility to various classes of fungicides and were classified into three MDR phenotypes. In all these strains, dicarboximides, anilinopyrimidines, and tolnaftate (an inhibitor of squalene epoxidase) were affected. This phenomenon extends to phenylpyrroles in MDR1 or to fenhexamid and sterol 14α-demethylase inhibitors (e.g., prochloraz, tebuconazole) in MDR2 (46). The MDR1 × MDR2 natural hybrid, called MDR3, displayed the broadest pattern of resistance (42, 47). These MDR strains are commonly found in Champagne and German vineyards. Their resistance patterns were recently found to be determined by overproduction of one to two membrane drug efflux proteins (42). Four representatives of each type were selected for this study: MDR1

TABLE 2. *In vitro* response of various *B. cinerea* phenotypes to fungicides affecting respiration

Fungicides affecting respiration	EC_{50} (μM)[a] for susceptible strains of *Botrytis* from group:		Resistance factor[b] of *Botrytis* group II strains with specific resistance to:						
			Strobilurins (StrR)[c]	Carboxamides					
	I	II		CarR1	CarR2	CarR3	CarR4	CarR5	CarR6
No. of strains	3	5	2	2	12	1	1	2	1
QoIs									
Azoxystrobin	0.12	0.33	>75	1.49					1.12
Dimoxystrobin	0.013	0.014	>100	1.30					1.00
Pyraclostrobin	0.012	0.014	>445	1.34					1.07
Trifloxystrobin	0.006	0.012	>500	1.40					0.85
QiI									
Antimycin A	0.0047	0.015	0.64	1.00	1.04	1.28	1.03	1.09	1.22
Uncoupler									
Fluazinam	0.0097	0.026	1.04	1.08	1.22	1.67	1.25	0.92	1.25
Multisite inhibitor									
Tolylfluanid	0.067	0.31	1.85	1.48	1.10	1.85	1.39	1.30	1.06
SDH inhibitor									
TTFA	76.6	68.5	0.89	1.11	1.05	1.32	1.32	0.92	0.95

[a] Mean EC_{50}s calculated from three strains of *Botrytis* group I (1 to 3) and from five strains of *Botrytis* group II (4 to 8) (see Table 1).
[b] Within *Botrytis* group II, for each phenotype, the resistance factor was calculated as the ratio of the EC_{50} of resistant strains to the mean EC_{50} of sensitive strains.
[c] Mean RF values obtained with two StrR strains (9 and 10); similar values were recorded with strobilurins for the CarR strains cumulating resistance toward this class of QoIs and carboxamides (see Table 1).

(strains 11 to 14), MDR2 (strains 15 to 18), and MDR3 (strains 19 to 22) (Table 1).

Resistance to inhibitors of mitochondrial complex III. In the presence of SHAM, low concentrations of azoxystrobin, dimoxystrobin, pyraclostrobin, trifloxystrobin, and antimycin A inhibited germ tube elongation of susceptible strains of *Botrytis* group II. Moreover, *Botrytis* group I strains were generally more susceptible than group II strains to these inhibitors of complex III, in particular to antimycin A and azoxystrobin (Table 2; see also Table SA3 in the supplemental material).

Three groups of *Botrytis* group II strains were defined on the basis of the EC_{50}s recorded for pyraclostrobin: susceptible (EC_{50} below 0.025 μM), weakly resistant (EC_{50} between 0.05 and 0.3 μM), and highly resistant ($EC_{50} > 10$ μM) (see Table SA3 in the supplemental material). All the weakly resistant strains were multidrug resistant, and cross-resistance to other tested strobilurins (i.e., azoxystrobin, dimoxystrobin, trifloxystrobin) and to antimycin A was recorded only for MDR3 strains (Fig. 1). Moreover, these MDR3 strains were less susceptible toward the uncoupler fluazinam and the multisite inhibitor tolylfluanid. In strains highly resistant to pyraclostrobin, positive cross-resistance was observed with azoxystrobin, dimoxystrobin, and trifloxystrobin but not with antimycin A, fluazinam, and tolylfluanid (Table 2). These strains are referred to as StrR strains (Table 1; see also Table SA3 in the supplemental material).

Molecular analysis of *cytb*. The full sequence of the mitochondrially encoded cytochrome *b* (characteristics in Table 3) was determined for three susceptible strains (7, 8, and 35) and three strobilurin strains (10, 25, and 32) from the *Botrytis* group II species. Strains 1, 2, and 3 were sequenced for comparison with the *Botrytis* group I species. No nucleotide polymorphism differentiating between the two *Botrytis* species was found in *cytb* sequences from the susceptible strains. Within *Botrytis* group II, very little polymorphism was observed in comparison to the available sequences of strains T4 and B05.10. The predicted amino acid sequence revealed high similarity to the cytochrome *b* sequences of other fungal species, namely, 84% identity to *Aspergillus niger*, 89% to *Venturia inaequalis*, 62% to *S. cerevisiae*, and 86% to *Mycosphaerella graminicola.* In the strobilurin-resistant strains, a mutation at codon 143 replacing a glycine residue by an alanine residue (G143A) was identified (see Table 6; Fig. 2).

We developed a CAPS test using the SatI restriction enzyme, which recognizes, within a 560-bp fragment amplified with the Qo13ext and Qo14ext primers, the 5′-GC↓GC-3′ nucleotide pattern, for rapid detection of the G143A alteration in the population data set from Courteron and Vandières. In the presence of the mutation, a typical two-band profile was observed, with fragments of 318 bp and 242 bp detected. No amplification was observed with DNA from grapevine *Vitis vinifera* or from several fungi and oomycetes usually present on this plant, such as *Erysiphe necator*, *Plasmopara viticola*, and *S. cerevisiae*, confirming the high level of specificity of this diagnostic tool (Fig. 3). This test showed the G143A substitution to be present at a frequency of 5.9% (10 isolates) in the field populations collected in Champagne. This substitution was found only in *Botrytis* group II strains (Table 4). Moreover, in the data set shown in Table 1, the StrR phenotype was always associated with G143A (Table 4; see also Table 6).

Variable occurrence of an intron in *cytb*. Regarding the size of the *cytb* gene, two categories were identified: strains with a 5,403-bp *cytb* gene and strains with a 6,608-bp sequence (Table 3). No isolate presented both fragments in the CAPS test.

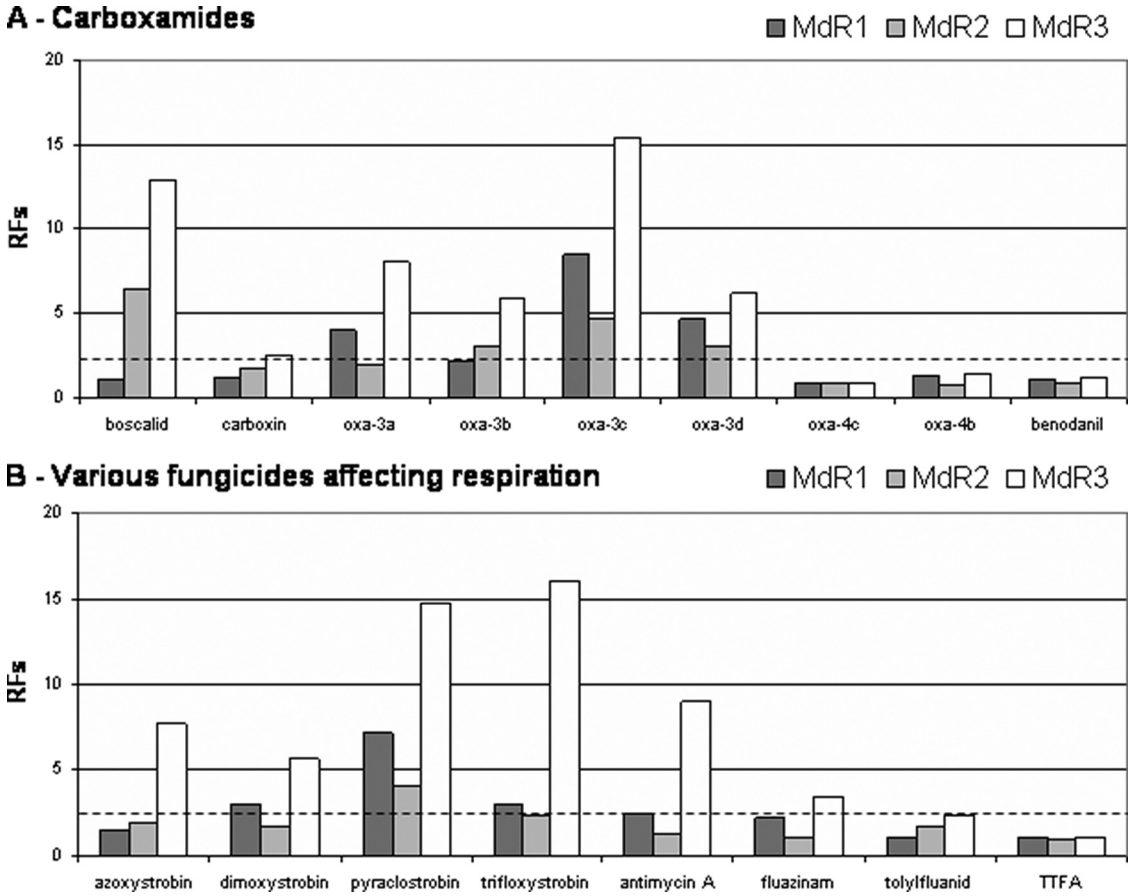

FIG. 1. *In vitro* responses of multidrug-resistant strains from *Botrytis* group II toward respiratory inhibitors. For given MDR strains, the RF equals the ratio of the EC_{50}s of MDR strains to the mean EC_{50} of the wild type (see Materials and Methods). Mean RF values were calculated using four strains for each MDR category (Table 1).

Sequencing revealed this difference to be due to the variable presence of a 1,205-bp intron located after codon 143 in both *Botrytis* group II (strains 7 and 8) and *Botrytis* group I (strains 1 and 2) strains (Fig. 3). This intron was not predicted in the two available *Botrytis* genome sequences (strains B05.10 and T4). In our population sample, 23.1% of the strains had this intron: 9.2% of *Botrytis* group II strains and 96.3% of *Botrytis* group I strains (Table 4). The G143A substitution was only found in *Botrytis* group II strains that did not harbor this intron.

We analyzed the evolution of intron-containing and intron-free versions of *cytb* in *Botrytis* groups I and II by investigating the relationship between the *cytb* genotypes and checking whether the presence of the type I intron discriminated between subpopulations. For this, field populations collected from Champagne vineyards were genotyped with the eight microsatellites listed in Table SA1 in the supplemental material. *Botrytis* groups I and II could be distinguished on the basis of the private allele 86 at the Bc6 locus, which was typical of *Botrytis* group I species. The *cytb* genotype was determined based on the length of the Qo13ext/Qo14ext PCR product and the result of the CAPS test. The four categories of strains (*Botrytis* group II with and without the intron after codon 143 and *Botrytis* group I with and without the intron after codon 143) were highly diverse, with more than 85% unique microsatellite profiles (G/N) and high levels of genotypic diversity. Only a few clones with identical microsatellite profiles were identified, consistent with the populations being in Hardy-Weinberg equilibrium, with recombination and/or migration occurring regularly (Table 4). As expected, pairwise calculations of the population differentiation (Fst) (71) between *Botrytis* group I and group II strains harboring the intron indicated high levels of differentiation between the two species (e.g., Fst = 0.488, highly significant after Bonferroni correction; a difference of the same order of magnitude was observed when *Botrytis* group I and group II strains were compared globally, independently of the presence of the intron). In contrast, the pairwise Fst between the two populations harboring different *cytb* genotypes within a single species was not significant (e.g., Fst = 0.032, not significant after Bonferroni correction, between *Botrytis* group II strains with and without the intron). These data indicate unrestricted gene flow between strains with intron-containing and intron-free *cytb*.

We calculated the Da genetic distance between all pairs of individuals and used it to reconstruct a neighbor-joining tree for distance (see Fig. SA2 in the supplemental material). The tree highlights the differentiation of strains from the two species, with the longest branch isolating the *Botrytis* group I

TABLE 3. Characteristics of the genes sequenced in *Botrytis* groups I and II

Gene	Coverage (bp)[a]	Gene size (bp)[b]	No. of introns[c]	% identity[d]	% similarity[e]	No. of polymorphisms[f] (%)	Accession no.[g]
Cytochrome *b*	5,830 or 7,035	5,403 or 6,608	3 or 4	62	80	0 (0)	FJ217740, FJ217741, FJ217742, FJ217743, FJ217744
Succinate dehydrogenase subunit A	2,560	2,209	6	73	86	49 (1.9)	FJ217745, FJ217746
Succinate dehydrogenase subunit B	1,021	953	1	70	83	10 (1.0)	AY726618, AY726619, GQ253444, GQ253445, GQ253446, GQ253447, GQ253448, GQ253449
Succinate dehydrogenase subunit C	1,139	712	2	32	51	11 (1.0)	GQ253442, GQ253443
Succinate dehydrogenase subunit D	1,175	683	2	35	52	8 (0.7)	GQ253439, GQ253440, GQ253441

[a] Size of the sequenced contig.
[b] Size of the gene, from methionine to the stop codon, including introns.
[c] Number of introns according to the Broad Institute prediction (B05.10 genome). For *cytb*, sequenced genomes (strains B05.10 and T4) don't exhibit the additional intron after codon 143.
[d] Amino acid sequence identity between the strobilurin- or carboxamide-susceptible group II sequence and the corresponding *Saccharomyces cerevisiae* gene sequence.
[e] Amino acid sequence similarity between the strobilurin- or carboxamide-susceptible group II sequence and the corresponding *Saccharomyces cerevisiae* gene sequence.
[f] Number of polymorphic sites between *Botrytis* group II and *Botrytis* group I.
[g] Accession number in the GenBank database. *S. cerevisiae* reference sequences had the accession numbers X84042, M86746, J05487, X73884, and L26333 for *cytb*, *sdhA*, *sdhB*, *sdhC*, and *sdhD*, respectively.

strains. This is mostly due to the group I private alleles in the Bc3, Bc4, and Bc6 microsatellites (see Table SA1 in the supplemental material). Finally, within a given species, no clear differences were found between strains with and without the type I intron after codon 143, this intron being randomly distributed in different lineages.

Resistance to inhibitors of mitochondrial complex II. Boscalid, the only carboxamide authorized for use against gray mold, prevented *B. cinerea* spore germination fully only at high concentrations, whereas it inhibited germ-tube elongation at low concentrations, particularly if biological tests were conducted in media containing succinate rather than glucose as the carbon source (data not shown). The tested strains of *B. cinerea* could be classified into categories on the basis of the EC_{50}s for germ tube elongation. The most susceptible strains (EC_{50}s below 0.2 μM) belonged to *Botrytis* group I (strains 1 to 3). The values for the wild-type (4 to 8), StrR (9 to 10), and MDR1 (11 to 14) strains in *Botrytis* group II were similar. Strains with EC_{50}s between 0.5 and 2 μM included the multidrug-resistant MDR2 (15 to 18) and MDR3 (19 to 22) strains. A slight decrease in susceptibility to carboxin was observed only in MDR3 strains, whereas resistance to 3′-substituted carboxin analogs was observed for all MDR strains. Moreover, the other tested carboxamides had similar effects on wild-type and MDR strains (Fig. 3; see also Table SA3 in the supplemental material). Other strains were either moderately (EC_{50}s of 2.5 to 10 μM) or highly (EC_{50}s above 30 μM) resistant to boscalid. They were subdivided into various carboxamide resistance (CarR) classes on the basis of the effects *in vitro* of the other carboxamides from Table 5, such as the benzamides (i.e., benodanil and flutolanil) and 3′-benzyl derivative of carboxin oxa-3b. We considered hypersusceptibility when the RF was less than 0.5, normal susceptibility when the RF was greater than or equal to 0.5 and less than 2, weak resistance when the RF was greater than or equal to 2 and less than 10, moderate resistance when the RF was greater than or equal to 10 and less than 100, and strong resistance when the RF was greater than or equal to 100.

CarR1 had moderate resistance to boscalid, weak resistance to oxa-3b, and hypersusceptibility (i.e., negative cross-resistance) to benzamides (strains 23 to 32). CarR2 had moderate resistance to boscalid, weak resistance to oxa-3b, and an absence of negative cross-resistance to benzamides (strains 33 to 44). CarR3 had moderate resistance to boscalid and normal sensitivity to oxa-3b (strain 45). CarR4 had moderate resistance to boscalid and to oxa-3b (strain 46). CarR5 had strong resistance to boscalid and moderate resistance to oxa-3b (strains 47 and 48). CarR6 had strong resistance to boscalid and normal susceptibility to oxa-3b (strains 49 and 50).

Cross-resistance between boscalid and carboxin (oxathiin derivative) occurred in all phenotypes. Experimental oxathiins, with substitutions at position 3′ in the phenyl ring (oxa3a to -c; see Fig. SA1 in the supplemental material), were 5 to 13 times more toxic to wild-type strains than carboxin. Overall resistance toward carboxamides was stronger in the CarR4 and CarR5 strains than in the CarR3 and CarR6 strains, with no cross-resistance (RFs between 0.5 and 2.0) to the 3′-benzyl derivatives oxa-3b in CarR3 and CarR6 and to oxa3d in CarR6. The two oxathiin derivatives substituted at position 4′ (oxa-4a and -b; see Fig. SA1 in the supplemental material) were less toxic than the derivatives described above and were most active against CarR6 strains (Table 5).

The other carboxamide fungicides tested (i.e., furcarbanil, methfuroxam, pyracarbolid, thifluzamide) were moderately toxic to wild-type strains ($EC_{50} > 7$ μM). Positive cross-resistance between these compounds and boscalid was recorded in all CarR strains (except for furcarbanil in CarR6 strains), generally with low RFs (Table 5).

Finally, TTFA, a noncarboxamide inhibitor of the succinate

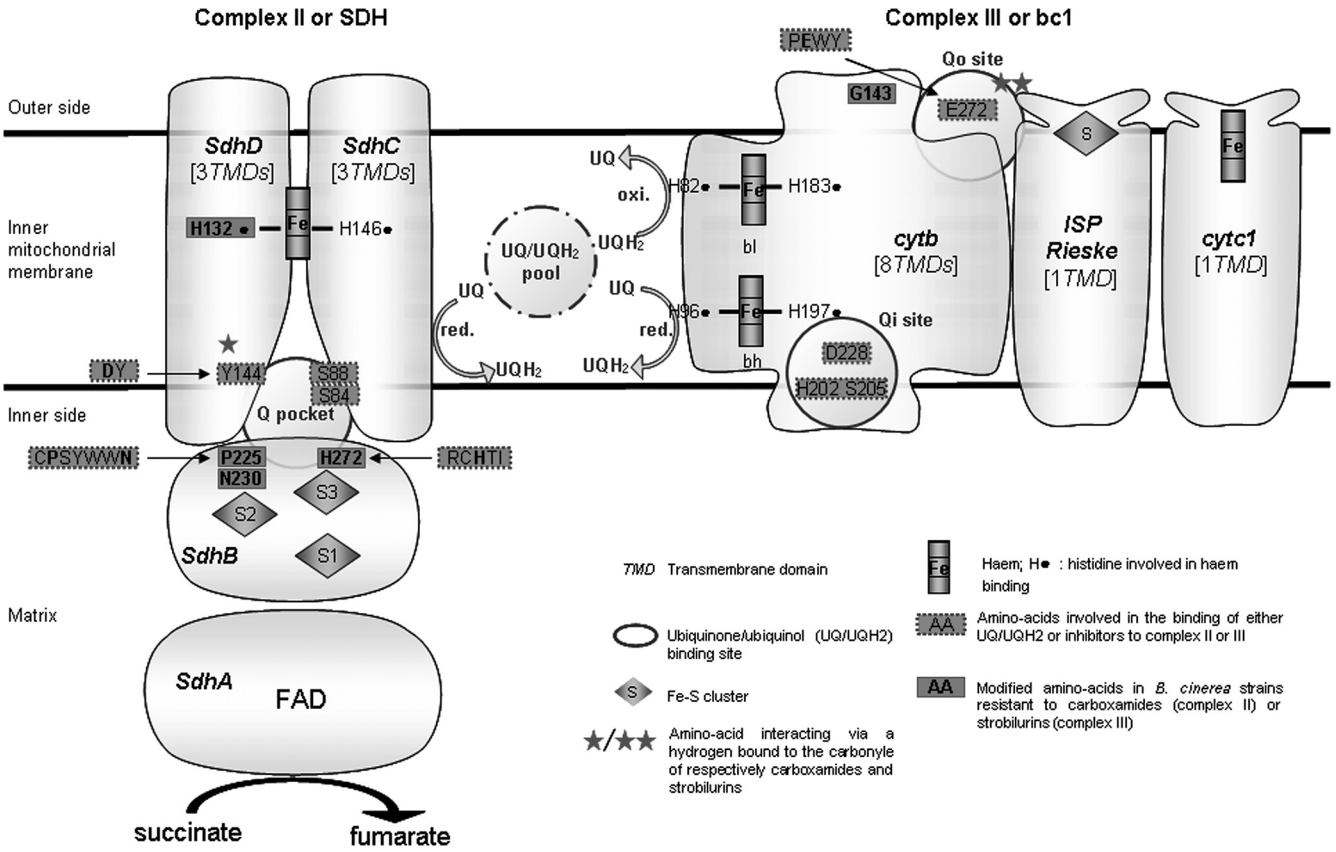

FIG. 2. Schematic structure of mitochondrial complexes II and III indicating the main amino acids of the different subunits involved in binding of inhibitors and in resistance. Complex II contains four subunits, whereas complex III consists of 10 to 11 polypeptides. For complex III, only three main subunits (i.e., Cytb, cytochrome *b*; ISP Rieske, iron sulfur protein with a Rieske-type cluster; cytc1, cytochrome c_1) directly involved in the electron transfer are shown. The amino acids involved in the binding of substrates (i.e., ubiquinone or ubiquinol) or of inhibitors were identified in crystallographic studies involving complex II or III from various origins (20, 28, 33, 35, 36, 59, 75). Amino acids are numbered according to *B. cinerea* sequences.

dehydrogenase complex, displayed only weak toxicity against all the *B. cinerea* strains tested (Table 2).

Molecular analysis of genes encoding succinate dehydrogenase subunits. The genes encoding the four subunits, *sdhA*, *sdhB*, *sdhC*, and *sdhD*, were sequenced in the two *Botrytis* species, and their main characteristics are presented in Table 3.

Numerous polymorphic locations were identified in each of the four genes but only between, not within, *Botrytis* group I and group II strains. Most of these polymorphisms were located in the introns or flanking sequences and did not affect the amino acid sequence, except for the P40S (Table 6) substitution identified in SdhB, which may be associated with the greater susceptibility of *Botrytis* group I strains to boscalid (Table 5).

Within *Botrytis* group II, most of the changes observed occurred within the second (S2) and third (S3) cysteine-rich clusters of the iron-sulfur protein, SdhB (Table 6; Fig. 2). Isolates harboring a mutation in codon 272, affecting the third Fe-S cluster region, were the most frequent in our sampling, and three potential resistant alleles associated with different phenotypes were identified. All CarR1 strains displayed the replacement of the histidine residue by a tyrosine residue at position 272 (H272Y). The replacement of this histidine residue by an arginine residue (H272R) was observed in nine of the 12 CarR2 strains, and the H272L substitution was associated with CarR5 strains. Changes in the second cysteine-rich cluster at position 225 were observed in one CarR3 strain (P225T) and in two CarR6 strains (P225L). A third site of alteration was found in the third exon of *sdhB* in the CarR4 strains (N230I). The phenotype-genotype correlation was not complete for CarR2 strains: 2 strains (36 and 41) had wild-type sequences for the four subunits of succinate dehydrogenase, and strain 35 harbored the H132R substitution in the SdhD anchor subunit.

Other fungicides affecting respiration. Low concentrations of the uncoupler fluazinam inhibited conidial germination and germ tube elongation (in media containing glucose and in the absence of SHAM). The most susceptible strains were found among *Botrytis* group I species and the least susceptible strains were the multidrug-resistant MDR1 and MDR3 strains. Smaller differences between these strains were observed for multisite inhibitors, such as captafol, chlorothalonil (data not shown), and tolylfluanid (Table 2; see also Table SA3 in the supplemental material; Fig. 3).

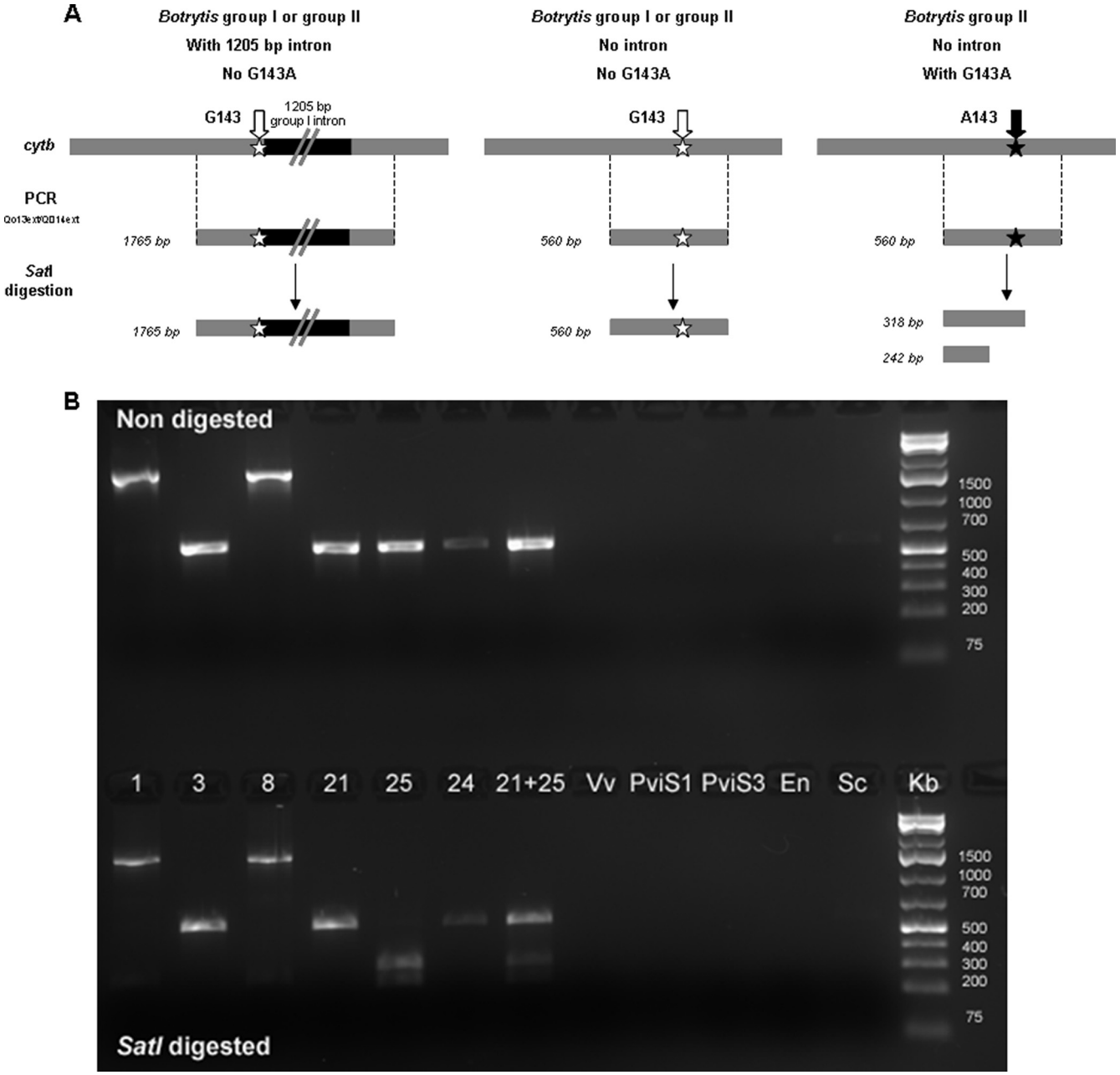

FIG. 3. Molecular polymorphism of the gene encoding cytochrome *b* (*cytb*) in strains of *Botrytis* group I and group II. (A) Possible structure of *cytb* according to the presence of the 1,205-bp group I intron and/or the G143A change and possible results observed after CAPS analyses. Presence of the intron changes the length of the PCR fragment, either in *Botrytis* group I or II. The G143A change was found only in *Botrytis* group II strains without the 1,205-bp intron. The mutation in *cytb* is recognized by the SatI restriction enzyme and leads to a two-band pattern after digestion of the PCR fragment. (B) Ethidium bromide-stained gel of PCR fragments subjected to electrophoresis after Qo13ext/Qo14ext amplification of the cytochrome *b* gene in *Botrytis* group I and group II (first row) and after *Sat*I digestion of these fragments (second row) (CAPS test). Strains 1 and 3, *Botrytis* group I with and without intron, respectively; strains 8, 21, 24, and 25, *Botrytis* group II. Strain 25 is strobilurin resistant (Table 1). Strain 8 exhibits an intron after codon 143. Vv, *Vitis vinifera* (grapevine); PviS1 and PviS3, *Plasmopara viticola* (downy mildew; PViS3 is strobilurin resistant); En, *Erysiphe necator* (powdery mildew); Sc, *Saccharomyces cerevisiae* (yeast).

DISCUSSION

Specific resistance to strobilurins is correlated with a single mutation in *cytb*. Up to 77 sites within *cytb* may be altered in eukaryotes and prokaryotes (10). In phytopathogenic fungi, only the F129L, G137R, or G143A substitution has been recorded in field strains resistant to strobilurins (21). In our data for *B. cinerea*, only the G143A alteration was found in all resistant phenotypes, either by direct sequencing or by CAPS analysis. It was identified in up to 27 phytopathogenic fungi listed by the Fungicide Resistance Action Committee

TABLE 4. Distribution of occurrence of a 1,205-bp intron in the *cytb* gene of a *Botrytis* sp. population sample from a Champagne vineyard

Group	Intron	No. of strains	% frequency	No. of StrR strains	G[a]	G/N[b]	No. of alleles/loci[c]	Genotypic diversity[d]
B. cinerea group II	+	13	7.7	0	13	1.00	5.38	1.000
	−	129	76.3	10	111	0.86	10.00	0.997
Botrytis group I	+	26	15.4	0	22	0.85	4.00	0.985
	−	1	0.6	0	1	1.00	1.00	1.000
Total group II		142	84.0	10	122	0.86	10.63	0.997
Total group I		27	16.0	0	23	0.85	4.25	0.985
Total		169	100	10	145	0.86	11.75	0.998

[a] G, number of multilocus genotypes, i.e., number of unique combinations of microsatellite alleles.
[b] G/N, frequency of unique multilocus genotypes in each category of isolates.
[c] Mean number of microsatellite alleles per locus. Calculated with GenClone software.
[d] Genotypic diversity calculated as the probability that two individuals taken at random have different genotypes. Calculated with Multilocus software, using 1,000 iterations.

(FRAC) (http://www.frac.info/frac/index.htm) and in reference 21. In susceptible strains, the glycine in position 143 probably contributes to the binding of the benzene ring linker of strobilurin fungicides to the Qo site of cytochrome *b* (Fig. 2) (20, 28). In our study, there was a complete correlation between the StrR phenotype and the G143A substitution. We therefore suggest that target alteration was the only mechanism of resistance operating in our data set. However, these results differ from those of a Japanese survey, which reported the absence of the typical G143A mutation from many StrR isolates collected from strawberries or citrus crops (38), consistent with an unknown mechanism of resistance, as reported for other fungi (22, 67, 70). Moreover, in our fungicide susceptibility test, strobilurins were

TABLE 5. *In vitro* susceptibility of various *B. cinerea* phenotypes to carboxamide fungicides

Carboxamides subgroup[a]	EC_{50} (μM)[b] for susceptible strains of *Botrytis* from group:		Resistance factor[c] of *Botrytis* group II strains with specific resistance to:						
			Strobilurins (StrR)	Carboxamides					
	I	II		CarR1	CarR2	CarR3	CarR4	CarR5	CarR6
No. of strains	3	5	2	10	12	1	1	2	2
Pyridine									
Boscalid	0.034	0.10	0.86	40.0	44.1	57.1	91.4	>350	>350
Benzamides									
Flutolanil	27.9	26.3	1.29	0.18	0.97	1.41	1.76	1.76	1.53
Benodanil	9.60	10.8	0.77	0.34	1.49	2.29	2.00	2.14	2.00
Furans									
Furcarbanil	6.03	7.44	1.00	2.06	2.00	1.56	4.69	5.00	0.75
Methfuroxam	13.0	13.1	0.83	3.67	2.83	4.00	4.00	4.00	5.00
Thiazoles									
Thifluzamide	3.80	7.58	1.25	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0
Dihydropyran									
Pyracarbolid	30.9	28.6	1.05	>3.3	>3.3	>3.3	>3.3	>3.3	>3.3
Oxathiin									
Carboxin	1.30	2.04	1.15	7.48	6.56	2.50	18.8	>21	3.33
oxa-3a	0.16	0.16	1.40	6.00	7.00	8.00	18.0	70.0	8.00
oxa-3b	0.12	0.18	1.33	8.33	10.0	1.67	23.3	41.7	0.83
oxa-3c	0.29	0.42	1.15	19.2	16.2	11.5	61.5	76.9	3.85
oxa-3d	0.25	0.40	1.15	4.62	3.85	3.85	11.5	6.15	1.54
oxa-4a[d]	4.20	7.37	1.09	>1.1	>1.1	>1.1	>1.1	>1.1	0.22
oxa-4b	6.52	7.49	1.09	1.74	1.52	0.43	1.74	1.74	0.35

[a] See the structure of the molecules in Fig. SA1 in the supplemental material.
[b] Mean EC_{50}s calculated from three strains of *Botrytis* group I (strains 1 to 3) and five strains of *Botrytis* group II (strains 4 to 8) (see Table 1).
[c] Within *Botrytis* group II, for each phenotype, resistance factors were calculated as the ratio of the EC_{50} of resistant strains to the mean EC_{50} of susceptible strains.
[d] Due to its low water solubility, this compound cannot be tested at a concentration above 8 μM.

TABLE 6. Alterations found in *cytb*, *sdhB*, and *sdhD* in various phenotypes of *B. cinerea* resistant to respiration inhibitors

Strain no.	*Botrytis* group[a]	Phenotype[b]		Molecular *cytb* characterization		Alteration in:	
		Str	Car	Intron after codon 143[c]	G143A[d]	*sdhB*	*sdhD*
1	I	S	S	+	−	P40S	
2	I	S	S	+	−	P40S	
3	I	S	S	−	−	P40S	
5	II	S	S	−	−		
7	II	S	S	+	−		
8	II	S	S	+	−		
10	II	R	S	−	+		
11	II	S	S	−	−		
12	II	S	S	−	−		
13	II	R	S	−	+		
17	II	S	S	−	−		
18	II	S	S	−	−		
19	II	S	S	−	−		
21	II	S	S	−	−		
23	II	S	R1	−	−	H272Y	
24	II	S	R1	−	−	H272Y	
25	II	R	R1	−	+	H272Y	
26	II	R	R1	−	+	H272Y	
27	II	R	R1	−	+	H272Y	
28	II	R	R1	−	+	H272Y	
29	II	R	R1	−	+	H272Y	
30	II	R	R1	−	+	H272Y	
31	II	R	R1	−	+	H272Y	
32	II	R	R1	−	+	H272Y	
33	II	R	R2	−	+	H272R	
34	II	R	R2	−	+	H272R	
35	II	R	R2	−	+		H132R
36	II	R	R2	−	+		
37	II	R	R2	−	+	H272R	
38	II	R	R2	−	+	H272R	
39	II	R	R2	−	+	H272R	
40	II	R	R2	−	+	H272R	
41	II	R	R2	−	+		
42	II	R	R2	−	+	H272R	
43	II	R	R2	−	+	H272R	
44	II	R	R2	−	+	H272R	
45	II	R	R3	−	+	P225T	
46	II	R	R4	−	+	N230I	
47	II	R	R5	−	+	H272L	
48	II	R	R5	−	+	H272L	
49	II	S	R6	−	−	P225L	
50	II	R	R6	−	+	P225L	

[a] *Botrytis* group I and II strains were differentiated on the basis of genotype and their response to sterol biosynthesis inhibitors, including fenhexamid and fenpropidin (25, 46).
[b] S, sensitive; R, resistant.
[c] +, 1,205-bp intron present after codon 143; −, absence of this intron.
[d] +, Qo13ext-Qo14ext PCR fragment digested with SatI (two-band pattern), indicating the presence of the G143A change; −, Qo13ext-Qo14ext PCR fragment undigested (single-band pattern), indicating the lack of mutation.

active against susceptible strains only if SHAM was added to inhibit the alternative oxidase. This suggests that the alternative oxidase is constitutively expressed *in vitro* in wild-type strains, as previously reported (68), and therefore cannot mediate the resistance of StrR isolates. This finding may also account for the low field efficacy of strobilurins against gray mold and explains why they are not approved for gray mold control in vineyards. However, the fungicides of this family have been widely used to control downy mildew due to *P. viticola* and powdery mildew due to *E. necator* on grapevine, through one to three treatments per year, since their introduction in 2001 in France. As a consequence, the G143A mutation is now widespread in most French populations of *P. viticola*, within two different *cytb* haplotypes (15), resulting in poor disease control when strobilurins are used alone. In contrast, resistance associated with the same *cytb* alteration took longer to be selected in *E. necator* and was first recorded in France in 2008 (19), highlighting the

dependence of the risk of resistance on the biology of the fungus. Thus, in grapevine, strobilurin resistance in *B. cinerea* populations may be the result of nontarget spraying unintentionally selecting the G143A alteration in *cytb*.

Variations in *cytb* structure. Strobilurin resistance was found only in *Botrytis* group II strains without the 1,205-bp intron after codon 143 of *cytb*. This intron, frequently found in fungal mitochondrial genes, was described as a type I intron (24). Such variations in the size of the cytochrome *b* gene have already been reported for *S. cerevisiae* (58), for which a deletion of 3.4 kb has been detected in some isolates. It was recently shown that the structure of *cytb*, including the presence or absence of an intron after codon 143, differs between species (30, 31). In many rusts and in some *Alternaria* and *Agrocybe* spp. (55), together with *Pyrenophora teres* (63), an intron was found after codon 143 that prevented the selection of G143A in *cytb*, owing to a defect in pre-mRNA splicing leading to the creation of an intron encoding a maturase, as described in yeast (14, 51). However, other mutations conferring lower levels of resistance may be selected in these fungi, such as F129L in *P. teres* (30). These findings may account for the presence of the G143A substitution only in strains lacking the intron in *Botrytis* group II in our sample and in Californian and Chinese isolates (40) and in Japanese strains isolated from various host plants for which no fitness cost associated with strobilurin resistance could be found (7). In our population data set, G143A was not found in any of the 27 group I strains. Finally, in our data set from the Champagne vineyard, we found that the *cytb* 143/144 intron was carried by different lineages within each species and that it was more frequent in *Botrytis* group I populations than in group II populations. Within a single species, the presence of this intron does not seem to be a barrier to gene flow and should not prevent strains of different *cytb* genotypes from mating. Based on the findings of an ongoing phylogenetic study, *Botrytis* group II species may be derived from *Botrytis* group I species (data not shown). Recurrent independent losses of the intron from *B. cinerea* group II populations, according to the retro-deletion mechanism previously described in yeast (27), may have occurred on several occasions after divergence from *Botrytis* group I, facilitating the subsequent acquisition of the G143A substitution in these lineages, in response to fungicide selection pressure. This hypothesis could be tested by sequencing this gene in several strains from different *Botrytis* species.

Specific resistance to carboxamides is correlated with various mutations in *sdh* genes encoding Sdh subunits. Resistance to carboxamides was first described some years ago for those effective against *Basidiomycetes*: field resistance to carboxin was observed in *Ustilago nuda* growing on barley (45) and was found to be partially dominant (57), whereas resistance to oxycarboxin was recorded in the causal agent of chrysanthemum rust, *Puccinia horiana* (1). More recently, resistance to boscalid has been observed in field strains of *B. cinerea* and of various phytopathogenic fungi from *Ascomycota* (65). Double resistance to these carboxamides and to strobilurins has been reported in many strains of *B. cinerea* (Table 1) and is also commonly found in *Alternaria alternata* (5). Resistance to carboxamides in field or laboratory mutants of many fungi and bacteria is generally determined by mutations in genes encoding the succinate dehydrogenase subunit SdhB, SdhC, or SdhD. These three subunits contribute to the binding of ubiquinone and carboxamide fungicides (Fig. 2) (29, 35, 36, 59, 75).

The highly conserved histidine residue (equivalent to H272 in *B. cinerea*) in iron sulfur cluster S3 of SdhB (Fig. 2) probably interacts with the O3 methoxy group of ubiquinone via hydrogen bonding. A similar phenomenon probably occurs with a heteroatom of most carboxamides (e.g., oxygen for oxathiin, furan, and pyran derivatives; nitrogen for pyrazole, pyridine, and thiadiazole derivatives) (35). The behavior of benzamide derivatives, which contain no such heteroatom (see Fig. SA1 in the supplemental material), remains to be determined. In many laboratory mutants of bacteria and fungi, this histidine residue has been replaced by a tyrosine, an asparagine, or a leucine residue (12, 34, 49, 53, 62, 64). More recently, field isolates of phytopathogenic fungi resistant to boscalid and carrying a mutation at this codon have been detected in populations of *Corynespora cassiicola* on cucurbits in Japan (H278Y) (37, 54) and of *A. alternata* on pistachios in the United States (H277Y/R) (6).

For *Botrytis* group II, we found that the three possible alleles, H272Y, H272R, and H272L, seemed to induce different phenotypes. The H272Y substitution, found within the CarR1 phenotype, is the most frequent genotype in French and German vineyards, and negative cross-resistance leads to an increase in susceptibility to the benzamides tested in this (i.e., flutolanil, benodanil) and other (i.e., fluopyram, mepronil) studies (43). This phenomenon has already been reported for some *U. nuda* field mutants of unknown genotype (45). These observations suggest that the binding of benzamides to succinate dehydrogenase is increased by the H272Y substitution, indirectly confirming the specific interaction of this class of carboxamides with SdhB. The other substitutions, H272R and H272L, found, respectively, in CarR2 and CarR5 strains, did not determine negative cross-resistance to any carboxamides. Moreover, the highest RFs were recorded for CarR5 strains. A similar laboratory mutant of *Ustilago maydis* harboring the H257L substitution was found to be highly resistant to carboxin and to display no negative cross-resistance to other carboxamides (41, 73).

All currently identified modifications affecting the second iron-sulfur complex (S2) of SdhB seem to be *Botrytis* specific. Most concern the proline residue in position 225, which is located within the highly conserved C<u>P</u>SYWWN motif. This proline residue and the two tryptophan residues probably contribute to carboxamide binding through hydrophobic interactions of the aniline group. Moreover, the tryptophan SdhB W229 seems to be a direct ligand of a carbonyl oxygen from ubiquinone or carboxamide (Fig. 2) (36, 59, 75). The substitutions P225T and P225L were observed, respectively, in CarR3 and CarR6 strains. A third possible allele, with a P225F modification at this codon, has also been identified in German isolates highly resistant to boscalid (65). In strains harboring the P225T or P225L substitution, resistance to carboxin was weak and some carboxin derivatives bearing phenyl substitutions at positions 4′ led to an increase in susceptibility. Such negative cross-resistance has also been reported for some laboratory mutants of *U. maydis* and *Aspergillus nidulans* of unknown genotype displaying moderate resistance to carboxin (72, 73). Finally, we identified, in a single CarR4 strain, a point

mutation at codon 230 of SdhB (N230I) not found in the carboxamide-resistant mutants of any other fungus. The asparagine residue in position 230 of SdhB belongs to the highly conserved CPSYWWN motif described above but is not thought to be involved in carboxamide binding to SdhB (Fig. 2) (35).

The anchor subunit SdhC contributes to the binding of ubiquinone probably via hydrogen bound between its O4 carbonyl group and the highly conserved serine residue in position 84 in *B. cinerea.* A strong interaction of carboxin with the corresponding amino acid residue in *Escherichia coli* was recorded (35). Moreover, in the same domain, the highly conserved arginine (SdhC R88 in *B. cinerea*) contributes to the binding of ubiquinone (Fig. 2) (35, 59). Mutations in *sdhC* were previously found in several laboratory and field mutants (29, 39, 62). Moreover, a recent report indicated that the H134R mutation is prevalent in SdhC of *A. alternata* collected from pistachios (65). This histidine (position 144 in *B. cinerea*) is a highly conserved residue involved in the iron coordination of heme *b* (Fig. 2) (59, 75); a second axial histidine residue is supplied by the SdhD subunit (SdhD H132 in *B. cinerea*; see below).

In the anchor subunit, SdhD, the highly conserved tyrosine residue at position 144 in *B. cinerea* is a major binding partner for ubiquinone and carboxamides. The carbonyl group of these fungicides (see Fig. SA1 in the supplemental material) and the O1 carbonyl atom of ubiquinone interact with this tyrosine residue via hydrogen bonds (35, 36). In laboratory mutants of *Paracoccus denitrificans* (52) and *Aspergillus oryzae* (62) selected on carboxin, the conserved aspartate flanking the previous tyrosine (codon 143 in *B. cinerea*) was replaced either by a glycine or a glutamate (52, 62). In *B. cinerea*, the replacement of a histidine residue in position 132 by an arginine residue was found in one strain with the CarR2 phenotype. Moreover, the same H132R substitution was recently found in a boscalid-resistant strain of the related species *Sclerotinia sclerotiorum* collected on rapeseed in France (29). As stated above, the H132 residue of SdhD and the H144 residue of SdhC are involved in the iron coordination of heme *b* (Fig. 2). Their role in electron transfer remains unclear and they do not seem to be involved directly in ubiquinone reduction (60). However, as arginine is not considered to be involved in iron coordination, its presence (in SdhC or SdhD) may result in some rearrangement indirectly affecting the binding of carboxamides. It would be interesting to investigate the functional consequence of these substitutions in either SdhC or SdhD in fungal mutants.

More information is required to improve our understanding of the relationship between the location of mutations, their effect on subunit structure, interactions with the various carboxamides, and the fitness of the various mutants. Further studies are also required to identify the resistance mechanism occurring in the two CarR2 strains with wild-type copies of the four *sdh* genes.

Multiple drug resistance is not linked to target gene alteration and induces a broad spectrum of resistance. Multidrug resistance (MDR) was demonstrated in our data set as the simultaneous resistance of organisms to a number of unrelated toxic compounds. MDR is commonly found in human fungal pathogens but has rarely been described for field strains of phytopathogenic fungi (16). In agriculture, the first case of MDR was recorded in field isolates of *B. cinerea*, with three possible phenotypes as a function of the fungicide resistance spectrum (48). In MDR1 strains, the ABC transporter AtrB is constitutively overexpressed due to mutations in the gene encoding a transcription factor that controls expression of the gene encoding this transporter (16, 42). In MDR2 strains, an MFS transporter has been implicated in the underlying mechanism, through the insertion of a transposon into its promoter. In MDR3, which has the broadest spectrum of fungicide resistance, both transporters are overexpressed (42). As previously reported for various classes of fungicides, the levels of resistance to respiratory inhibitors recorded in MDR strains were lower than those induced by changes in the target site. The highest levels of resistance were attributed to MDR3 strains. Based on the phenotypic responses of MDR1 and MDR2 strains, some respiratory inhibitors seem to display a particular affinity for ABC transporters (e.g., antimycin A, fluazinam, dimoxystrobin), for MFS transporters (e.g., boscalid), or for both transporters (e.g., 3′ derivatives of carboxin, pyraclostrobin, trifloxystrobin). This phenomenon applies to different extents to molecules of the same mode of action, with some fungicides, such as the carboxamide carboxin and the strobilurin azoxystrobin, little affected. Similar results have been reported for squalene epoxidase inhibitors: both MDR1 and MDR2 phenotypes are resistant to the thiocarbamate tolnaftate but susceptible to the alkylamines naftifine and terbinafine (46). The relationship between the physicochemical properties (for example, lipophilia) of the various respiratory inhibitors and their affinity to ABC or MFS transporters remains to be determined.

Respiratory inhibitors induce highly diverse resistance mechanisms in fungi, thereby complicating resistance management. Our survey of strains from French and German vineyards showed that at least two different mechanisms—target alteration and efflux pump overexpression—are responsible for resistance to respiratory inhibitors in *B. cinerea*, the causal agent of gray mold. Resistance concerned either a single (specific resistance) or several (multidrug resistance) classes of fungicides. In cases of specific resistance, amino acid substitutions in the target proteins probably reduced fungicide binding. Typical qualitative resistance to strobilurins was recorded, with full cross-resistance (monogenic and monoallelic), whereas a more complex situation suggestive of quantitative resistance with partial cross-resistance was observed with carboxamides (multigenic and multiallelic). Fungicides with several modes of action (e.g., anilinopyrimidines, phenylpyrroles, dicarboximides) used to control gray mold may have been responsible for the selection of some or all of the MDR phenotypes identified (42). MDR was rarely associated with specific resistance to botryticides (Table 1), with the exception of benzimidazoles, resistance to which has been generalized throughout Champagne for more than 20 years (48). This may indicate that there is a fitness cost attached to the association of MDR with specific resistance other than to benzimidazoles. However, some combinations of specific resistances seemed to be frequent in our data set, such as multiple resistances to fenhexamid and carboxamides or strobilurins and carboxamides (Table 1). Thus, in the Cham-

pagne vineyard, which is subject to intense but diverse selective pressures, several mechanisms of resistance may have evolved independently, resulting in a complex situation in terms of fungicide resistance, which may well become increasingly difficult to manage, given the decrease in the number of available active ingredients likely to be effective (48).

Moreover, as MDR tends to result in low to moderate levels of resistance and as strobilurins do not target *B. cinerea*, the greatest source of concern is resistance to carboxamides. The higher diversity of genotypes recorded for *B. cinerea* populations than for other fungi is surprising (65). Indeed, the selection pressure in French and German vineyards was restricted to a maximum of one full-dose application of boscalid per year—the mean number of boscalid treatments per season in the Champagne vineyard varied between 0.53 at its introduction and 0.34 in 2008. Moreover, a large number of molecules with alternative modes of action are available, making it possible to vary the class of fungicide used, thereby varying selection pressure (48). As observed in several phytopathogenic fungi (6, 37), the SdhB H272Y/R genotypes predominate in the emerging carboxamide-resistant populations of *B. cinerea*. However, various other mutations reducing the efficacy of boscalid have nonetheless been selected in *B. cinerea*. This suggests that (i) not all genotypes are of equal fitness, as already shown for *sdhB*, *sdhC*, and *sdhD* laboratory mutants whose succinate dehydrogenase activity was differently affected (62), and (ii) new carboxamides with different biological and chemical properties, which are likely to become available soon (29), may again increase the diversity of *sdh* genotypes or, at least, readjust the balance in their frequencies. *B. cinerea* management should therefore include resistance risk assessment in populations in which carboxamide resistance is emerging. The alternation of SDHI molecules and long rotations of fungicides from different groups with different modes of action might delay the selection of carboxamide-resistant strains (48). However, it might also favor the development of MDR, the impact on field efficacy of which remains limited (61). Finally, although mixtures of inhibitors displaying negative cross-resistance have improved field efficacy in some resistance situations (e.g., mixtures of carbendazim and diethofencarb used to control carbendazim-resistant gray mold populations in the 1980s in the Champagne vineyard (44), this strategy, based for example on the combined use of boscalid and a benzamide derivative such as fluopyram (see Fig. SA1 in the supplemental material), may be less effective for carboxamides, because not all the resistant phenotypes display negative cross-resistance. The use of mixtures of SDHIs with fungicides with another mode of action may help to delay the development of this specific resistance. However, such mixtures might lead to the selection of doubly resistant mutants or MDR.

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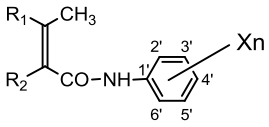
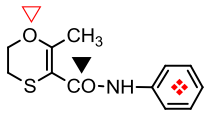
A : "Cis-crotonanilides" and related carboxamides

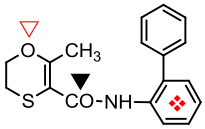


basic structure

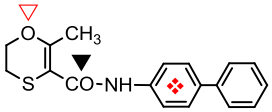
A_1 : oxathiin-carboxamides



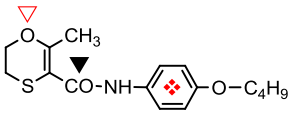
carboxin



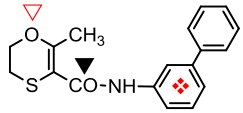
2'-phenylcarboxin (F427 [b])



4'-phenylcarboxin (oxa4a)



4'-butoxycarboxin (oxa4b)



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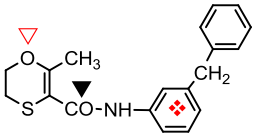

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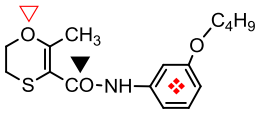
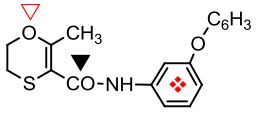
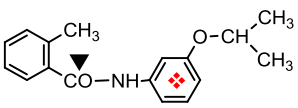


3'-butoxycarboxin (oxa3c)

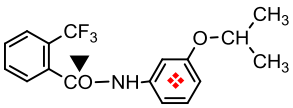


3'-hexyloxycarboxin (oxa3d)

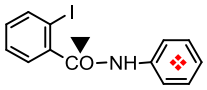
A_2 : benzamides



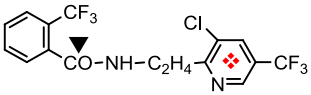
mepronil [b]



flutolanil

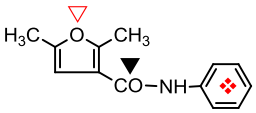


benodanil

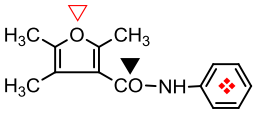


fluopyram [b]

A_3 : furan-carboxamides

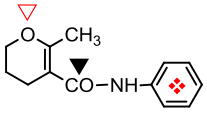


furcarbanil



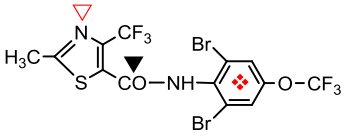
methfuroxam

A_4 : dihydropyran-carboxamide



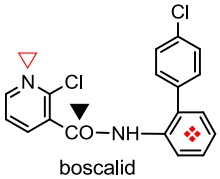
pyracarbolid

A_5 : thiazole-carboxamide



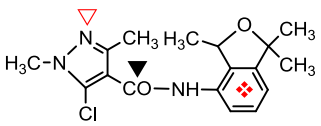
thifluzamide

A_6 : pyridine-carboxamide

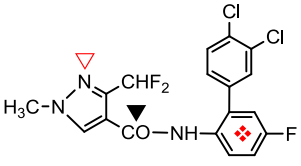


boscalid

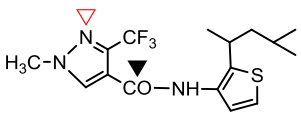
B : N-methylpyrazole carboxamides



furamethpyr [b]



bixafen [b]



penthiopyrad [b]

Figure SA1: Chemical structure of representative carboxamide fungicides[a]

[a] Structural features: ▲ carbonyl group interacting with a tyrosine residue in SdhD through hydrogen bonding (position 144 in *B. cinerea*); Δ oxygen or nitrogen atoms from heterocycles interacting, probably by hydrogen bonding, with a SdhB histidine (position 272 in *B. cinerea*); ❖ phenyl group interacting, probably through hydrophobic contacts, with a proline and two tryptophane residues from SdhB (positions 225, 228 and 229 in *B. cinerea*) (Figure 3) (1, 2, 3)

[b] molecules not tested in this study

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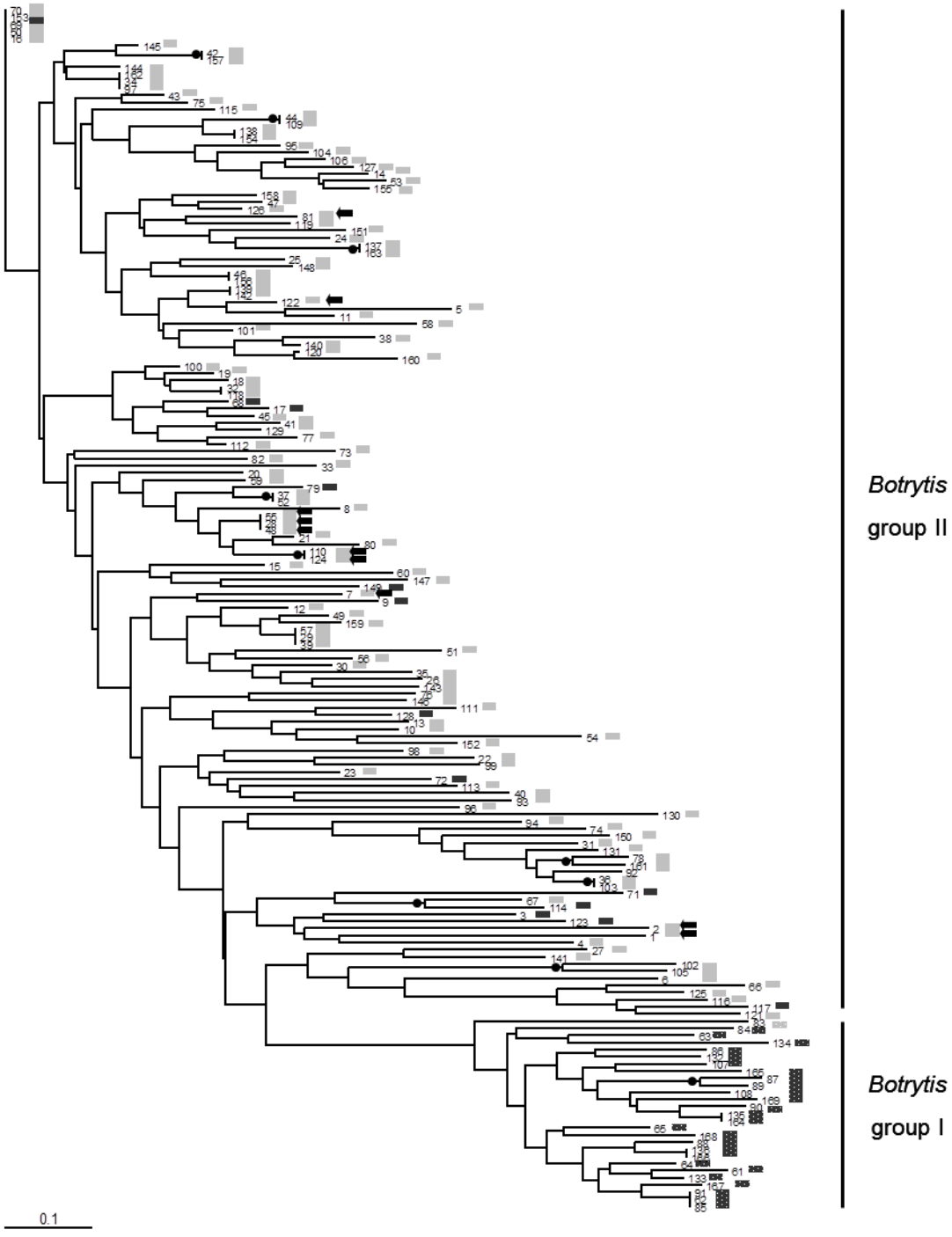
Botrytis
group II
Botrytis
group I
0.1

Figure SA2: Tree of *Botrytis* individual strains generated by neighbor-joining analysis based on Da distance (1), showing the relationship between individual microsatellites and the presence or absence of the 143-144 intron in *cytb*.
Nodes with a bootstrap value (1000 repetitions) greater than 500 are indicated with a black circle.
Botrytis group I isolates are shown by dotted boxes, whereas *Botrytis* group II strains are not dotted.
Isolates with the 143-144 intron are shown in dark gray, whereas the isolates in light gray do not have this intron.
Black arrows indicate strobilurin-resistant strains.

1. **Nei, M., F. Tajima, and Y. Tateno.** 1983. Accuracy of estimated phylogenetic trees from molecular data. II Gene frequency data. Journal of Molecular Evolution **19:**153-170.

Table SA1: Characteristic of the Champagne strains used for population analysis

Isolate	Location	Collection date	Host plant	Species[a]	Str[b]	Intron[c]	Bc1	Bc2	Bc3	Bc4	Bc5	Bc6	Bc7	Bc10
D340	Courteron	June 2006	Brambles	II	R	-	215	161	227	125	157	134	131	175
D342	Courteron	June 2006	Brambles	II	R	-	225	185	215	125	157	126	125	147
D359	Courteron	June 2006	Brambles	II	S	+	245	153	219	125	157	124	119	147
D367	Courteron	June 2006	Brambles	II	S	-	209	163	219	125	157	118	129	175
D368	Courteron	June 2006	Brambles	II	S	-	219	171	219	128	165	120	119	173
D369	Courteron	June 2006	Brambles	II	S	-	227	139	219	116	157	124	113	175
D373	Courteron	June 2006	Brambles	II	R	-	219	181	219	125	147	120	121	181
D374	Courteron	June 2006	Brambles	II	S	-	231	167	219	125	161	120	113	185
D377	Courteron	June 2006	Brambles	II	S	+	235	181	219	125	161	120	113	181
D380	Courteron	June 2006	Brambles	II	S	-	217	167	217	125	159	120	113	183
D383	Courteron	June 2006	Brambles	II	S	-	219	171	219	125	165	120	119	183
C200	Courteron	September 2005	Grapevine	II	S	-	231	171	219	125	159	120	119	181
C201	Courteron	September 2005	Grapevine	II	S	-	239	167	217	125	159	120	119	183
C202	Courteron	September 2005	Grapevine	II	S	-	217	167	221	125	157	120	121	167
C203	Courteron	September 2005	Grapevine	II	S	-	217	171	217	125	147	120	119	181
C204	Courteron	September 2005	Grapevine	II	S	-	217	167	219	125	147	120	119	181
C205	Courteron	September 2005	Grapevine	II	S	+	217	169	219	125	157	120	117	181
C206	Courteron	September 2005	Grapevine	II	S	-	217	167	219	125	147	120	117	189
C207	Courteron	September 2005	Grapevine	II	S	-	217	167	219	125	147	120	117	183
C209	Courteron	September 2005	Grapevine	II	S	-	231	191	219	125	147	120	119	181
C210	Courteron	September 2005	Grapevine	II	S	-	231	167	219	125	147	120	117	185
C211	Courteron	September 2005	Grapevine	II	S	-	235	161	223	125	163	120	119	183
C212	Courteron	September 2005	Grapevine	II	S	-	231	171	219	125	157	120	119	183
C213	Courteron	September 2005	Grapevine	II	S	-	217	171	221	125	147	120	119	183
C214	Courteron	September 2005	Grapevine	II	S	-	217	171	219	125	159	120	119	167
C217	Courteron	September 2005	Grapevine	II	S	-	235	167	217	125	159	120	113	181
C218	Courteron	September 2005	Grapevine	II	S	-	219	169	217	116	147	120	113	183
C219	Courteron	September 2005	Grapevine	II	R	-	231	167	219	125	147	120	119	185
C221	Courteron	September 2005	Grapevine	II	S	-	231	181	219	125	159	120	119	181
C222	Courteron	September 2005	Grapevine	II	S	-	231	167	219	125	159	120	113	181
C223	Courteron	September 2005	Grapevine	II	S	-	225	167	213	125	153	130	117	167
C224	Courteron	September 2005	Grapevine	II	S	-	217	167	219	125	147	120	117	185
C228	Courteron	September 2005	Grapevine	II	S	-	215	167	215	125	161	120	119	181
C229	Courteron	September 2005	Grapevine	II	S	-	217	167	219	125	163	120	119	181
C230	Courteron	September 2005	Grapevine	II	S	-	231	167	223	125	159	120	121	181
C231	Courteron	September 2005	Grapevine	II	S	-	239	167	213	125	153	130	123	167
C232	Courteron	September 2005	Grapevine	II	S	-	231	167	219	125	159	120	119	185
C233	Courteron	September 2005	Grapevine	II	S	-	217	171	223	125	165	120	121	181
C234	Courteron	September 2005	Grapevine	II	S	-	231	181	219	125	159	120	119	181
C235	Courteron	September 2005	Grapevine	II	S	-	231	161	233	125	157	120	121	183
C236	Courteron	September 2005	Grapevine	II	S	-	217	155	219	125	145	120	117	181
C237	Courteron	September 2005	Grapevine	II	S	-	217	183	219	125	157	120	119	181
C243	Courteron	September 2005	Grapevine	II	S	-	217	167	219	125	147	120	119	185
C244	Courteron	September 2005	Grapevine	II	S	-	217	167	219	125	157	120	121	167
C246	Courteron	September 2005	Grapevine	II	S	-	217	171	219	125	157	120	117	181
C247	Courteron	September 2005	Grapevine	II	S	-	217	171	219	125	163	120	119	181
C248	Courteron	September 2005	Grapevine	II	S	-	217	181	219	125	165	120	119	183

C251	Courteron	September 2005	Grapevine	II	R	-	231	167	219	125	147	120	119	185
C252	Courteron	September 2005	Grapevine	II	S	-	231	181	219	125	159	120	117	181
C253	Courteron	September 2005	Grapevine	II	S	-	217	167	219	125	147	120	119	181
C255	Courteron	September 2005	Grapevine	II	S	-	231	161	217	125	163	120	113	181
C257	Courteron	September 2005	Grapevine	II	S	-	231	167	219	125	159	120	119	185
C258	Courteron	September 2005	Grapevine	II	S	-	217	167	221	125	258	120	121	167
C259	Courteron	September 2005	Grapevine	II	S	-	233	171	217	125	163	140	113	183
C270	Courteron	September 2005	Grapevine	II	R	-	231	167	219	125	147	120	119	185
C271	Courteron	September 2005	Grapevine	II	S	-	231	171	219	125	165	120	113	181
C272	Courteron	September 2005	Grapevine	II	S	-	231	181	219	125	159	120	119	181
C274	Courteron	September 2005	Grapevine	II	S	-	239	141	219	116	165	120	119	181
C299	Courteron	September 2005	Grapevine	II	S	-	231	167	219	125	147	120	119	189
C300	Courteron	September 2005	Grapevine	II	S	-	231	141	217	125	147	120	119	173
C216	Courteron	September 2005	Grapevine	I	S	+	221	161	199	0	147	86	113	159
C240	Courteron	September 2005	Grapevine	I	S	+	221	167	199	0	147	86	115	159
C249	Courteron	September 2005	Grapevine	I	S	+	221	181	199	0	165	86	119	159
C250	Courteron	September 2005	Grapevine	I	S	+	221	167	199	0	147	86	117	159
C254	Courteron	September 2005	Grapevine	I	S	+	225	167	199	0	147	86	119	159
D203	Courteron	June 2006	Grapevine	II	S	-	229	139	215	116	155	128	113	175
D210	Courteron	June 2006	Grapevine	II	S	-	233	167	219	125	151	124	125	185
D212	Courteron	June 2006	Grapevine	II	S	-	217	171	219	125	147	120	117	181
D215	Courteron	June 2006	Grapevine	II	S	+	217	167	219	125	147	120	119	181
D225	Courteron	June 2006	Grapevine	II	S	-	217	167	219	125	147	120	119	181
D226	Courteron	June 2006	Grapevine	II	S	+	211	153	221	125	151	124	117	167
D227	Courteron	June 2006	Grapevine	II	S	+	241	155	219	125	157	120	117	183
D228	Courteron	June 2006	Grapevine	II	S	-	229	167	219	128	147	120	119	183
D235	Courteron	June 2006	Grapevine	II	S	-	227	171	213	125	159	130	117	167
D236	Courteron	June 2006	Grapevine	II	S	-	217	167	219	125	165	120	119	185
D238	Courteron	June 2006	Grapevine	II	S	-	217	181	213	125	159	120	117	183
D241	Courteron	June 2006	Grapevine	II	S	-	217	167	221	125	163	120	117	181
D261	Courteron	June 2006	Grapevine	II	S	-	241	181	213	125	153	130	123	167
D265	Courteron	June 2006	Grapevine	II	S	+	231	181	219	125	159	120	119	185
D270	Courteron	June 2006	Grapevine	II	S	-	231	167	217	125	147	120	117	185
D279	Courteron	June 2006	Grapevine	II	R	-	217	181	219	125	151	120	119	185
D331	Courteron	June 2006	Grapevine	II	S	-	243	167	219	125	147	120	115	181
D230	Courteron	June 2006	Grapevine	I	S	-	241	157	199	0	157	86	129	161
D231	Courteron	June 2006	Grapevine	I	S	+	235	161	199	0	159	86	113	159
D233	Courteron	June 2006	Grapevine	I	S	+	221	167	199	0	147	86	115	159
D248	Courteron	June 2006	Grapevine	I	S	+	219	167	199	0	157	86	115	161
D256	Courteron	June 2006	Grapevine	I	S	+	227	157	199	0	151	86	115	159
D257	Courteron	June 2006	Grapevine	I	S	+	227	167	199	0	147	86	115	159
D296	Courteron	June 2006	Grapevine	I	S	+	227	181	199	0	151	86	115	159
D312	Courteron	June 2006	Grapevine	I	S	+	221	181	199	0	151	86	115	159
D320	Courteron	June 2006	Grapevine	I	S	+	221	167	199	0	147	86	115	159
C141	Vandières	September 2005	Brambles	II	S	-	239	171	213	125	153	130	123	167
C143	Vandières	September 2005	Brambles	II	S	-	239	181	221	125	157	120	121	185
C145	Vandières	September 2005	Brambles	II	S	-	241	171	213	125	165	130	119	181
C146	Vandières	September 2005	Brambles	II	S	-	217	167	219	125	163	120	121	183
C147	Vandières	September 2005	Brambles	II	S	-	243	169	219	125	151	120	113	173
C148	Vandières	September 2005	Brambles	II	S	-	217	167	219	125	163	120	119	181
C154	Vandières	September 2005	Brambles	II	S	-	231	171	219	125	163	120	117	167

C161	Vandières	September 2005	Brambles	II	S	-	235	171	221	125	163	120	117	183
C162	Vandières	September 2005	Brambles	II	S	-	217	167	219	125	147	120	117	181
C163	Vandières	September 2005	Brambles	II	S	-	217	181	219	125	165	120	119	181
C165	Vandières	September 2005	Brambles	II	S	-	231	181	217	116	161	124	117	173
C166	Vandières	September 2005	Brambles	II	S	-	239	167	213	125	153	130	123	167
C171	Vandières	September 2005	Brambles	II	S	-	217	167	221	125	163	120	119	183
C173	Vandières	September 2005	Brambles	II	S	-	231	157	217	116	161	124	119	173
C176	Vandières	September 2005	Brambles	II	S	-	217	167	221	125	163	120	121	181
C144	Vandières	September 2005	Brambles	I	S	+	219	167	199	0	165	86	115	159
C157	Vandières	September 2005	Brambles	I	S	+	221	171	199	0	157	86	115	159
D142	Vandières	June 2006	Brambles	II	S	-	217	167	219	125	157	120	121	167
D145	Vandières	June 2006	Brambles	II	R	-	231	167	219	125	147	120	121	185
D147	Vandières	June 2006	Brambles	II	S	-	229	167	217	125	159	120	117	185
D149	Vandières	June 2006	Brambles	II	S	-	217	167	219	125	163	120	117	181
D161	Vandières	June 2006	Brambles	II	S	-	247	157	219	125	155	120	121	183
D163	Vandières	June 2006	Brambles	II	S	+	233	157	219	125	151	124	117	185
D164	Vandières	June 2006	Brambles	II	S	-	217	167	219	125	159	120	119	189
D170	Vandières	June 2006	Brambles	II	S	-	229	167	213	116	155	128	127	173
D171	Vandières	June 2006	Brambles	II	S	+	229	139	213	116	155	128	123	173
D173	Vandières	June 2006	Brambles	II	S	+	217	167	219	125	147	120	117	185
D177	Vandières	June 2006	Brambles	II	S	-	217	181	219	125	163	120	119	189
D179	Vandières	June 2006	Brambles	II	S	-	217	181	219	125	165	120	121	181
D180	Vandières	June 2006	Brambles	II	S	-	229	147	213	116	155	128	113	173
D181	Vandières	June 2006	Brambles	II	R	-	217	171	219	125	165	120	119	183
D182	Vandières	June 2006	Brambles	II	S	+	215	215	219	125	157	124	127	173
D190	Vandières	June 2006	Brambles	II	R	-	231	167	219	125	147	120	121	185
D193	Vandières	June 2006	Brambles	II	S	-	229	139	219	116	155	128	113	173
D196	Vandières	June 2006	Brambles	II	S	-	217	181	219	125	147	120	119	183
D197	Vandières	June 2006	Brambles	II	S	-	217	167	221	125	163	120	121	183
D201R	Vandières	June 2006	Brambles	II	S	+	221	167	219	125	159	120	117	185
D204R	Vandières	June 2006	Brambles	II	S	-	217	155	219	125	163	120	117	181
D207R	Vandières	June 2006	Brambles	II	S	-	239	155	211	125	153	132	123	169
D209	Vandières	June 2006	Brambles	II	S	-	241	167	213	125	153	130	123	167
D165	Vandières	June 2006	Brambles	I	S	+	225	167	199	0	157	86	115	159
D176	Vandières	June 2006	Brambles	I	S	+	221	167	199	0	147	86	113	159
D184	Vandières	June 2006	Brambles	I	S	+	221	139	199	0	115	86	155	159
D195	Vandières	June 2006	Brambles	I	S	+	221	181	199	0	147	86	115	159
D200R	Vandières	June 2006	Brambles	I	S	+	227	167	199	0	147	86	113	159
C109	Vandières	September 2005	Grapevine	II	S	-	217	171	221	125	157	120	119	167
C110	Vandières	September 2005	Grapevine	II	S	-	217	167	219	125	157	120	119	167
C111	Vandières	September 2005	Grapevine	II	S	-	217	171	219	125	165	120	119	181
C112	Vandières	September 2005	Grapevine	II	S	-	217	171	219	125	165	120	121	181
C114	Vandières	September 2005	Grapevine	II	S	-	231	169	217	116	159	120	113	181
C116	Vandières	September 2005	Grapevine	II	S	-	217	171	219	125	165	120	119	181
C117	Vandières	September 2005	Grapevine	II	S	-	239	167	217	125	159	120	121	181
C118	Vandières	September 2005	Grapevine	II	S	-	217	167	219	125	165	120	119	181
C120	Vandières	September 2005	Grapevine	II	S	-	217	167	219	125	157	120	119	181
C121	Vandières	September 2005	Grapevine	II	S	-	217	181	219	116	159	120	117	181
C122	Vandières	September 2005	Grapevine	II	S	-	239	169	217	125	147	120	117	181
C123	Vandières	September 2005	Grapevine	II	S	-	217	169	219	125	163	120	119	167
D105	Vandières	June 2006	Grapevine	II	S	+	231	167	213	125	147	120	117	181

D107	Vandières	June 2006	Grapevine	II	S	-	251	171	213	125	153	130	121	167
D109	Vandières	June 2006	Grapevine	II	S	-	217	181	221	125	147	120	119	189
D127	Vandières	June 2006	Grapevine	II	S	-	231	171	217	125	159	120	113	183
D136	Vandières	June 2006	Grapevine	II	S	-	217	167	219	125	147	120	119	181
D140	Vandières	June 2006	Grapevine	II	S	-	217	167	219	125	157	120	119	167
D22	Vandières	June 2006	Grapevine	II	S	-	217	167	221	125	163	120	121	167
D49	Vandières	June 2006	Grapevine	II	S	-	217	171	219	125	163	120	119	181
D72	Vandières	June 2006	Grapevine	II	S	-	217	183	219	125	157	120	119	181
D81	Vandières	June 2006	Grapevine	II	S	-	217	183	219	125	147	120	119	183
D84	Vandières	June 2006	Grapevine	II	S	-	231	181	219	125	159	120	113	181
D92	Vandières	June 2006	Grapevine	II	S	-	217	181	219	116	165	120	121	181
D94	Vandières	June 2006	Grapevine	II	S	-	239	181	213	125	153	130	123	167
D98	Vandières	June 2006	Grapevine	II	S	-	217	167	219	125	163	120	119	181
D99	Vandières	June 2006	Grapevine	II	S	-	217	171	221	125	157	120	119	167
D114	Vandières	June 2006	Grapevine	I	S	+	221	181	199	0	147	86	115	159
D117	Vandières	June 2006	Grapevine	I	S	+	201	181	199	0	165	86	115	159
D124	Vandières	June 2006	Grapevine	I	S	+	227	167	199	0	147	86	113	159
D21	Vandières	June 2006	Grapevine	I	S	+	221	167	199	0	147	86	115	179
D37	Vandières	June 2006	Grapevine	I	S	+	225	167	199	0	163	86	113	159
D7	Vandières	June 2006	Grapevine	I	S	+	221	169	199	0	153	86	115	159

[a] *Botrytis* species: II is *Botrytis* group II and I is *Botrytis* group I.

[b] *cytb* genotype, in regard to the G143A change. Determined by digestion of the Qo13ext/Qo14ext PCR fragment with the restriction enzyme *Sat*I. Strains with a double-band pattern were considered R (resistant to strobilurins).

[c] Absence (-) or presence (+) of the 143-144 1205 bp intron, determined based on the length of the Qo13ext/Qo14ext PCR fragment

Table SA2: Primers used to sequence *cytb, sdhA, sdhB, sdhC* and *sdhD* in *Botrytis* group I and group II.

Primer	Sequence (5'-3')	Orientation
Cytochrome *b*		
Qo1	ACCGAATGGTGGGATCAATA	F
Qo2	TCCGTAGGTTTCCTGCTGAT	R
Qo3	TTTAGCAATGCACTACAACCCTA	F
Qo4	AAAAATCTTCGACAATAAAAGAACA	R
Qo5	ATTTAAAATAAGTTTGCATATTGACGA	F
Qo6	CATAAACCTTCAGTAATAAAGCCATTC	R
Qo7	GCAATAGCTAACACAGATGCTTT	F
Qo8	TCACGCATAATCAAACTCTGC	R
Qo9	GAGCGTCTAGGGATTAGACCA	F
Qo10	TTCCACTTAAACCTCTTGTGTCTTT	R
Qo11	GGGGATATTGTACAAGTTGTGAATTC	F
Qo12	ACCTTTTATATGGCCGACGA	R
Qo13	ACCCGACGGGGTTATAGAAT	F
Qo14	CCATCTCCATCCACCATACC	R
Qo15	GAGTGCTGTACCATGAATTGGA	F
Qo16	ACCTTCAGCCATTGTGAACC	R
Qo17	GGAAAGATCTGCCTTCTGTTTT	F
Qo18	TCGTTGAACGTTCTTGATTCC	R
Qo19	TCATTTGCAGCGAAGTTTGA	F
Qo20	AGCACTTAACATAGCAATAACACCT	R
Qo21	AATGCAAACACCACCAGCTA	F
Qo22	TCCGAGATACCAGTAGCGTTTA	R
Qo13ext	GGTATAACCCGACGGGGTTATAGAATAG	F
Qo14ext	AACCATCTCCATCCACCATACCTACAAA	R
Succinate dehydrogenase subunit A		
SdhA1	ATCGAGCCATCATCGCTATT	F
SdhA2	TCTCCCAACCAATCTGAACC	R
SdhA3	AAGAGCAGCATTCGGTCTTG	F
SdhA4	ACCCTGGGAGTTGAGGAGAT	R
SdhA5	TGGTTACGGACGTGCCTACT	F
SdhA6	TGCATGGCTAGACGAACATC	R
SdhA7	GCGATAACTTCGAACCAGGA	F
SdhA8	CTCCAATTCCCAACCAGCTA	R
Succinate dehydrogenase subunit B		
IpBcBeg	CCACTCCTCCATAATGGCTGCTCTCCGC	F
IpBcEnd2	CTCATCAAGCCCCCTCATTGATATC	R
Succinate dehydrogenase subunit C		
SdhC1	ATCACGTGCCAGATTTCCTT	F
SdhC2	TTACAAGCCCTCCAAATTGC	R
SdhC3	CCAAATAAAGGCGAAGCAAG	R
SdhC4	TGCCTCCCAAGACAGATACC	F
SdhC5	AGGTGGGATCTTGGGGTTAT	R
Succinate dehydrogenase subunit D		
SdhD1	TGATGACCTTGCACGTGACT	F
SdhD2	AGCAGCATGTGTTTGACAGC	R

Table SA3: *In vitro* effects of fungicides on germ-tube elongation in *B. cinerea* strains (EC_{50} in µM)

Strain number	QoIs		QiIs	Carboxamides			SDHI inh.	Decoupler	Multisite inh.
	Azoxystrobin	*Pyraclostrobin*	*Antimycin A*	*Boscalid*	*Flutolanil*	*Carboxin*	*TTFA*	*Fluazinam*	*Tolylfluanid*
1	0.10	0.015	0.006	0.029	24.8	1.3	63.1	0.011	0.058
2	0.15	0.010	0.004	0.029	27.8	1.5	76.6	0.009	0.086
3	0.12	0.010	0.004	0.044	31.0	1.1	90.0	0.009	0.058
4	0.25	0.018	0.019	0.15	31.0	2.1	76.6	0.032	0.43
5	0.37	0.013	0.019	0.087	27.8	1.7	76.6	0.032	0.23
6	0.50	0.015	0.017	0.073	23.2	2.1	67.6	0.022	0.26
7	0.25	0.013	0.009	0.087	27.8	2.6	76.6	0.022	0.35
8	0.30	0.013	0.009	0.12	18.6	1.7	45.0	0.022	0.29
9	>25	>10	0.009	0.087	31.0	2.1	72.1	0.022	0.43
10	>25	>10	0.009	0.087	37.2	2.6	49.5	0.032	0.72
11	0.50	0.13	0.028	0.087	26.3	2.1	45.0	0.043	0.29
12	0.50	0.077	0.056	0.12	37.2	2.6	54.1	0.065	0.43
13	>25	>10	0.028	0.15	37.2	2.6	90.0	0.054	0.23
14	>25	>10	0.028	0.10	31.0	2.6	90.0	0.075	0.35
15	0.74	0.052	0.022	0.73	31.0	3.8	54.1	0.022	0.72
16	0.62	0.077	0.019	0.73	27.8	3.0	90.0	0.032	0.58
17	0.74	0.052	0.019	0.44	37.2	3.4	72.1	0.022	0.43
18	0.50	0.052	0.015	0.73	27.9	3.8	54.1	0.032	0.35
19	2.0	0.15	0.093	1.75	27.9	5.1	76.6	0.086	0.86
20	3.0	0.23	0.093	1.17	34.0	6.0	76.6	0.075	0.72
21	2.5	0.21	0.112	0.87	34.0	4.7	76.6	0.086	0.72
22	2.7	0.26	0.131	1.46	37.2	5.1	67.6	0.108	0.58
23	0.50	0.021	0.013	5.0	3.7	15.7	76.6	0.022	0.23
24	0.50	0.015	0.015	5.8	5.3	16.2	90.0	0.022	0.29

25	>25	>10	0.013	5.8	4.6	17.0	67.6	0.022	0.58
26	>25	>10	0.011	5.0	4.3	17.8	72.1	0.032	0.58
27	>25	>10	0.019	4.4	6.2	14.9	67.6	0.032	0.72
28	>25	>10	0.017	3.5	4.6	13.6	72.1	0.022	0.35
29	>25	>10	0.015	2.9	5.6	14.9	58.6	0.043	0.43
30	>25	>10	0.013	2.9	4.6	12.8	76.6	0.022	0.58
31	>25	>10	0.015	2.9	4.6	19.1	54.1	0.043	0.58
32	>25	>10	0.015	2.6	4.6	10.6	76.6	0.022	0.29
33	>25	>10	0.015	4.4	24.8	15.7	76.6	0.043	0.58
34	>25	>10	0.013	2.9	21.7	17.0	67.6	0.043	0.35
35	>25	>10	0.015	2.9	31.0	12.8	58.6	0.022	0.29
36	>25	>10	0.015	2.9	15.5	14.9	81.1	0.022	0.26
37	>10	>2.5	0.019	6.4	21.7	10.6	76.6	0.032	0.43
38	>10	>2.5	0.015	4.4	23.3	12.8	67.6	0.043	0.43
39	>10	>2.5	ND	5.8	37.2	10.6	58.6	ND	ND
40	>10	>2.5	0.015	4.4	31.0	10.6	67.6	0.026	0.35
41	>10	>2.5	ND	4.4	23.3	17.2	90.0	ND	ND
42	>10	>2.5	0.015	5.8	21.7	17.2	58.6	0.022	0.58
43	>10	>2.5	ND	5.2	27.8	8.5	90.0	ND	ND
44	>10	>2.5	0.019	6.4	21.7	12.8	90.0	0.043	0.29
45	>10	>2.5	0.019	5.8	37.2	5.1	90.0	0.043	0.58
46	>10	>2.5	0.015	9.3	46.4	38.3	90.0	0.032	0.43
47	>25	>10	0.017	>36	52.6	>43	67.6	0.026	0.43
48	>25	>10	0.015	>36	40.2	>43	54.1	0.022	0.58
49	0.37	0.015	0.019	>36	43.3	8.5	72.1	0.032	0.23
50	>10	>2.5	0.017	>36	37.2	5.1	58.6	0.032	0.43

Conclusions

- La résistance aux QoIs est présente à forte fréquence dans toutes les populations de *B. cinerea*, alors que ce fongicide n'est pas homologué pour lutter contre la pourriture grise (mais contre d'autres maladies de la vigne). Cette résistance est déterminée par une mutation conduisant au changement G143A dans le cytochrome b (complexe respiratoire III).

- Chez *Botrytis cinerea*, la résistance aux QoIs est observée uniquement pour les souches ne comportant pas d'intron après le codon 143. Cette résistance a probablement une origine multiple. Chez *B. pseudocinerea,* cette résistance n'est jamais sélectionnée car la majorité des souches possèdent cet intron dans le cytochrome b. Ces deux espèces phytopathogènes sont le seules pour lesquelles deux versions du cytochrome b co-existent dans la nature.

- La résistance spécifique aux SDHIs est émergente dans les populations françaises de *B. cinerea* collectées sur vigne. Six phénotypes sont identifiés, se distinguant par leurs spectres de résistances croisées entre les différentes sous-familles de SDHIs et leurs niveaux de résistance. Les phénotypes corrèlent presque parfaitement avec des génotypes altérés pour la sous-unité B de la succinate deshydrogénase (cible des SDHIs), au niveau des acides aminés assurant l'interaction avec l'ubiquinone. Certaines souches portent également une mutation dans la sous-unité D (ligand du fer hémique) ou aucune mutation dans les quatre sous-unités de la SDH.

- La MDR concerne la plupart des inhibiteurs respiratoires. Au sein d'une classe de mode d'action, les molécules les plus lipophiles sont les plus affectées.

Perspectives

- La validation fonctionnelle des mutations responsables de la résistance aux SDHIs, identifiées au sein des sous-unités B et D, pourrait être apportée par la production de mutants inactivés.

- La valeur sélective des différents mutants pourrait être mesurée *in vitro* sur ces mutants inactivés, de fonds génétiques identiques.

- L'impact des mutations sur l'activité de la SDH pourrait également être mesuré sur ces mutants.

Ces différents travaux font l'objet de la thèse d'Anaïs Lalève, que j'ai co-encadrée ces trois dernières années (soutenance le 31 mai 2013). L'ensemble des travaux des deux thèses permettra de réfléchir et d'adapter les stratégies anti-résistance au SDHIs, en particulier dans le cadre de l'homologation prochaine du fluopyram (benzamides).

- Cette étude mériterait d'être complétée par la caractérisation phénotypique des mutants SdhB P225F, détectés récemment au champ.

- La recherche du mécanisme de résistance des souches ne portant pas de mutations au sein des sous-unités de la succinate deshydrogénase constitue un projet de recherche en soi.

- Des mesures de binding et d'absorption pourraient également être entreprises pour caractériser l'interaction entre les transporteurs impliqués dans l'efflux et les différentes molécules, pour comprendre pourquoi les composés les plus lipophiles sont principalement concernés par la MDR.

- Une étude portant sur l'évolution moléculaire du cytochrome b au sein du genre *Botrytis* et des espèces proches pourrait permettre de comprendre pourquoi *B. cinerea* et *B. pseudocinerea* sont les deux seules espèces de champignons phytopathogènes disposant d'au moins deux variants (et également d'un très grand polymorphisme intronique (Yin *et al.* 2012)) du cytochrome b et d'évaluer les conséquences pour l'efficacité des QoIs et la gestion de la résistance chez ces espèces.

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French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould)

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Abstract

Resistance to fungicides is an evolutionary process resulting from the selection of advantageous genotypes in naturally diverse populations. Seven fungicide modes of action are authorised to control grey mould caused by *Botrytis cinerea* on grapevine in France, and five of them have encountered specific resistance, with variable frequencies in populations and possible consequences for field fungicide efficacy. Moreover, multidrug resistance is caused by fungicide efflux and allows a weak resistance towards six unrelated modes of action. Here, a review is given of the fungicide resistance status of *B. cinerea* in France, particularly in the vineyards of Champagne, which are the most affected. Recently developed resistance and recent findings concerning the associated resistance mechanisms are focused upon in particular. Finally, antiresistance strategies are presented, and examples of managed resistance are discussed in a more general manner with the aim of extending this knowledge to other crops and countries undergoing similar resistance problems.

Keywords: *Botrytis cinerea*; grey mould; fungicide resistance; resistance mechanism; resistance monitoring; resistance management

1 INTRODUCTION

Selection, along with mutation, migration and genetic drift, is one of the four evolutive forces that drives variation in natural populations.[1] The selection of resistance in a fungal population subjected to selective pressure due to fungicides is then an evolutionary mechanism that promotes advantageous genotypes, i.e. genotypes adapted to their current environment, which then become observable in populations because of their higher frequencies (i.e. proportions).[1] Resistant genotypes are different from wild type in the sense that they bear changes (mutation, deletion or another kind of rearrangement) in one of several genes in the fungal genome. These changes modify the initial (wild-type) phenotype, such that the fungus becomes less susceptible (potentially even fully resistant) to the fungicide in question and then gains an 'advantage' in competing with other strains in field populations, this change being stable and transmitted to the progeny.[2,3] At the individual level, i.e. for a given strain of a given phenotype, this biological resistance is characterised by the following:[2]

1. The strength of the resistance, or the resistance level (syn. resistance factor RF), estimated as the ratio EC_{50} tested (resistant) strain/EC_{50} reference (susceptible) strain, the EC_{50} values being calculated experimentally as the fungicide dose that reduces the growth (measured from mycelium, germination or germ-tube elongation) of the strain by 50%.
2. The pattern of cross-resistance, or the simultaneous resistance of the strain to fungicides with similar or unrelated modes of action.

The frequency of resistant strains tends to increase over time in populations under selective pressure owing to the acquired benefits of resistance over the wild-type phenotype. The speed of this increase depends especially on the intensity of the selective pressure (i.e. the intrinsic risk associated with the active ingredients, the number of applications and the strategy of use) and the biological characteristics of the pathogen (particularly the number of generation cycles within an agricultural season, the occurrence of sexual reproduction and the relative fitness of the resistant individuals with respect to the wild type).[4] Below a given frequency, resistance may be difficult to detect (depending on the detection threshold of the diagnostic tool used) and may have little effect on fungicide efficacy, giving the impression that no resistance management is needed. Field resistance, also referred to as practical resistance, is observed in populations in which the frequency and resistance factor of resistant strains are high enough for significant resistance to be observed, resulting in poor fungicide control.[2] Thus, biological resistance does not necessarily result in field resistance, but careful monitoring of field resistance is essential from the first detection of biological

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resistance, to ensure that appropriate antiresistance strategies are implemented to prevent or delay a breakdown of fungicide efficacy.

Botrytis cinerea (tel. *Botryotinia fuckeliana*) and *Botrytis pseudocinerea* (tel. *Botryotinia pseudofuckeliana*) together cause grey mould on diverse crops, and on grapevine in particular. These two species were long confused but have recently been more clearly delimited.[5] *B. pseudocinerea* is the minority member of this complex of cryptic species (population frequency below 10%). It is found principally in Europe, on dead floral debris, and its frequency is highest in spring. Grapevine damage after veraison is caused mostly by *B. cinerea*, as rotten berries both decrease crop yield and lead to the development of muddy aromas in wines. Grey mould control and prevention is necessary to limit these qualitative and quantitative consequences. Prophylactic measures include reducing vine vigour (e.g. adapted rootstock and grape varieties, controlled fertilisation, mostly nitrogen, permanent grass cover between the rows), grape aeration (e.g. pruning, desuckering, lifting, leaf removal in the grape cluster zone or green harvest) and the limitation of berry wounding due to insects and especially berry moths, powdery mildew or mechanical tools (''Note Nationale Vigne 2013'' http://www.afpp.net/apps/accueil/autodefault.asp?d=5121). Biocontrol agents, mainly the filamentous fungi *Trichoderma*, *Gliocladium* and *Ulocladium*, the bacteria *Bacillus* and *Pseudomonas* and the yeasts *Pichia* and *Candida* are also available to prevent the disease.[6] In France, *Bacillus* and *Aurebasidium* preparations are registered in organic and conventional farming systems (Table 1). Lastly, chemical control is also a major measure in any global protection strategy. As symptoms differ between years (because of climate) and fields (because of the local environment), and as resistance and residue issues are of great concern, chemical protection is adapted according to the susceptibility of the plants in the plot concerned and the risk considered acceptable for the vine grower. In France, in 2013, ten active ingredients, with seven different modes of action, two microbial agents and one mineral preparation are authorised for grey mould control on vine (Table 1). Anti-*Botrytis* programmes may include between zero (southern vineyards) and a maximum of three sprays per season (northern vineyards, e.g. Champagne and Burgundy). This modulates the selective pressure exerted by the fungicide over the country. Moreover, application strategies vary among vineyards, but the fungicide groups mostly used are phenylpyrroles, hydroxyanilides and anilinopyrimidines (Fig. 1). As a result of these regional contrasts, higher frequencies of resistance are encountered in northern vineyards, even if specific resistance, recorded for five of the seven modes of action (Table 1), is present in most vineyards. Resistance monitoring is therefore essential for detecting the emergence of new resistances, observing the spatial and temporal dynamics of resistance and estimating resistance frequencies regionally. All this information is important for the appropriate local adaptation of resistance management.

Here, a review is given of the fungicide resistance status of *B. cinerea* in French vineyards, as this specific example is representative of resistance problems possibly occurring with this species in other countries and/or other crops. Recently developed resistance and recent findings concerning the associated resistance mechanisms are focused upon in particular. Finally, resistance management options are presented, and examples of managed resistance are discussed in a more general manner, with the aim of extending this knowledge to other crops and countries undergoing similar resistance problems.

2 MONITORING OF FUNGICIDE RESISTANCE IN *BOTRYTIS* POPULATIONS

Monitoring protocols, including sampling time, sampled material and analysis procedure, should be adapted to answer specific questions, generally aiming either to follow the evolution of resistance in time and space (at national, regional or local scales) in production plots or to measure selective pressure in relation to fungicide efficacy in trials. In the present work, the objective was to estimate the general evolution of resistance in each French vineyard, as selective pressure may vary greatly locally (see Section 1 and Fig. 1), and to detect the emergence of new phenotypes in real time. Therefore, long-term sample collections (starting back in 1982 for the oldest modes of action) were organised, which also made it possible to observe resistance frequencies after some molecules had become unauthorised, and then to estimate the possible fitness penalties of some phenotypes in populations. Most botryticides were tested from the date of their first registration or a few years before. It was decided to measure resistance after the seasonal selective pressure, i.e. after botryticides had been applied and when symptoms had peaked, in the autumn, just before vintage (from early September to late October, according to the vineyards). All samples were collected in growers' fields (not trials) chosen for their representativeness of real production practices from a given area in a given year. The monitoring presented in this paper was organised as follows.

A general survey was carried out yearly by the French authorities in most vineyards. For this national monitoring, between 36 and 279 plots were sampled between 2004 and 2011 in various French vineyards (including Aquitaine, Poitou-Charentes, Burgundy, the Loire Valley, Midi-Pyrénées and the Rhône Valley) by field technicians working for the Direction Générale de l'Alimentation (part of the French Ministry of Agriculture). A specific survey was also conducted in Champagne, which is considered to be at high risk of *Botrytis* epidemics, by the local Comité Interprofessionnel du Vin de Champagne (CIVC). Since the early 1980s, this survey has included a minimum of 200 plots representative of the diversity of the vineyards in terms of grape varieties, geography, climate and soil composition. Samples from both networks were analysed by the same protocol[7] at the laboratories of ANSES-RPP (national monitoring) and INRA-BIOGER (Champagne monitoring). Unlike other work that has analysed a limited number of pure strains collected in a minimum number of fields per area, the decision was made to work with bulked populations, which made it possible to collect many more samples from the field and then give a more accurate view of resistance evolution.

Conidia bulks representative of local populations were constituted by shaking contaminated berries or swabs (minimum 20 per plot, collected from different clusters and vines) obtained from field plots in sterile water. Antibiotics (streptomycin and penicillin, final concentration 50 mg L^{-1} each) and discriminating doses of fungicides, the concentrations of which were adapted to allow only the resistant strains to grow, were introduced into molten PG agar medium (10 g of glucose, 2 g of K_2HPO_4, 2 g of KH_2PO_4 and 12.5 g of agar in 1 L of deionised water) and dispensed into 5.5 cm petri dishes. For SDHI fungicides, the glucose was replaced with 4 g of the dibasic hexahydrate of sodium succinate. The fungicides were kindly provided by the manufacturers as technical products, diluted in ethanol, and were added to the medium such that the volume of ethanol never exceeded 0.25% of the total volume of the medium, including for the control. The final concentrations of fungicides

Table 1. Antifungal agents used to control grey mould caused by *Botrytis cinerea* in French vineyards

				Detection in France of strains with reduced susceptibility			
					Specific resistance[e]		
Mode of action[a]	Group name[a]	Chemical group[a]	Active ingredient	Multidrug resistance (MDR)[d]	Detection	Resistant phenotype name	Resistant genotype[f]
Multisite contact activity	Dithiocarbamates	Dithiocarbamates	Thiram[b]	–	–		
β-tubulin assembly	MBC fungicides (methyl benzimidazole carbamates)	Thiophanates	Thiophanate-methyl	+	+	BenR1	*Mbc1* E198A
		Benzimidazoles	Carbendazim[b]	+	+	BenR1	*Mbc1* E198A
		N-Phenylcarbamates	Diethofencarb[b]	+	+	BenR2	*Mbc1* F200Y
		Pyridine-carboxamides	Boscalid	+	+	CarR1-CarR7	*SdhB* H272Y/R/L, N230I, P225L/T/F
							SdhD H132R and others unknown
Respiration (complex II or succinate dehydrogenase)	SDHIs (succinate dehydrogenase inhibitors)	Pyridinyl-ethyl-benzamides	Fluopyram[c]	+	+	CarR3-CarR7	*SdhB* H272L, N230I, P225L/T/F
Respiration (uncoupler of oxidative phosphorylation)	Pyridinamines	2,6-Dinitro-anilines	Fluazinam	+	–		
Sterol biosynthesis (SBI)	Hydroxyanilides (SBI class III)[g]	Hydroxyanilides	Fenhexamid	+	+	HydR3	*Erg27* F412S/I/V and others
C4-demethylation							
MAP/histidine kinase in osmotic signal transduction	Dicarboximides	Dicarboximides	Iprodione, procymidone,[b] vinchlozolin[b]	+	+	ImiR1	*Daf1*
							I365N/R/S and others
	PP fungicides (phenylpyrroles)	Phenylpyrroles	Fludioxonil	+	–		
Methionine biosynthesis	AP fungicides (anilino-pyrimidines)	Anilino-pyrimidines	Pyrimethanil, mepanipyrim, cyprodinil	+	+	AniR1	Unknown
Microbial disrupters of pathogen cell membranes	Microbial (*Bacillus* sp.)	*Bacillus subtilis* and fungicidal lipopeptides produced	*Bacillus subtilis* (strain QTS 713)	–	–		
Competition for space and nutrients	Microbial	–	*Aureobasidium pullulans* (strains DSM 14940 + DSM 14941)	–	–		
Inorganic compound	–	Mineral salt	Potassium bicarbonate	–	–		

[a] After FRAC classification (www.frac.info).
[b] No longer authorised for use in France.
[c] To be registered in the near future.
[d] Three MDR phenotypes identified. For characterisation, see Leroux *et al.*[36–38]
[e] All phenotypes described be Leroux *et al.*[8,12] Strains highly resistant to strobilurins (or QoIs, inhibitors of respiration, complex III) were also discovered (phenotype StrR; see Leroux *et al.*[8]). However, the compounds of this group are registered and used for the control of diseases other than grey mould in vineyards and are therefore not included in this table. Nevertheless, mixtures of QoIs and SDHIs are registered to control grey mould in other countries.
[f] When specific resistance occurs, a mutant genotype is described with the name of the altered gene and the change(s) observed in it.
[g] The oxo-pyrazole fenpyrazamine belongs to this class of SBI and exhibits cross-resistance with fenhexamid; it is not yet registered in France.

were as follows: procymidone 10 mg L^{-1}, carbendazim 1 mg L^{-1}, diethofencarb 10 mg L^{-1}, carbendazim + diethofencarb 1 + 10 mg L^{-1}, fludioxonil 0.2 mg L^{-1}, fluazinam 0.3 mg L^{-1}, pyrimethanil 1 mg L^{-1}, prochloraz 0.25 mg L^{-1}, fenhexamid 0.4 and 4 mg L^{-1}, tolnaftate 10 mg L^{-1}, azoxystrobin 10 mg L^{-1} and boscalid 3.3 and 7 mg L^{-1}. Moreover, as *B. cinerea* has an active alternative oxidase,[8] SHAM (0.5 mM) was added to test for azoxystrobin. A quantity of 0.3 mL of bulk spore suspension was spread over each of a set of petri dishes, which were then incubated in the dark at 20 °C. After 1 day of incubation, the dishes were observed under a microscope. For each discriminatory concentration, susceptible spores did not germinate or exhibited short germ tubes, whereas resistant ones produced long germ tubes (>50% control). Taking into account the percentage of

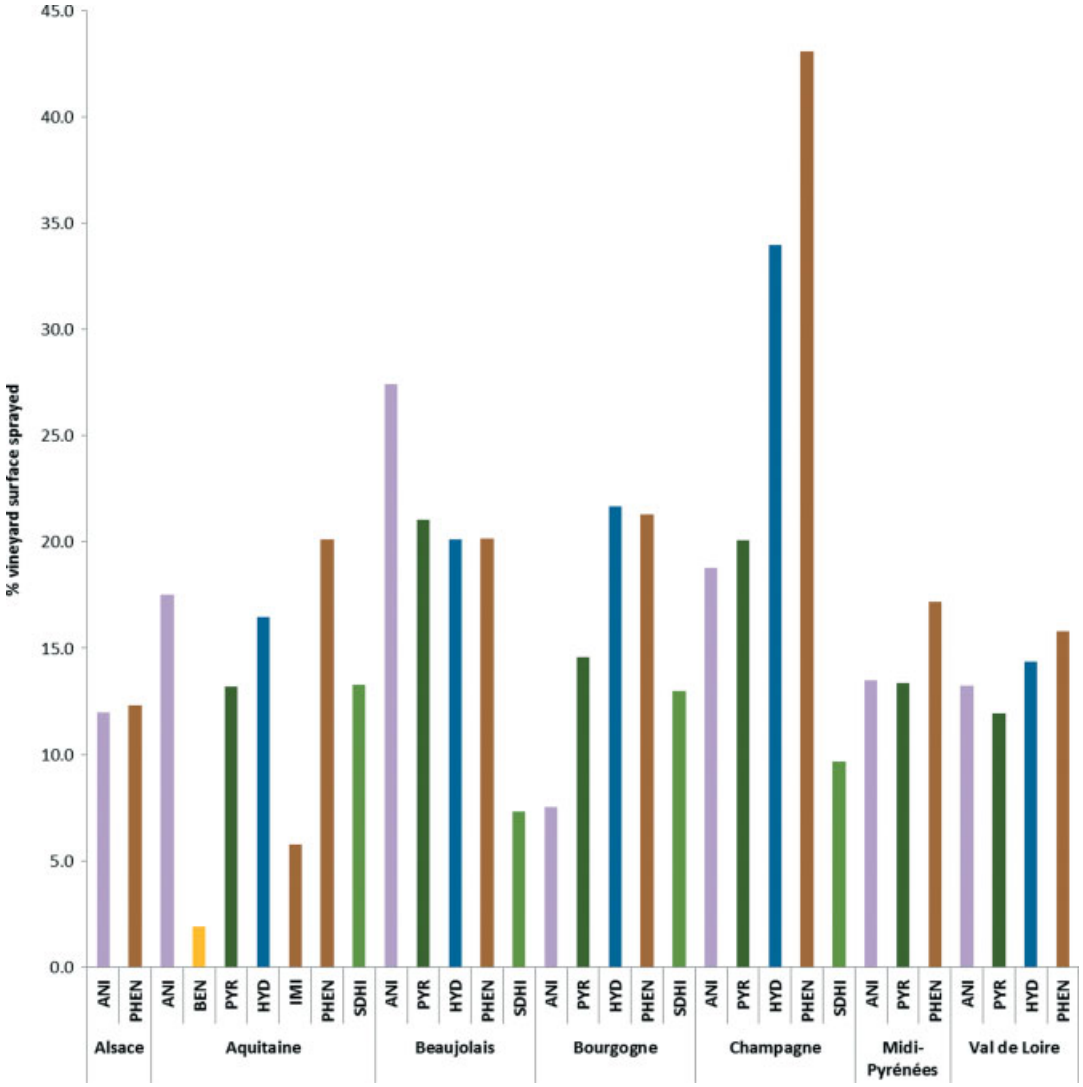

Figure 1. Proportion of vineyards sprayed with various botryticides in seven French vine-growing areas in 2010. Data from the French Ministry of Agriculture. Modes of actions were included in this survey if they were sprayed on at least 30 plots per vineyard. Between 300 to 600 plots per vineyard were used. ANI: anilinopyrimidines; PHEN: phenylpyrroles; BEN: benzimidazoles; PYR: pyridamines; HYD: hydroxyanilides; IMI: dicarboximides; SDHI: succinate dehydrogenase inhibitors.

germinated spores in the absence of fungicide, it was possible to estimate the proportion of each phenotype (see Figs 2 to 7 and the description in subsequent sections) within the tested populations. Mean resistance frequencies and the number of plots containing resistant strains are presented for France in Fig. 2 and for Champagne in Figs 3 to 7.

3 RESISTANCE TO FUNGICIDES AFFECTING OSMOREGULATION

Iprodione, vinclozoline and procymidone are dicarboximides, whereas fludioxonil, an analogue of the microbial toxin pyrrolnitrin, is a phenylpyrrole (Table 1). They interfere with the osmotic signal transduction pathway, resulting in an abnormal accumulation of glycerol.[9] The primary target site of dicarboximides and phenylpyrroles has yet to be established, but they are thought to interfere with a two-component histidine kinase, for which many mutations, causing especially I365R/N/S changes, have been described in the corresponding *Daf1* (syn. *BcOS1*) gene and associated with resistant phenotypes in field strains or laboratory mutants.[10] More specifically, structure modelling of the cytoplasmic linker domains (also named HAMP domains because of their presence in Histidine kinase Adenyl cyclases, Methyl accepting proteins and Phosphatases) recently revealed that the replacements of hydrophobic residues within these domains generally affected their helical structure, probably eliminating signal transduction.[11]

Dicarboximides were registered in the late 1970s and were used intensively (up to four sprays per season in Champagne) in many vineyards. Consequently, practical resistance with poor fungicide efficacies was rapidly encountered[7] owing to selection of the ImiR1 phenotype (Fig. 3 and Table 1) (see the description in Leroux *et al.*[12]) of specific resistance to dicarboximides (but not phenylpyrroles).[12] Brief interruption of dicarboximide use resulted in a large decrease in the frequency of ImiR1, suggesting that resistant strains incurred a high fitness penalty, even if their osmosensitivity was found to be normal in laboratory conditions.[12] Consequently, resistance to dicarboximides could be easily managed by limiting the number of applications and organising long rotations in

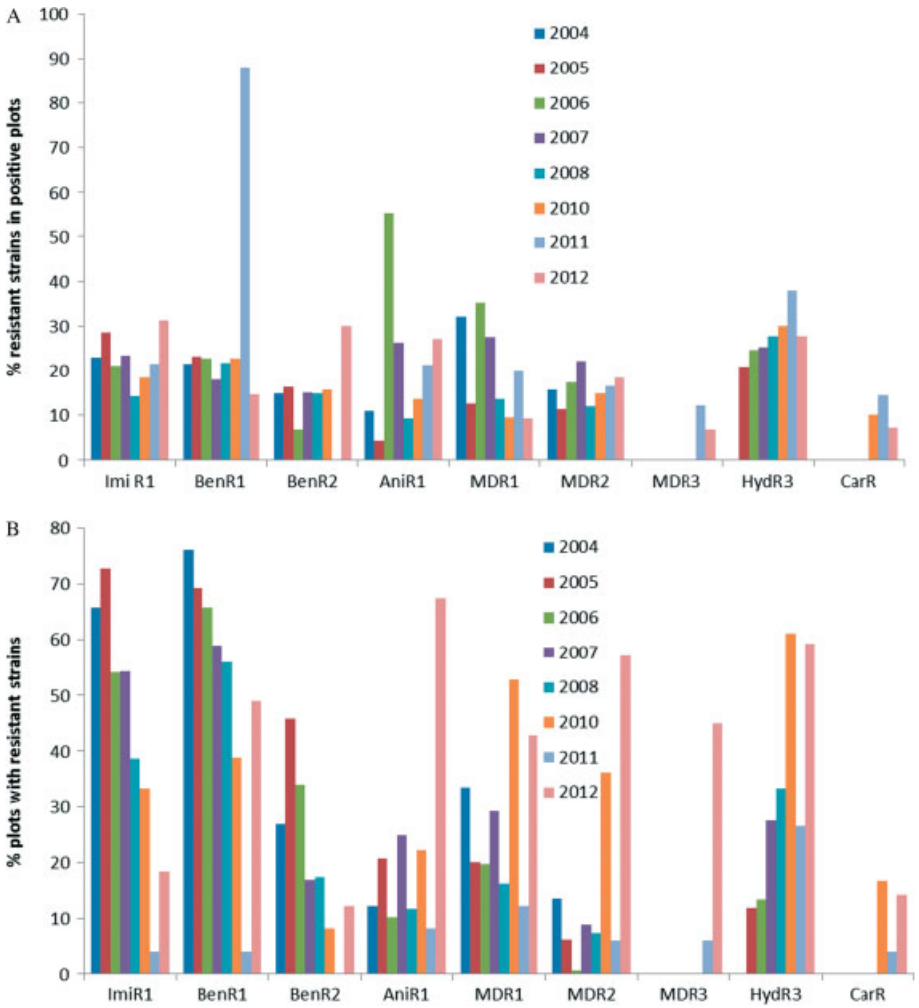

Figure 2. Evolution of resistance to fungicides in *Botrytis cinerea* in French vineyards: (A) mean frequency of a given resistant phenotype in all plots in which this resistance is found; (B) mean frequency of plots displaying a given resistant phenotype with respect to all tested populations. Phenotypes are abbreviated as follows: specific resistance to dicarboximides (ImiR1), antimicrotubules (BenR1 and BenR2), anilinopyrimidines (AniR1), hydroxyanilides (HydR3), carboxamides or SDHIs (CarR) and multidrug resistance (MDR1, MDR2 and MDR3). Description of these phenotypes is detailed in the text.

the use of this fungicide ("Note nationale Vigne 2013" http://www.afpp.net/apps/accueil/autodefault.asp?d=5121). Nevertheless, following the release onto the market of new fungicides with better intrinsic activity, the use of dicarboximides gradually decreased, together with the frequency of ImiR1 strains in all vineyards (Figs 2 and 3). Nowadays, only iprodione is still registered.

B. cinerea mutants highly resistant to dicarboximides and phenylpyrroles are easily induced under laboratory conditions but are susceptible to osmotic stress. This characteristic may account for the lack of development of strains resistant to phenylpyrroles in the field.[12] In spite of this low resistance risk, fludioxonil is recommended at only one spray per season and is also available as a solo product or as a mixture with cyprodinil.

4 RESISTANCE TO ANTIMICROTUBULE AGENTS

The benzimidazoles carbendazim and benomyl, as well as the related agent thiophanate-methyl, were the first systemic single-site fungicides to be introduced into agriculture in the 1970s. They inhibit germ-tube elongation and mycelial growth by binding to β-tubulin, preventing microtubule assembly.[13] In *B. cinerea*, as in many other fungi, resistance was selected soon after the introduction of fungicides with this mode of action.[14] This resistance was conferred by a mutation of the *Mbc1* gene, encoding β-tubulin, at codon 198 (E198A substitution).[14] The derived BenR1 phenotype displayed highly specific resistance to benzimidazoles, but remained susceptible to the *N*-phenylcarbamate diethofencarb (negative cross-resistance).[12] Carbendazim + diethofencarb mixture was widely used to control BenR1 strains, but this led to the rapid selection of BenR2 strains, which are highly resistant to both compounds[12] owing to the F200Y substitution in Mbc1, and to practical resistance (Fig. 4).

Since the 1990s, antimicrotubule fungicides have been replaced by fungicides with other modes of action, but the frequency of BenR1 in populations has remained high, particularly in Champagne (Figs 2 and 4): after more than 10 years during which the use of carbendazim + diethofencarb mixtures has been limited, the frequency of BenR1 strains remains at about 20%, indicating that the fitness penalty of these strains is small. By contrast, the frequency of BenR2 strains fell to low levels as soon as the use of the mixture ceased, suggesting high fitness penalties due to this second *Mbc1* mutation. Thus, the risk of resistance to benzimidazoles and thiophanate fungicides is lower when these

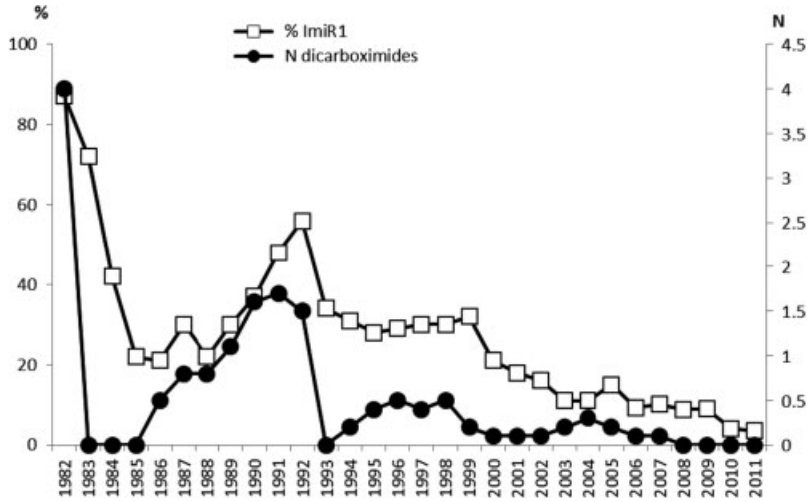

Figure 3. Evolution of *B. cinerea* resistance to dicarboximides (mean frequency of ImiR1 strains in the whole sample) in Champagne vineyards as a function of fungicidal selection pressure (*N* as mean number of sprays per season).

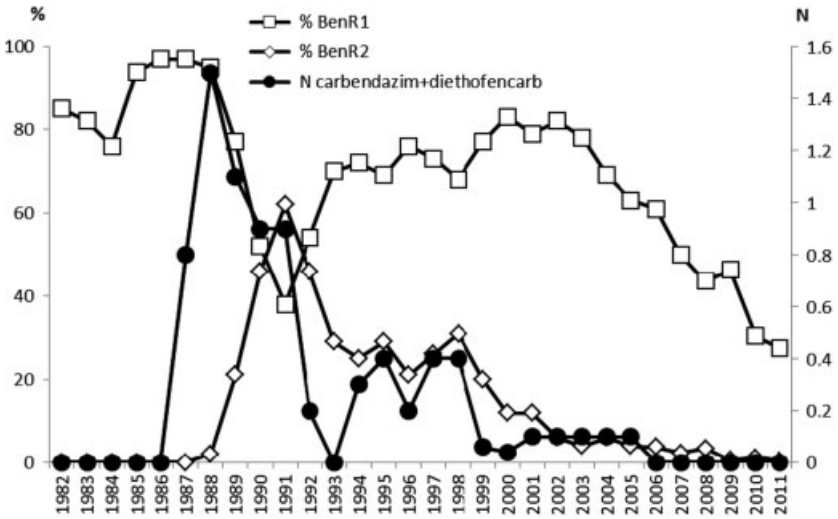

Figure 4. Evolution of *B. cinerea* resistance to antimicrotubule agents (mean frequency of BenR1 and BenR2 strains in the whole sample) in Champagne vineyards according to fungicidal selection pressure (mixture of carbendazim + diethofencarb; *N* as mean number of sprays per season).

compounds are used in mixtures with diethofencarb, favouring a phenotype of limited competitiveness. Thus, such mixtures could still be used occasionally if the diethofencarb partner were still authorised, in an appropriate rotation of fungicides with different modes of action.

5 RESISTANCE TO METHIONINE BIOSYNTHESIS INHIBITORS

The anilinopyrimidine fungicides pyrimethanil, mepanipyrim and cyprodinil were introduced into various European countries, for use as botryticides, in the mid-1990s. As several amino acids, including methionine in particular, had been shown to reverse anilinopyrimidine fungitoxicity when added to culture media,[15] it was suggested that the primary target site of these fungicides was an enzyme involved in methionine biosynthesis. Screening was carried out for the two candidates cystathionine β-lyase (encoded by *BcmetC*) and cystathionine γ-synthase (encoded by *BcmetB*), and their mutations in the emerging highly resistant AniR1 phenotypes (see the description in Leroux *et al.*[12]). Unfortunately, no mutation was found in *BcmetC*,[16] and the mutations detected in *BcmetB* were also found in susceptible strains.[17] Further studies are therefore required to identify the primary target of anilinopyrimidines and the associated resistance mechanism. It has been suggested that they could affect protein secretion pathways involving the Golgi complex.[12]

No field resistance has yet been recorded in commercial French vineyards sprayed with anilinopyrimidines (''Note Nationale Vigne 2013'' http://www.afpp.net/apps/accueil/autodefault.asp?d=5121), but control failure was observed in Swiss experimental plots intensively sprayed with anilinopyrimidines.[18] This is consistent with the low frequencies of AniR1 strains detected in France and in Champagne (Figs 2 and 5) and the poor stability of this phenotype observed in laboratory conditions (Leroux P., unpublished data), even if fitness penalty was not demonstrated *in vitro*.[19] Nevertheless, the proportion of plots harbouring these strains has increased considerably in recent years, and this resistance should therefore be carefully monitored. This mode of action is managed in French vineyards by limiting the number of treatments to one per season. A mixture of cyprodinil with

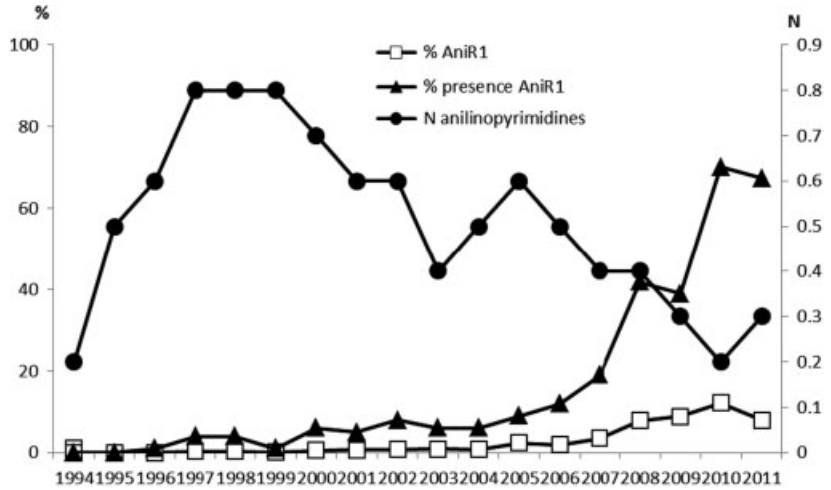

Figure 5. Evolution of *B. cinerea* resistance to anilinopyrimidines (mean frequency of AniR1 strains in the whole sample and frequency of plots displaying this resistance) in Champagne vineyards as a function of fungicidal selection pressure (*N* as mean number of sprays per season).

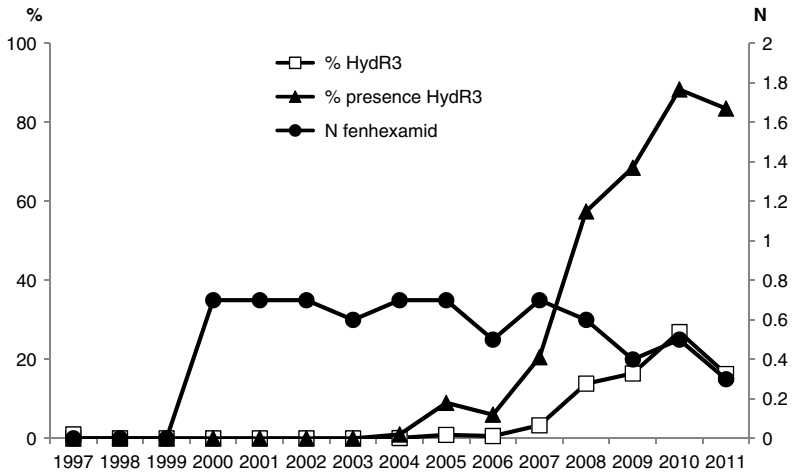

Figure 6. Evolution of *B. cinerea* resistance to hydroxyanilides (mean frequency of HydR3 strains in the whole sample and frequency of plots displaying this resistance) in Champagne vineyards as a function of fungicidal selection pressure (*N* as mean number of sprays per season).

fludioxonil is also available but is counted in strategies as bringing one treatment for each chemical family (anilinopyrimidines and phenylpyrroles respectively).

6 RESISTANCE TO STEROL BIOSYNTHESIS INHIBITORS (SBIS)

Fenhexamid is the only SBI fungicide registered for controlling *B. cinerea* in grapevine. It specifically inhibits the 3-ketoreductase enzyme encoded by *erg27*, which is involved in the C-4 demethylation of sterols.[20] Resistance to fenhexamid was first detected in Champagne, 4 years after its introduction. HydR3 resistant phenotypes were classified into two categories, according to the resistance factors at the germ-tube elongation or mycelial growth stages. HydR3$^-$ strains exhibit weak to medium RFs whereas HydR3$^+$ strains exhibit high RF values. Resistance was shown to be mediated by target alteration in both phenotypes,[21] with changes to *erg27* affecting only codon 412 in HydR3$^+$ strains (changes F412S/I/V) and codons 195, 309, 314, 336, 369 or 400 in HydR3$^-$ strains. Strains naturally resistant to fenhexamid were present in wild populations before the introduction of this fungicide and correspond to the minor species *B. pseudocinerea* (also referred to as phenotype HydR1[12]). This natural resistance can be little accounted for by changes to the target protein Erg27. The main resistance mechanism was demonstrated to be fenhexamid detoxification, characterised by synergy with DMI fungicides and determined by an alteration to the *cyp684* promoter and/or mutations in this gene, the biological function of which is still unknown. This constitutes one of the first examples of this resistance mechanism in phytopathogenic fungi.[22]

Since its introduction in vineyards in 2000, fenhexamid has been limited to one treatment per season at label dose. *B. pseudocinerea* strains are present at low frequencies in the population and do not seem to be subject to fenhexamid selection, as its frequency in populations follows previously described variation (unpublished data). The mean frequency of HydR3 phenotypes may reach 30%, but the abundance of this phenotype in Champagne now exceeds 60% of the plots (Figs 2 and 6). No significant failure of fenhexamid control has yet been reported, but this remains a matter of concern if no limits are placed on the use of this fungicide. A reduced fitness cost was recently demonstrated for HydR3 strains, possibly putting such strains at a disadvantage in winter conditions[23] and accounting for their moderate frequency in populations.

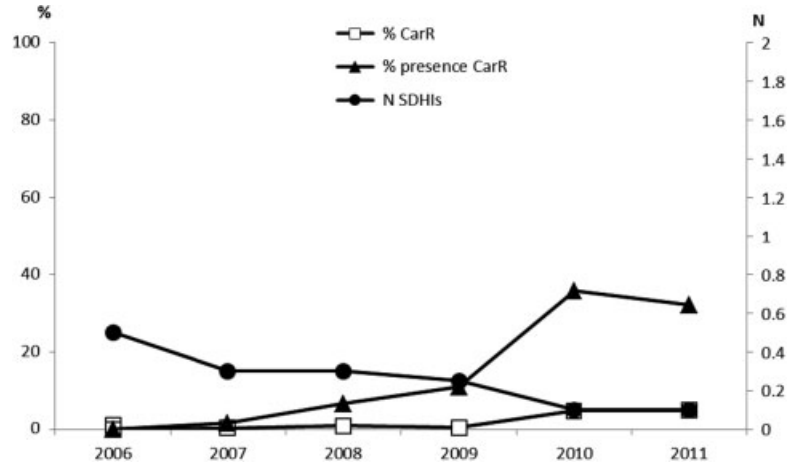

Figure 7. Evolution of *B. cinerea* resistance to SDHIs (mean frequency of CarR strains in the whole sample and frequency of plots displaying this resistance) in Champagne vineyards as a function of fungicidal selection pressure (*N* as mean number of sprays per season).

7 RESISTANCE TO RESPIRATORY INHIBITORS

7.1 Multisite inhibitors

Respiratory inhibitors are among the most widely used fungicides worldwide. They include the multisite toxicants that were among the first active ingredients available for the control of plant diseases. Thiram (a dithiocarbamate) was until recently the only molecule of this type registered as a botryticide on grapevine in France (still registered in other countries). However, its use was limited in practice because it was not very effective against *Botrytis* and penetrated poorly into the plant (contact inhibitor), resulting in plant protection being compromised by rain leaching. Its application on grapevine was also problematic because it slowed down must fermentation. Its effects were mostly preventive, through the inhibition of spore germination. Within the fungal cell, thiram, as with other multisite toxicants, targets several thiol-containing enzymes involved in spore respiration. No case of resistance to thiram has been reported in *B. cinerea*, whereas problems with dichlofluanid (a chloromethylmercaptan derivative no longer authorised for use in France) have been reported in other countries.[24] Thiram use was limited to one treatment per season, only at the flowering stage, to prevent fermentation problems.

7.2 Pyridinamines

Fluazinam (a pyridinamine) has a high level of intrinsic activity targeting the spores and mycelia of *B. cinerea*. It uncouples mitochondrial oxidative phosphorylation, the process leading to the biosynthesis of ATP, which supplies fungal cells with energy.[25] No strain with specific resistance to fluazinam has been detected in French populations since the introduction of this molecule in vineyards in 1999. Nevertheless, such resistance has been observed on Japanese bean crops,[26] suggesting that precautionary measures should be taken, including the imposition of limitations on the use of this single-site fungicide to one spray per season.

7.3 Quinone outside inhibitors (QoIs)

Strobilurins are powerful respiration inhibitors targeting the Qo active site of cytochrome *b*, a component of complex III of the mitochondrial respiration chain. They are used to control pathogens of various crops, but have not been authorised for use against grey mould in vineyards owing to inconsistent results. The heterogeneity of the results obtained may result from the constitutive expression of the terminal alternative oxidase in *B. cinerea*, allowing electrons to bypass the blockage of the cytochrome pathway caused by strobilurins.[27] Nevertheless, strains of *B. cinerea* highly resistant to QoIs (StrR phenotype) have been found in French vineyards, in spite of the absence of specific selective pressure.[8] This resistance, caused by a G143A substitution in the cytochrome *b* protein, is the main mechanism of resistance found in phytopathogenic fungi worldwide[28] and is thought to have resulted from unintentional selection following the widespread use of these molecules to treat powdery or downy mildew in many vineyards. In some countries and other crops, QoIs are also registered in mixture with SDHIs (see below). StrR strains were first collected in 2008 (mean frequency of 33% and presence in 88% of the plots), rapidly colonised the Champagne population and are now ubiquitous. The spread of this resistance is all the more surprising because some *B. cinerea* strains harbour an intron just downstream of codon 143 in the *cytb* gene encoding cytochrome *b*.[8] This intron has been shown to prevent the selection of the G143A mutation in this gene[29] and may therefore have delayed the selection of QoI resistance. In *B. pseudocinerea* populations, strains that mostly contain this intron, the QoI resistance was not detected.[8] No specific management policy for StrR strains is currently implemented in vineyards, because strobilurins are not used to target grey mould in practical conditions.

7.4 Succinate dehydrogenase inhibitors (SDHIs)

SDHIs (or carboxamides) were initially developed in the 1960s against Basidiomycetes (molecules from the oxathiin carboxamide subgroup, such as carboxin and oxycarboxin). In the last decade, several novel SDHIs exhibiting a wide spectrum of activity have been discovered. Among them, boscalid was the first pyridine-carboxamide to be introduced in 2005 and is effective against ascomycetes, including grey mould and powdery mildew agents. Moreover, the benzamide fluopyram is scheduled to be released onto the market in the near future. SDHIs target complex II (or succinate dehydrogenase SDH) in the mitochondrial respiratory chain. They prevent ubiquinone reduction by interfering with three subunits of SDH (i.e. SDHB, SDHC and SDHD).

In France, the first resistant strains (CarR strains) were identified 2 years after boscalid introduction. As in other fungi,[30] *B. cinerea* strains resistant to carboxamides exhibit a mutation in the gene encoding the SDHB (leading to the changes H272Y/R/L,

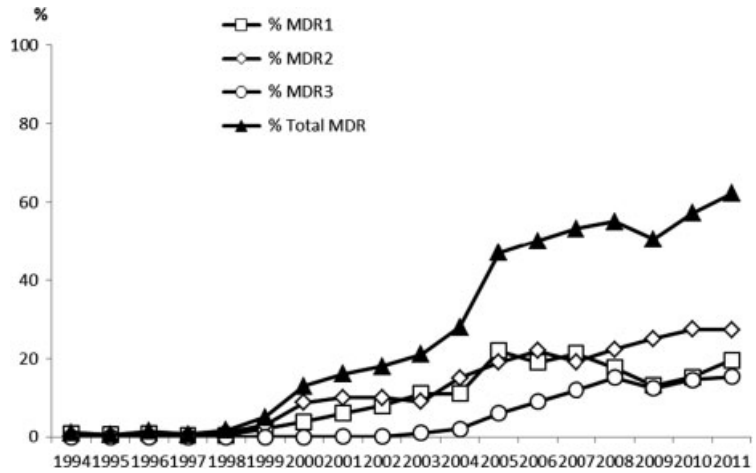

Figure 8. Evolution of *B. cinerea* MDR resistance (mean frequency of MDR strains in the whole sample) in Champagne vineyards.

N230I or P225L/F/T) or SDHD (change H132R).[8] Some strains moderately resistant to boscalid but not harbouring mutations in any of the SDH proteins have also been observed. The SDHB H272R/Y genotypes were the most frequently observed. They were moderately resistant to boscalid, but were susceptible or hypersusceptible to fluopyram respectively. The other CarR strains exhibited cross-resistance between these two SDHIs, with the highest resistance levels observed for the SDHB H272L and P225F/L genotypes. Since 2006, the frequency of CarR strains has remained below 10% in many vineyards (Fig. 2), but the proportion of plots containing such strains is steadily increasing (32% of the plots concerned in 2011 in Champagne) (Fig. 7). Monitoring in German and Canadian vineyards, in American apple orchards and in French, Greek and German strawberry fields has revealed higher frequencies of resistance, with the generalisation of resistant strains in some cases (unpublished data).[31–34] In conclusion, SDHI resistance may evolve very quickly, and strict resistance management and monitoring are therefore required. Boscalid is limited to one treatment per season in French vineyards and is used on its own, keeping CarR frequencies at a reasonable level (Fig. 7). The forthcoming introduction of fluopyram may soon increase the selection pressure due to SDHIs. It may also trigger a change in the structure of the resistant *B. cinerea* populations (dominated by the SDHB H272R/Y mutants), with a movement towards strains displaying positive cross-resistance to boscalid and fluopyram.

8 MULTIDRUG RESISTANCE

Strains exhibiting patterns of multiple resistance are often found in vineyards subjected to intense selective pressure and result from the accumulation, in a single cell, of different mutations conferring different distinct resistances. However, some strains in which simultaneous resistance towards independent modes of action was monogenic[35] arose in Champagne vineyards in the late 1990s. These strains were described as 'multidrug resistant' (MDR) and displayed at least three patterns of cross-resistance to fungicides: MDR1 (or AniR2), MDR2 (or AniR3) and MDR3 (or AniR2-R3).[36] Resistance factors were low to moderate, and resistance concerned almost all botryticides and several SBIs, to various degrees (Table 1).[37] In addition, all MDR strains were highly resistant to the sterol squalene epoxidase tolnaftate, used in medicine but clearly discriminating these strains in the present monitoring. An increase in fungicide efflux by overexpressed transporters was demonstrated in these strains in experiments with radiolabelled fungicide[38] and in synergy experiments with transporter modulators.[37] In MDR1 strains, the ATP-binding cassette (ABC) transporter encoded by *BcatrB* is upregulated owing to several different mutations in the gene encoding its transcription factor.[38] In MDR2 strains, the major facilitator superfamily (MFS) transporter gene *BcmfsM2* was found to be overexpressed, and two possible insertions in its promoter sequence were found to have occurred once and to have spread from French to German populations.[39] Finally, MDR3 strains have been described as natural hybrids, resulting from sexual recombination between MDR1 and MDR2 strains.

MDR strains have continued to increase in frequency and to spread in French populations (Figs 2 and 8), reaching a mean frequency of more than 60% in Champagne vineyards. These MDR phenotypes may have been selected by the repeated use of the same three-spray programmes (including at least fenhexamid and fludioxonil) in the 2000s. MDR rendered *B. cinerea* strains simultaneously resistant to sprayed fungicides of almost all modes of action. As resistance factors are low to moderate, no loss in field performance by this mechanism has ever been reported for these molecules.[40] Fungicide limitation was strongly recommended for the management of this resistance, and two-spray programme strategies have spread throughout Champagne in the last few years. The use of full doses of the individual products, as well as rotations of the molecules between seasons, is also recommended, to ensure that each type of fungicide remains effective. Transporter modulators could be good companions in a mixture with single-site fungicide, if they ever become cost effective and their registration possible. Actually, some synthetic modulators are used in human health, and some natural compounds (curcumin, flavonoids, etc.) are described to have these properties.[37]

9 RESISTANCE MANAGEMENT IN GREY MOULD POPULATIONS

Resistance to fungicides is an important issue for scientists, farmers and fungicide manufacturers, because of its possible consequences for farmer and industry income, the environment and human health. Fungicides with original modes of action, meeting safety requirements, are rarely discovered, in spite of intensive screening by companies, and it is therefore necessary to

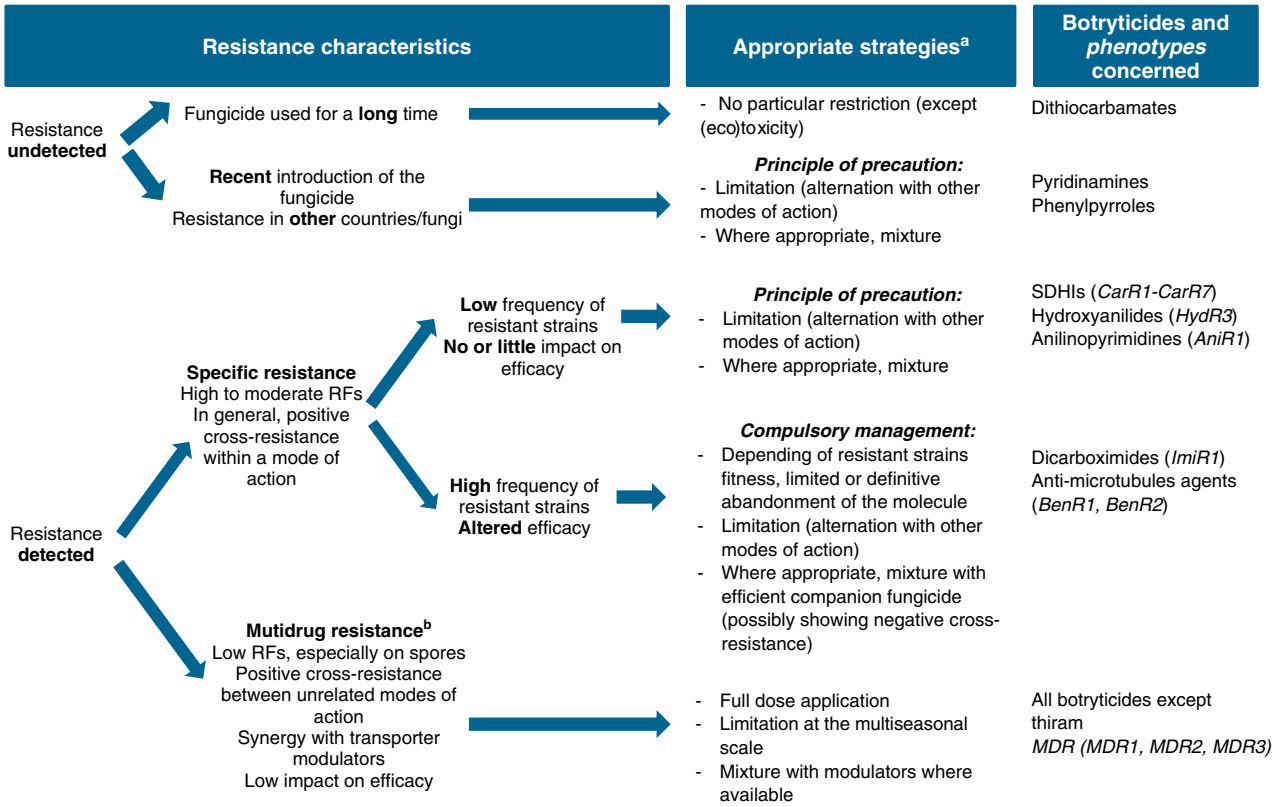

Figure 9. Argumented decision tree for strategies for managing fungicide resistance in *Botrytis cinerea* populations. Scheme valid for synthetic and natural antifungal agents. In all cases, resistance monitoring is a prerequisite for the identification of the right strategy, and its adaptation, if resistance continues to evolve. In all situations, prophylactic measures help to delay resistance. [a] Mixtures can be proposed to manage resistance only when active ingredients not displaying positive cross-resistance and/or not already encountering resistance are available and can be used at an appropriate dose. [b] MDR on its own generally induces low to moderate RFs. Nevertheless, when this mechanism is associated with other resistance mechanisms (for example, specific resistance due to target alteration), RFs may be greater for a mode of action. RF: resistance factor.

protect the available modes of action in effective antiresistance strategies.

Antiresistance strategies are based on the skilful deployment of tools (prophylaxis, plant resistance genes and antifungal compounds) to delay resistance. Fungicides may be alternated over time (at the seasonal or multiseasonal scale) or over space (at the farm or vineyard scale). They can also be used at the recommended dose or at a lower dose (for example, in mixtures). The ranking of these strategies (alternation, mixture and dose) on the basis of their efficacy to delay resistance may seem to be a conundrum. Pesticide and drug resistance evolution has been modelled by many authors, some of whom have compared the outputs of the various strategies, which have recently been reviewed.[41,42] Surprisingly, there is little consensus on how to combine selection pressure, the evaluation of a resistance management strategy requiring an accurate case-by-case definition of the expected strategy outcomes. Nevertheless, a mixture of molecules has proved to delay resistance better in some, but not all, situations, because it ensures multiple intragenerational killing. Nevertheless, this strategy may not be adapted to all situations (legislation on mixtures and residues), with mixtures of molecules possibly evolving resistance. In the particular case of fungicides, unisite molecules are often combined with multisite inhibitors to preserve efficacy, and therefore such mixtures were not in the range of the hypothesis tested by these authors.[41] Thus, their conclusion should be carefully interpreted, even if one recent model on a cereal pathogen demonstrated the benefit of multisite inhibitors in mixtures.[43] Recent work also focuses particularly on the impact of full or reduced fungicide doses on selection for resistance.[44,45] The authors conclude that, in experimental studies, selection for fungicide resistance is increased as long as the fungicide dose increases, with one exception. These results are confirmed by the modelling studies reviewed. The authors suggest that these results could be different for quantitative resistance, i.e. resistance with a polygenic mechanism, on account of which the pathogen develops partial resistance.[45] Finally, managing resistance can sometimes interfere with fungicide efficacy management, some strategies being highly efficient in controlling the disease but at high resistance risk. Therefore, resistance management has to be included in a long-term vision.

Various strategies have been used to deal with *Botrytis* (see above), based on information provided by research on fungus biology and resistance mechanisms. Population monitoring is also a key component in the establishment of control strategies, as it provides important information about the evolution of resistance in the field. In this work, a protocol has been provided to quantify resistances in bulk spore suspensions, but it should be adapted if crops other than grapevine are under investigation for resistance development. More particularly, samples should be collected after the selective pressure that needs to be evaluated, and at a crop stage that authorises the easy collection of symptoms and a good representativeness of the local population. The choice of the collected material will also depend upon the part of the crop (fruit, leaf, stem, flower, etc.) that is mostly affected by *Botrytis*

sp. Monitoring should also be undertaken over a long period of time, to minimise interpretation bias of evolution as a result of interannual natural variation and methodological errors in resistance frequency measurement.

The key elements of the actual management of fungicide resistance in *Botrytis* are summarised in Fig. 9, as a decision tree based on the observation of mechanism, frequency and phenotype of field resistant mutants. Derived from the authors' experience in French vineyards, this decision tree aims to reach a broader general audience and should find application in most situations where *Botrytis* resistance is a problem. Figure 9 presents strategies adapted to resistance situations that may occur in the field and links them with the appropriate resistance phenotypes described in this paper. As fungicides often have lower intrinsic activities against *Botrytis* than against other fungi, the mixture strategy may not be the most appropriate or should be restricted to the most powerful inhibitors. Limiting the use of each botryticide at the multi-seasonal scale and alternation of active ingredients with different modes of action seems to be a suitable suitable approach in many situations, particularly in cases of emerging resistance (e.g. resistance to SDHIs) or multidrug resistance. Given the demonstrated fitness cost in resistant strains, this previous strategy has been shown to decrease the frequency (e.g. ImiR1, BenR2) or to delay the emergence (e.g. HydR3, AniR1) of resistant strains. Finally, in France, as a result of the implemented strategies (Fig. 9), no dramatic control failure linked to resistance is nowadays recorded in vineyards, making possible a reasonable use of all modes of action listed in Table 1 (*Botrytis* 'Note Nationale', http://www.afpp.net/apps/accueil/autodefault.asp?d=5121).

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Conclusions

- L'usage des anti-*Botrytis* est variable selon les vignobles et les pressions épidémiques.
- La résistance aux fongicides anti-*Botrytis* est détectée au vignoble pour tous les modes d'actions, à l'exception des multisites, des pyridinamines et des phénylpyrroles.
- Le mécanisme moléculaire et le statut de la résistance à ces différentes familles sont détaillés en particulier pour la Champagne, depuis près de 20 ans. Les résistances MDR, aux benzimidazoles et aux QoIs sont généralisées. Les résistances aux anilinopyrimidines, hydroxyanilides et aux dicarboximides sont faiblement ou modérément implantées, du fait probablement de fitness altérées. La résistance aux SDHI est émergente et à surveiller. L'évolution des résistances au cours du temps, moyennée sur le vignoble, permet d'estimer la valeur sélective des différents phénotypes et de surveiller les pressions sélectives exercées par les différentes familles de fongicides.
- Ces informations sont intégrées pour raisonner un arbre de choix des stratégies anti-résistance permettant de choisir la stratégie la plus adaptée, au cas par cas.

Perspectives

- Ces réseaux de surveillance ont vocation à se poursuivre dans le temps, sur un échantillonnage représentatif et une méthodologie adaptée, pour construire une vision à long-terme de l'évolution des résistances et sécuriser les recommandations d'emploi.
- Un intérêt particulier doit être apporté aux MDR (plus de 60% des souches en moyenne), et à la résistance spécifique aux SDHIs, famille à risque fort de résistance, dont la pression de sélection risque d'être modifiée par l'introduction prochaine du fluopyram (contre-sélection des mutants SdhB H272Y/R au profit de mutants très résistants aux pyridines et aux benzamides ?).

CONTEMPORARY FUNGICIDE APPLICATIONS SIGN FOR SELECTION IN *BOTRYTIS CINEREA* POPULATIONS COLLECTED IN THE CHAMPAGNE VINEYARD (FRANCE)

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ABSTRACT

Populations of fungal pathogens may be subject to many selective pressures in agricultural environments. Among them, fungicides constitute one of the most powerful determinants of population adaptation acting in a short time span. Here, we investigated whether fungicides sprays applied yearly in the Champagne vineyard to control the grey mold causal agent *Botrytis cinerea* could shape population structure and evolution. We carried out a 2-year survey (4 collection dates) on three treated/untreated pairs of plots. We found that fungicides treatments had no or little on population subdivision at neutral loci, as well as on diversity or reproduction mode. Nevertheless, we found evidence of stronger genetic drift in some treated plots, consistent with the regular application of fungicides. Moreover, we observed spatial structure in resistance frequency for two loci under contemporary selective pressure, as reflected by cline patterns. At last, using a modeling approach, we estimated fitness costs of resistance to fungicides, responsible for resistance frequency decay during winter. Further work is needed to estimate parameters of positive selection and migration exerted on *B. cinerea* populations, and to disentangle the relative effect of the evolutionary forces at work.

Keywords: *Botrytis cinerea*, population structure, selection, migration, fitness cost, fungicides, diversity, cline, vineyard

INTRODUCTION

Despite the environmental (cultural practices) and genetic (host variety) uniformity of modern agricultural ecosystems, pesticides represent a major ecological constraint for pests (Stukenbrock and McDonald, 2008). Fungicide applications may reduce drastically both pathogen population sizes and migration between populations. As a drawback, most pest infecting crops got adapted and developed resistance (for example, roughly, 300 cases of resistance to 30 modes of action, in 250 species of phytopathogenic fungi; Fungicide Resistance Action Committee (FRAC) database; http//www.frac.info), possibly leading to significant yield or quality losses. Moreover, the evolution of resistance to pesticides is a major issue for agronomy and food production, because the number of molecules with distinct modes of action has proved to be limited. Generalization of resistance in pest populations often leads to greater pesticide consumption, which is costly for farmers and detrimental for the environment and human health.

Pesticide resistance is so successful because it promotes advantageous genotypes, *i.e.* genotypes adapted to their current environment, which become observable in populations because of their increase in frequency, as long as the selective pressure occurs (Barton et al., 2007). In comparison to wild-type, resistant genotypes gained changes (point mutation, deletion or another kind of rearrangement) in one or several genes of the genome that determine their decrease in susceptibility to the pesticide in question, these changes being stable and transmitted to the progeny (Anonymous, 1988; Milgroom et al., 1989; WHO, 1957). In the absence of such selective pressure (*i.e.* in untreated areas), pleiotropic effects potentially associated with the resistant allele may induce a fitness cost and penalize resistance evolution (Fisher, 1930). Therefore, evolution of pesticide resistance constitutes a textbook example of adaptation to environmental changes operating at a very short spatio-temporal scale, since it is governed by several evolutionary processes (Lenormand et al., 1999).

Several evolutionary forces may operate in heterogeneous landscapes where treated and untreated areas are embedded. In treated areas, positive selection exerted by pesticides may induce resistance increase. Genetic drift may also have a greater magnitude in populations of small size, which may lead to the fixation of rare alleles. In untreated areas, or in previously treated areas, counter-selection (*i.e.* negative selection or fitness cost) is supposed to decrease resistance frequency. Additionally, migration can possibly homogenize allele frequencies between treated and untreated populations. At last, the reproductive mode is a supplementary constraint in such systems: whereas asexual reproduction leads to linkage between alleles at neutral and selected loci, sexual reproduction breaks any association between the two types of loci. Therefore, asexual reproduction

may enhance the homogenizing effect of migration and negative selection and the adaptation after positive selection, whereas sexual reproduction counterbalances these effects. The relative importance of these forces determines the direction of population evolution, and depends on pest biology on one hand, and on the nature and the intensity of the environmental and anthropic constraints, on the other hand. Therefore, disentangling the respective magnitudes of the evolutionary forces at work in a given system, on which spatial heterogeneity of the selection is applied, is of major importance. Manipulating adaptation by controlling gene flow, negative or positive selection may have simple and direct applications, either to disrupt local adaptation of pests to pesticides (REX_Consortium, 2013) or to maintain local adaptation of agronomical or endangered species (Storfer, 1999).

Estimating the effects of the various forces at work first requires a description of the spatial distribution of the observed allele frequencies and their temporal evolution. The second step requires the development of a mathematical model that integrates the interaction between forces (*e.g.* gene flow and selection) in order to calculate allelic frequencies under different scenarios. A statistical approach (e.g., using maximum likelihood) is used to estimate parameters values from the theoretical model that best fits the described allelic frequencies. When clines, i.e., spatial gradients in allele frequencies can be observed, much information can be gained from analyses of their shape at one or a few dates using genetic cline theory (Endler, 1977; Haldane, 1948; Nagylaki, 1975). Cline shapes are expected to result from the interplay between evolutionary forces (*e.g.* migration and selection)(Figure 1). In the specific case of pesticide adaptation (exogenous selective pressure), clines are expected to be observed only at loci affected by the selection pressure (or loci linked to them, (Lenormand and Raymond, 2000)). Very few studies used the cline theory to understand the migration-selection equilibrium to understand the evolution of populations resistant to pesticides. One of these rare studies described the adaptation pattern of the mosquito *Culex pipiens* to organophosphate insecticides in Southern France (Lenormand et al., 1999; Lenormand et al., 1998; Lenormand and Raymond, 2000). In fungal pathogens, a recent article studied the spatio-temporal distribution and evolution of benzimidazole and strobilurin resistant populations of the banana pathogen *Mycosphaerella fijiensis* in Cameroon (Rieux et al.). Clines only form when migration is limited by distance. When migration is intense or of island type over the connected populations, clines will always be very abrupt and their shape will not be exploitable for inferences. Estimations will then require modeling the evolution of allelic frequencies for a locus supposed to be selected in a subdivided population with island migration (Crow and Kimura, 1970; Dempster, 1955; Levene, 1953), as proposed e.g., by (Milgroom et al., 1989) and (Ennos and McConnell, 1995)) for an

applications to *Crumenulopsis sororia* populations). It will also require that the temporal change in allelic frequencies is measured over a sufficient number of points in time.

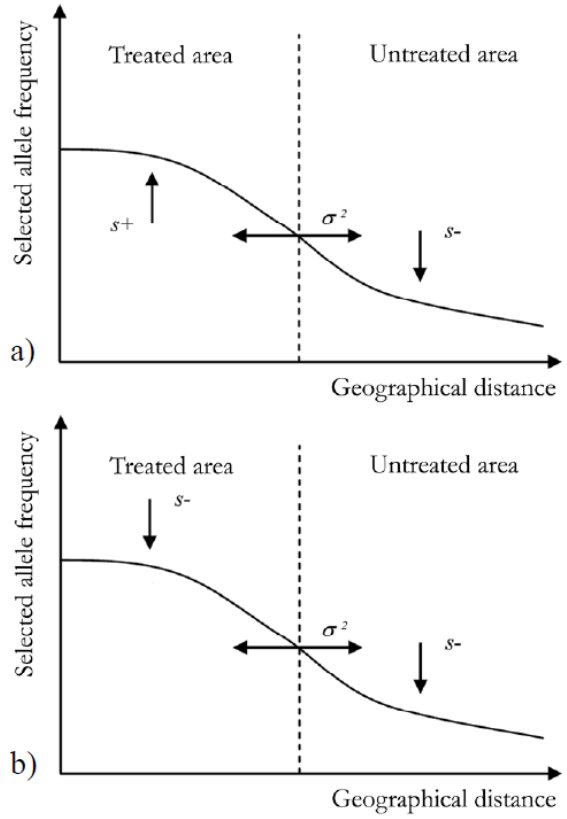


Figure 1 : Forces at work in studies populations (after (Rieux et al.)).

Positive (*s*+) and negative (*s*-) (*i.e.* fitness cost) are supposed to diversify populations, whereas migration (measured by the variance of parent-descendant dispersal distances σ^2) between treated and untreated areas are supposed to homogenize them.

(A) When fungicides are sprayed in the treated area, during spring and summer, positive selection occurs whereas negative selection due to fitness cost operates in the untreated area, leading to a migration-selection equilibrium.

(B) During autumn and winter, where grapevine plots are not treated, only negative selection occurs, which potentially leads to a diminishing pattern of cline.

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is a necrotrophic ascomycete fungus responsible for grey mold on more than 220 host plants (Elad et al., 2004), possibly infecting dead or living plant material (Martinez et al., 2003). It has deleterious consequences on crop yield and quality, and especially on perennial hosts, such as grapevine. In French vineyards, control is mainly achieved by fungicide sprays (0 to 3 per year, according to the vineyard). Consequently, resistance arose to most fungicide modes of action, either *via* specific resistance and/or multidrug resistance, which is related to increased drug efflux (Kretschmer, 2012; Walker et al., 2013a). *B. cinerea* is a highly polymorphic fungus, as observed from its mycelium color and aspect and from sclerotia formation (Martinez et al., 2003). In addition, grey mold was recently found to be caused by a complex of two cryptic species, *B. cinerea* and *B. pseudocinerea*, the last one being the minority species, most frequently found in spring (Walker et al., 2011). More recently, a distinct genetic entity (group *s*) was also characterized on several hosts and especially on strawberry (Leroch et al., 2013). *B. cinerea* is recognized as genetically highly variable (Fournier and Giraud, 2008; Giraud et al., 1997) and geographical structure is identified only at the continental scale (Isenegger et al., 2008), unless

geographical barriers occur within a country (Karchani-Balma et al., 2008). Until recently, only differentiation due to host-plant was found at a country scale (Fournier and Giraud, 2008; Karchani-Balma et al., 2008). Nevertheless, more exhaustive samplings of this fungus in sympatric ecological niches demonstrated that populations were in fact highly structured, (1) by the cropping system (indoor *vs* outdoor), the host plant and, to a lesser extent, by geography (Walker et al., 2013b). The authors also demonstrated that, at the French scale, *B. cinerea* populations could be divided into five genetic clusters: one in *Solanum lycopersicum* (tomato) greenhouses, one on *Rubus fruticosus* (bramble), a wild rosaceous plant frequently found in the neighborhood of cultivated plots, and three on *Vitis vinifera* (grapevine), one of which suffering temporal isolation and therefore, submitted to asynchronous gene flow and sexual reproduction.

In the present study, we aimed to address the relative influence of genetic drift, gene flow and selection on both the neutral and selected spatial genetic structure of *B. cinerea* populations at a regional scale. To achieve this objective, we analyzed the population structure and diversity from three pairs of treated/untreated grapevine plots in the Champagne vineyard, in the North-East of France, following the evolution of resistance frequencies for four different loci and of neutral allele frequencies at eightmicrosatellite loci. Because this vineyard suffers important grey mold epidemics, it receives two to three fungicide sprays targeting *B. cinerea* per season (Walker et al., 2013a). Therefore, it constitutes a place of choice to measure the evolution of resistance over time and space. Using descriptive genetic population and cline analysis, we revealed significant evolution of resistance frequencies over the studied period and a likely important migration between treated and untreated areas. At last, we estimated the magnitude of negative selection after modeling resistance evolution during winter.

MATERIAL AND METHODS

STUDIED AREA AND ISOLATE SAMPLING

Samples were collected at four dates: September 2005, June 2006, September 2006 and June 2007. These represent either the beginning of the cropping season (June collections), *i.e.* after presumed sexual reproduction occurring in winter, or at the end of the cropping season, just before vintage (September collections), *i.e.* after asexual multiplication on the host. Samples were collected on grapevine (*Vitis vinifera*) in three locations (Courteron, Hautvillers and Vandières) in Champagne (France), distant from each other from 20 km to 208 km (Table 1). In each location, we worked in

commercial plots divided in two parts. One part never received fungicides since at least three years before the start of the experiment, and the other part received yearly a three-spray program; otherwise, the two parts were identical from the agronomical point of view (same grape variety, climatic and soil conditions and cultural practices). The two parts were separated by a minimum of four rows where no samples were collected. The disease chemical protection in the treated part included, for the three plots and the two years of study: fenhexamid, at the end of flowering, fludioxonil, at bunch closure and pyrimethanil or fluazinam, at veraison. All fungicides were applied at recommended dose. *B. cinerea* was collected using cotton swabs from berry sporulating lesions in September and from asymptomatic flower caps in June. In this last case, *B. cinerea* strains were collected following plant material incubation in a moisture chamber at room temperature until sporulation was conspicuous. All samples were located in the plot they were collected. The grapevine plot was used as a grid to establish strain coordinates (number of the row since the left border of the plot; number of the vine stock in this row).

		September 2005	**June 2006**	**September 2006**	**June 2007**	**Total**
Courteron	Untreated	26 / 57	30 / 37	50 / 49	27 / 33	133 / 176
	Treated	27 / 46	20 / 45	48 / 49	3 / 4	98 / 144
Hautvillers	Untreated	45 / 58	23 / 36	55 / 56	44 /48	167 / 198
	Treated	48 /59	36 / 45	53 / 55	3 / 8	140 / 167
Vandières	Untreated	31 / 53	44 / 49	44 / 46	10 / 10	129 / 158
	Treated	32 / 49	40 / 54	41 / 49	19 / 21	132 / 173
Total		209 / 322	193 / 266	291 / 304	106 / 124	799 / 1016

Table 1: Number of strains sampled for this study on grapevine, according to their collection date and site.

The first figure indicates the number of strains genotypes for the eight SSR markers (Fournier et al., 2002). The second figure indicates the number of strains phenotyped for fungicide resistance (Walker et al., 2013a).

STRAINS ISOLATION, STORAGE AND PHENOTYPING

Single spore cultures were isolated for all strains. Stocks of spore suspensions were kept for each strain at -80°C in 20% glycerol until use. When needed, single-spore strains were grown on malt-yeast-agar (MYA) medium (20g.l^{-1}malt extract, 5 g.l^{-1} yeast extract and 12.5 g.l^{-1} agar) and incubated at 19°C-21°C, under continuous illumination, to induce sporulation.

Resistance phenotype was established for each strain (Table 1) after its exposition to discriminating doses of fungicides in synthetic media and after reading spore germination or germ

tube elongation at 24h-48h, as previously described (Walker et al., 2013a). Resistance profile was checked for specific resistances towards five fungicide families, for which resistant mutants are already known in the field, after alteration of the target protein (Leroux et al., 2002):

- Benzimidazoles (*e.g.* carbendazim), inhibitors of microtubules; phenotype BenR1
- Hydroxyanilides (*e.g.* fenhexamid), inhibitors of sterol C4-demethylation; phenotype HydR3
- Dicarboximides (*e.g.* iprodione, pyrimethanil), inhibitors of osmotic signal transduction; phenotype ImiR1
- Anilinopyrimidines (*e.g.* pyrimethanil, cyprodinil), inhibitors of methionine biosynthesis; phenotype AniR1
- Carboxamides (*e.g.* boscalid), inhibitors of succinate dehydrogenase; phenotype CarR.

We also identified multidrug resistant strains, referred as non-specific increased efflux of drugs, caused by either a monogenic alteration of the transcription factor of an ABC-transporter gene (phenotype MDR1), or an insertion in the promoter of a MFS-transporter gene (phenotype MDR2) (Kretschmer et al., 2009; Leroux and Walker, 2013b). At last, we used chi-square statistics (χ^2) to test for significant differences in frequencies overtime between untreated *vs* treated plots.

MOLECULAR ANALYSES

Total genomic DNA was extracted after robot extraction with the DNeasy adapted kit (Qiagen). All strains were genotyped with 8 SSR markers Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7 and Bc10 (Fournier et al., 2002), organized in multiplex PCR, as already described (Leroux et al., 2010). We excluded strains of the cryptic species *B. pseudofuckeliana*, morphologically similar to *B. fuckeliana*, on the basis of a previously described diagnostic allele at locus Bc6 and of its specific resistance phenotype towards fenhexamid (Leroux et al., 2002; Walker et al., 2011). Only isolates genotyped at all SSR markers were included in the study (Table 1).

ANALYSIS OF POPULATION STRUCTURE

The genetic structure of populations sampled in the three sampling sites, either in among the treated and untreated part of each site, was tested for each collection dates using a hierarchical analysis of molecular variance (AMOVA), as implemented in the ARLEQUIN V3.5 software (Excoffier and Lischer, 2010). Geography and treatment were used as grouping factors. The significance of the

fixation indices was calculated at each level of analysis using a non-parametric permutation approach, as implemented in the software.

In addition, the level of genetic differentiation at SSR loci between treated and untreated populations within a location over time was estimated with F_{ST} (Weir and Cockerham, 1984), using GENEPOP V4.1 run on the web (Raymond and Rousset, 1995). The significance of F_{ST} was tested using Fisher's exact tests implemented in the software (Markov chain parameters: dememorization number=2000, number of batches=250, number of iterations per batch= 2000).

At last, population subdivision was investigated using the Bayesian clustering method implemented in STRUCTURE V2.3.3 (Pritchard et al., 2000), with the admixture model and correlated allele frequencies. Burn-in length was set at 100,000 Markov Chain Monte Carlo iterations, followed by a run phase of 500,000, with the number of clusters *K* ranging from 1 to 10 and 10 independent replications for each *K*. The amount of additional information explained by increasing *K* was determined using the Δ*K* statistic (Evanno et al., 2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt, 2011).

ANALYSIS OF POPULATION DIVERSITY

Calculations were performed for each population, defined by its sampling site and time and disease control strategy (fungicide treated *vs* untreated). GENETIX (http://www.genetix.univ-montp2.fr/genetix/intro.htm) was used to estimate within-population variability based on genic diversity estimated from the expected heterozygosity (*He*, unbiased estimate (Nei, 1978)) and allelic richness (*Ar*), defined as the mean number of alleles over the eight loci. We also used the rarefaction procedure implemented in HP-RARE V1.0 (Kalinowski, 2005) to estimate private allelic richness (PA_R). This index allowed calculating the number of alleles that are detected in one population but absent in the other. The number of unique multilocus genotypes (G) and clonal richness (G/N) were calculated with the software MULTILOCUS V1.3b (Agapow and Burt, 2001). We also used this software to calculate the $\bar{r}_D$ index, an estimate of the multilocus linkage disequilibrium. Contrarily to the I_A index, $\bar{r}_D$ is corrected for the number of studied loci, and ranges between 0 (complete panmixia) and 1 (strict clonality). The significant deviation of this estimator from 0 was tested after 1000 randomizations (Agapow and Burt, 2001). We also evaluated the significance of pairwise linkage disequilibria using contingency tests (with default parameters) implemented in GENEPOP V4.1 (Raymond and Rousset, 1995). For each population, we also scored the proportion of pairs of loci whose linkage was significant at each collection dates. At last, we used χ^2 statistics or the non-

parametric Wilcoxon-Mann-Whitney test to test for significant differences over time between untreated *vs* treated plots for the proportion of paired loci, or the *He, Ar,* PA_R, $\bar{r}_D$ and G/N indexes, respectively.

CLINE FITTING ANALYSIS

We used the CFIT V7 program (http://www.cefe.cnrs.fr/ecogev/siteGB/CFitpage.htm) (Gay et al., 2008; Rieux et al.) to fit genetic cline models using allelic count data for each location and sampling time. For each of the four bi-allellic loci involved in resistance to fungicides (resistance to benzimidazoles BenR1, to dicarboximides ImiR1 and multidrug resistances MDR1 and MDR2), the frequency of resistant allele was fitted as a decreasing function, according to the geographical distance. We considered a scaled logit function (Rieux et al.), characterized by four parameters:

$$h_1 + h_p\,(1-h_1)\frac{e^{b(c-x)}}{1+e^{b(c-x)}}$$

where h_1 is the lower asymptotic frequency, h_p determines h_2 (the higher asymptotic frequency) as $h_2 = h_1 + h_p\,(1-h_1)$, b is related to the maximum slope of the cline (the slope being $-h_p(1-h_1)b/4$) and c is the center of the cline. Fits were realized without constraining any parameter in 1D, taking into account the distance between the grapevine row in which the samples were collected and the row on the left border of the plot. We used likelihood-ratio tests (LRT) to test for significant patterns of clines by comparing the likelihoods of the full cline model with that of a null model assuming a constant frequency over space.

ESTIMATION OF FITNESS COSTS FROM YEARLY DIFFERENCES IN ALLELIC FREQUENCIES

The available data make it possible, in principle, to infer the evolutionary parameters determining the dynamics of resistance frequencies in treated and untreated areas (Lenormand and Raymond, 2000). To do so, one needs modeling the evolution of allelic frequencies over time at resistance loci. We will consider that distinct processes govern allelic frequencies between September and June (hereafter called 'winter') and between June and September (hereafter called 'summer') (Figures 2 and 3). This assumption naturally arises from the seasonality of temperate cultures. It is also motivated by the observed oscillations in allelic frequencies at resistance loci and at neutral loci. Indeed, at resistance loci, resistant alleles tend to become less frequent during 'winter' and increase again in frequency during 'summer'. Similar evolution of gene diversity (H_e) was

also observed at neutral loci. Hence, it is likely that multiple processes combine during summer to act on allele frequencies (namely: linkage created by asexual reproduction, migration between treated and untreated adjacent patches, migration from the wild compartment…). Thus, exploiting 'summer' data first commits an adequate modeling of the processes in action during summer, for any estimate to be valid. Without this modeling phase, exploiting 'summer' data is challenging. Therefore, we will here only focus on 'winter' data as a first step. In addition to specific climatic conditions, 'winter' is, in the present context, characterized by a drastic diminution of host availability at the beginning of the period (vintage), and an absence of treatments even in usually treated fields. Sexual reproduction is suspected to take place during this period (Beever and Weeds, 2004; Walker et al., 2013b). Exchanges between plots are suspected limited during most of the period, but some exchanges just before June samplings cannot be fully excluded. We also observe that resistance becomes less frequent between September and June in many situations (Figure 3). Such decay can reasonably be attributed to two main processes: the cost of resistance, by which resistant strains would die with a higher mortality rate or multiply less intensively at sexual reproduction than sensible strains, and migration from untreated areas with weak resistance frequencies.

To simplify, we will first consider that the observed pattern can entirely be attributed to the cost of resistance. Migration either from resistance-free areas or from the adjacent patch is neglected. For each patch, we consider that the initial resistance frequency is p_{sept}. As no treatment is conducted, susceptible strains multiply at a rate w and resistant strains multiply at a rate w $(1 - c)$ where c measures the cost of resistance over the whole season. In June, the frequency of resistant strains is simply (Crow and Kimura, 1970):

$$p_{June} = \frac{p_{Sept} w(1-c)}{p_{Sept} w(1-c) + (1-p_{Sept}) w} , \quad (1)$$

which can also be written:

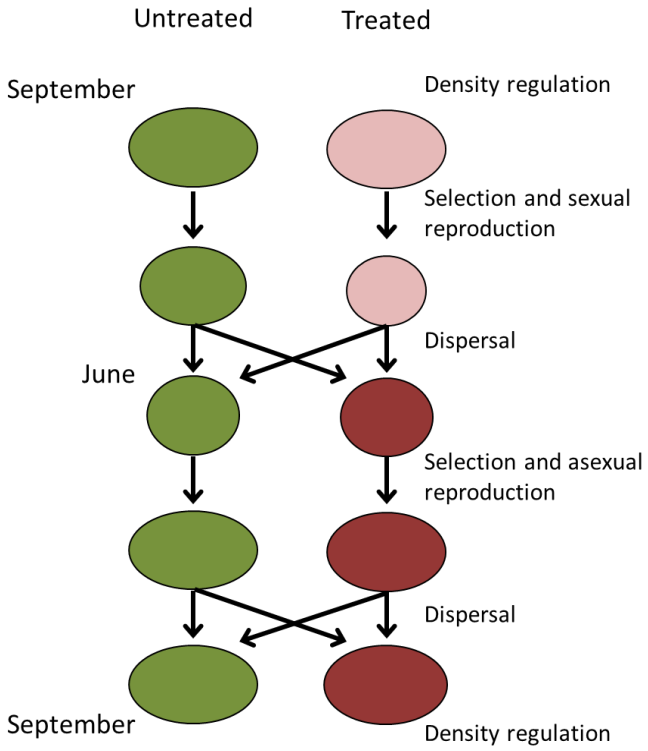


Figure 2: Schematization of *B. cinerea* life cycle and forces at work in treated (red) and untreated (green) populations infecting grapevine.

Between September and June, the fungus reproduces sexually. Migration *via* asexual spores is limited and the pathogen is present in the field as dormant sclerotia and/or mycelium. Therefore, only negative selection due to fitness cost acts on resistant strains; their frequency is low (light red) and the population size decreases (small ellipse). Between June and September, the fungus propagates asexually and double-sense migration between treated and untreated plots occurs due to asexual spores (conidia), whose quantity increases gradually with grape berries maturation, until its maximum at vintage. During this period, a positive selective pressure (fungicide sprays) is exerted three times in treated areas and leads to an increase in resistance frequency (dark red).Population size increases because of resistant strains (large ellipses). In September, vintage induces a drastic density regulation.

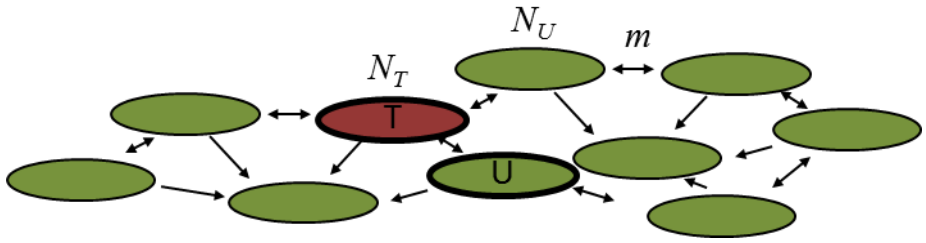


Figure 3: Ecological context of the study

Treated (in red: T; effective size N_T) populations exchange migrant individuals with population in the untreated plot (in green: U; thick line) as well as with other local untreated populations (in green; narrow line), constituted of wild host plants, for example *Rubus fruticosus* ((Walker et al., 2013b). All untreated populations (from untreated plots or from the wild) have an effective size N_U. m is the migration rate between population from the treated patch and all other untreated populations (from either untreated or wild patches).

$$p_{June} = \frac{(1-c)p_{Sept}}{1-cp_{Sept}}. \quad (2)$$

Solving for *c*, one gets:

$$c = \frac{p_{Sept} - p_{June}}{p_{Sept}\left(1-p_{June}\right)}. \quad (3)$$

This quantity can be computed for all observed transitions (n = 12) and then averaged. Assuming that *c* does not vary between localities and years, *c* may be further estimated by a nonlinear regression between frequencies in September and frequencies in June the year after (using equation 2). This value simply represents the cumulated effects of the cost of resistance of the 182 days of the season.

As a complement, one may seek to account for variability in sampling effort among localities and dates. To do so, we computed the likelihood of the 12 'winter' transitions (3 localities x 2 patches x 2 years) under the model defined by equation (2). The observed frequencies were considered as resulting from a binomial sampling so that the likelihood of a given transition is:

$$V\left(p_{Sept}, N_{June}, R_{June}, c\right) = C_{N_{June}}^{R_{June}} \left(\frac{(1-c)p_{Sept}}{1-cp_{Sept}}\right)^{R_{June}} \left(1-\frac{(1-c)p_{Sept}}{1-cp_{Sept}}\right)^{N_{June}-R_{June}}, \quad (4)$$

where N_{June} and R_{June} are the number of strains tested and the number of resistant strains found, respectively. The likelihood of the whole dataset is the product of the 12 transition likelihoods. The likelihood was maximized with regards to *c* using the function Maximize of Mathematica 7.0 (Wolfram Research, Inc.). Support limits were estimated within 2 units around the maximum likelihood value.

As said above, *c* accounts for the effect of resistance cost over the whole season. For the sake of comparison with other studies, it is necessary to express this cost on a daily basis. From a classical continuous-time model of resistance evolution in closed populations (Crow and Kimura, 1970; Milgroom et al., 1989), the dynamics of the frequency of resistant alleles *p* can be modeled as:

$$\frac{dp}{dt} = (r_S - r_R)p(1-p), \quad (5)$$

where r_S and r_R are the fitness of susceptible and resistant strains, respectively. Let c_{inst} denote the fitness differential between susceptible and resistant strains $r_S - r_R = c_{inst}$. Solving equation 5 between September and June leads to:

$$\frac{p_{Sept}}{1-p_{Sept}} = \frac{p_{June}}{1-p_{June}} e^{-c_{inst}t} \quad (6)$$

.

By comparing with equation 2, one finds that:

$$1-c = e^{-c_{inst}t}. \quad (7)$$

Equivalently, the instantaneous cost of resistance is:

$$c_{inst} = -\frac{Ln(1-c)}{t}. \quad (8)$$

ESTIMATION OF FITNESS COSTS FROM LONG-TERM EVOLUTION IN ALLELIC FREQUENCIES

At last, we also calculated estimates of resistance cost using mean resistance frequencies data previously collected in surveys undertaken in the Champagne vineyard (150-200 locations analyzed each year, representative of the diversity of the agronomic situations)(Walker et al., 2013a). We used data collected between 2002 and 2011 and 1999 and 2011 for the BenR1 and ImiR1 phenotype, respectively. These periods are correlated with significant decay of resistance in the vineyard and very limited use of benzimidazoles and dicarboximides. Such calculations were not possible for MDR1 and MDR2, because their mean resistance frequencies still increase under contemporary selective pressures. Using equation 6, we used a linear regression of $\mathrm{Ln}\left(p/(1-p)\right)$ against time to infer the instantaneous cost of resistance (here obtained as the slope of the regression).

RESULTS

EVOLUTION OF FUNGICIDE RESISTANCE IN POPULATIONS

We performed the systematic identification of resistance profile for the 1016 strains collected at four different dates, in order to obtain an accurate overview of resistance status in our

study sites from Champagne. The overall frequencies of specific resistances towards hydroxyanilides, carboxamides and anilinopyrimidines (respectively, phenotypes HydR3, CarR and AniR1 detailed in (Walker et al., 2013a) were all very weak (below 5%), disabling any further detection of selection at the corresponding loci. On the contrary, we measured higher overall frequencies of phenotypes exhibiting specific resistance towards benzimidazoles (BenR1 = 61.2%), dicarboximides (ImiR1 = 10.1%) and also of multidrug resistant phenotypes (MDR1 = 23.8% and MDR2 = 11.7%).

As expected, resistance frequency was higher in some but not all treated plots (Figure 4). Significant differences between the two plots were detected through χ^2 tests only for the MDR1 and MDR2 phenotypes in the three locations and for one sampling date in Vandières for the ImiR1 phenotype (Figure 4). Moreover, resistance frequency varied over time in the treated plots as well as in the untreated plots. The magnitude of this temporal evolution was similar for the BenR1 and ImiR1 phenotypes, but was greater in the treated plots for MDR1 and MDR2, the lower frequencies being observed in spring, before the starting of the chemical protection (Figure 4).

POPULATION GENETIC STRUCTURE

The relative contributions of the “sampling location” and “treatment strategy” to the genetic variance were estimated for each sampling date with hierarchical analyses of molecular variance (AMOVA) while using these parameters successively as grouping factors (Table S1). In all situations, the within-group variation explained most of the total variance (98.3% in mean; range 96.1% - 100%; -0.001<F_{ST}<0.040). The effect of sampling site was never significant when it was used as a grouping factor (1.3% in mean; range -0.2% - 3.3%; -0.002<F_{CT}<0.032). Nevertheless, it had a significant effect within treatments for two dates when the fungicide treatment was used as a grouping factor (1.8% in mean; range 0.7% - 3.9%; 0.007<F_{SC}<0.039). Similarly, the effect of the fungicide treatment was never significant when it was used as a grouping factor (-0.2% in mean; range -1.0% - 0.9%; -0.010<F_{CT}<0.009) but it was significant within locations for two situations when location was used as a grouping factor (0.6% in mean; range -2.4% - 2.6%; -0.024<F_{SC}<0.027). Altogether, these results

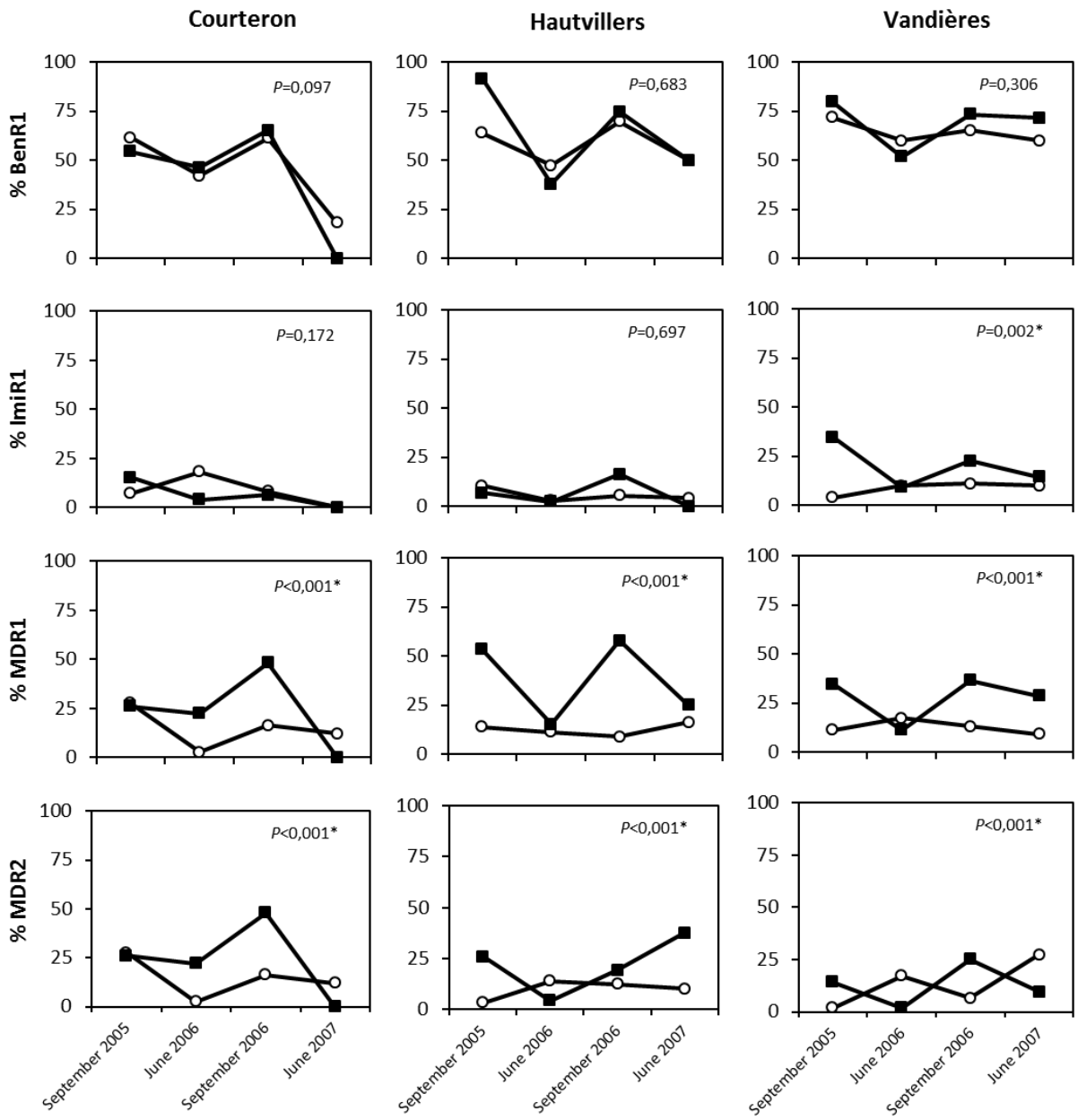


Figure 4: Evolution of resistance over time in untreated or treated field populations of *Botrytis cinerea.*

Figures indicate the evolution of frequency of four resistant phenotypes in treated (black squares) and untreated (white dots) grapevine plots from three locations in Champagne (France). Phenotypes are described in (Walker et al., 2013c) and concern two specific resistances (ImiR1 and BenR1) and two cases of multidrug resistance (MDR1 and MDR2). Statistical difference between treated and untreated plots was calculated with a χ^2 test (df=3). *P*-value marked with an asterisk * are significant at the 5% confidence level.

indicate that the effects of fungicide treatment and sampling site on neutral population subdivision are always weak over time and detectable only occasionally, probably due to stochastic phenomena.

Differentiation calculated between the treated and untreated plots with the F_{ST} index was always low, for all situations and for each of the eight markers (range -0.0001, 0.1549; mean F_{ST}=0.0007). Nevertheless, F_{ST} values were significant for half of the calculations (Table 2). Multilocus F_{ST} calculated with the eight markers gave similar results (range -0.0015, 0.0870; mean F_{ST}=0.0074 significant in 11 out of 12 situations).

At last, the results of the Bayesian population structure analyses did not reveal any meaningful population subdivision over the sampled area. The best values of K that maximize the likelihood of the data as determined by Evanno's method (Evanno et al., 2005) were K=6, K=4, K=8 and K=2 for September 2005, June 2006, September 2006 and June 2007, respectively (Table S2). In a previous study that includes these samples among others from other French regions (Walker et al., 2013b), we found that isolates sampled on *Vitis* were assigned to three different genetic clusters. We thus checked the congruence between this previous assignment and the assignment calculated by STRUCTURE for K=3 in this analysis, for the same individuals. We found a good congruence between the two clustering analyses (Figure S1), which demonstrates that in this dataset populations are not structured regionally, or by fungicide treatments, but rather by fine host adaptation on *Vitis*.

POPULATION RECOMBINATION AND DIVERSITY IN TREATED *VS* UNTREATED PLOTS

In the three locations, the proportion of unique multilocus genotypes was always greater than 0.72, in treated as well as in untreated plots. Linkage disequilibrium estimated with the $\bar{r}_D$ was always below 0.18 and in the same range of magnitude for treated and untreated plots, if we exclude Courteron and Hautvillers populations with two few individuals in June 2007 (Table 1). Similarly, the proportion of SSR linked pairs is always below 50%, in all situations (Figure 5). Altogether, these results are in agreement with the regular occurrence of recombination, probably due to sexual reproduction, preferentially in winter between September and June, as suggested by the $\bar{r}_D$ variations in Hautvillers. The reproduction mode was similar in treated and untreated plots in each location, as confirmed by the non-significant *P* values for the linkage disequilibrium and clonal richness indexes after the Wilcoxon-Mann-Whitney-test.

Locus	F_{ST} treated *vs* untreated											
	Courteron				**Hautvillers**				**Vandières**			
	September 2005	June 2006	September 2006	June 2007	September 2005	June 2006	September 2006	June 2007	September 2005	June 2006	September 2006	June 2007
Bc1	0.0322	-0.0118*	-0.0075**	-0.1168	-0.0001*	0.1549**	0.0031**	0.2446*	-0.0127**	0.0031	0.0045**	0.0376**
Bc2	0.0575**	0.0071**	-0.0057**	-0.1285	0.0186**	0.0275**	0.0491**	-0.0893	0.0196**	-0.0092	-0.0128**	-0.0007**
Bc3	0.0095	-0.0108*	0.0116**	-0.1178	0.0070*	0.0631**	0.0118**	-0.0515	-0.0224	-0.0153	-0.0092**	-0.0410
Bc4	-0.0239	0.0404	-0.0076	-0.1368	0.0327*	0.0030	-0.0120	-0.1267	-0.0266	0.0060	-0.0105	0.0373
Bc5	-0.0120	-0.0288	0.0464**	-0.0291	0.0026*	0.0955**	-0.0073	0.0153	0.0380**	-0.0029**	0.0067**	0.0043
Bc6	-0.0239	0.0253	0.0259	-0.1157	0.0022**	0.0203**	0.0084	-0.1267	-0.0269	0.0258**	0.0065	-0.0690
Bc7	-0.0004	0.0146	0.0186**	-0.0714*	0.0637**	0.0540**	-0.0121	-0.0165	0.0041*	0.0144**	-0.0083	-0.0766
Bc10	0.0118**	-0.0010	0.0225**	0.0666*	0.0306**	0.1476**	-0.0045	-0.0275**	0.0207**	0.0062**	-0.0111	-0.0706
All loci	0.0035**	-0.0015**	0.0152**	-0.0680	0.0226**	0.0870**	0.0062**	0.0111*	0.0087**	0.0011**	-0.0042**	-0.0222**

Table 2: Pairwise values of genetic differentiation (F_{ST}) between fungicide treated and untreated plots in Champagne vineyard

Fisher's exact test: $^{*}P<0.05$; $^{**}P<0.01$

Multilocus gene diversity, H_e , ranged between 0.25 and 0.61 in untreated and treated plots from the three locations. H_e was higher in untreated as compared to untreated plots from Courteron and Hautvillers (even if the difference was not significant). Similar results were observed for the allelic richness A_r (range 1.6 – 6.9 alleles/locus) and the private allelic richness PA_r (range 0.1 – 5.4 alleles/locus) indexes, which were also significantly higher in untreated plots as compared to treated plots from Courteron (P=0.081 for A_r; P=0.027 for PA_r) and Hautvillers (P= 0.029 for A_r; P=0.029 for PA_r) (Figure 5). Altogether, these results suggest a greater effect of genetic drift in treated plots, at last in two locations, as expected if the selective pressure due to fungicides treatments is acting.

At last, we verified that the 8 SSR loci used here could be considered as neutral markers. This is of great importance for further mathematical modeling aiming at estimating migration, fitness cost, and selection parameters (see below). One way to verify the neutrality of markers is to plot F_{ST} against H_e for each locus (Olivieri, 2010): outliers from the resulted point cloud are defined as non neutral loci. For each situation (n=12) and for each SSR locus (n=8), we plotted the F_{ST} value of each pair of treated *vs* untreated plots against the H_e value calculated after pooling the two populations (Figure 6). For each SSR locus, all points were grouped together with no outlier. This indicate that all loci measure H_e and F_{ST} of similar magnitudes, whether the populations are treated or not, which is an evidence of their neutrality. We also mapped these neutral and selected markers on the *B. cinerea* genome recently established (Amselem et al., 2011)(Figure S2) and found that all markers were situated on different linkage groups or at distant positions (> 2 000 kbases) within a contig; this ensures that no physical linkage exists between SSR and selected loci.

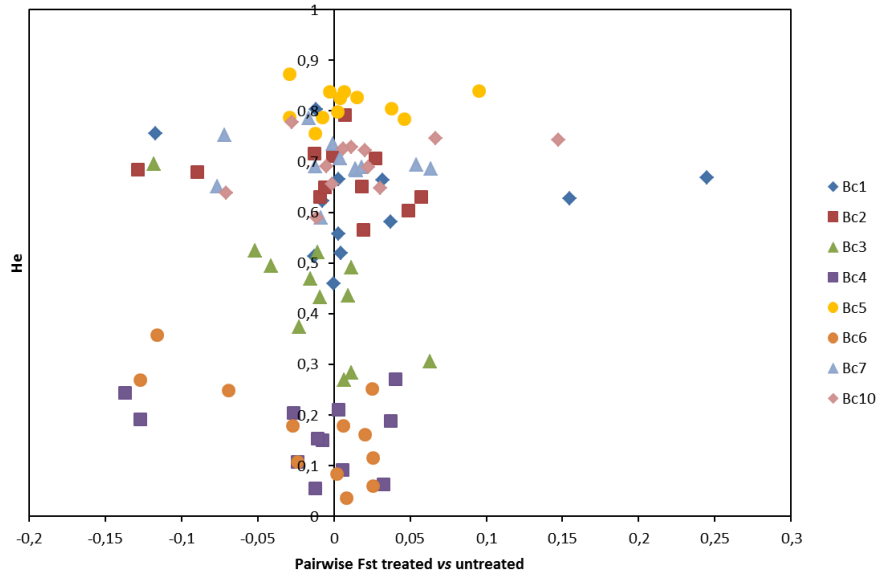


Figure 6: Estimation of SSR markers neutrality

For each SSR marker, the pairwise F_{ST} between each pair of untreated *vs* untreated populations was plotted against the heterozygoty H_E calculated for the two previous populations pooled together. Each point corresponds to a single situation (3 sites x 4 collection dates).

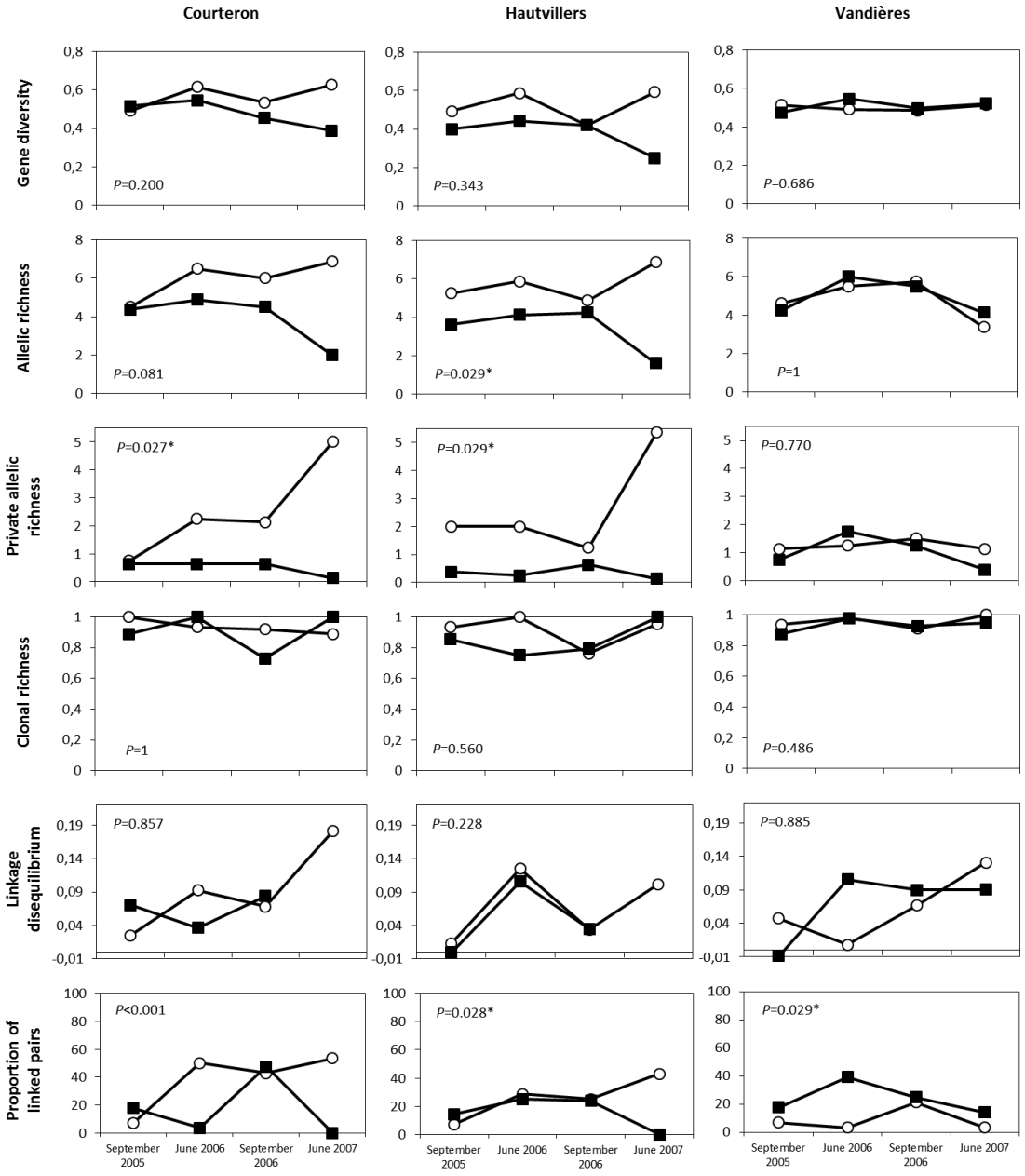


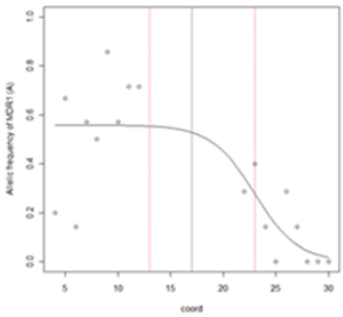
Figure 5: Evolution over time of diversity and recombination indexes in *Botrytis cinerea* populations from three Champagne locations with untreated (white circles) and treated (black squares) plots.

Gene diversity estimated from the expected heterozygoty (H_e, unbiaised estimate calculated following (Nei, 1978); allelic richness (A_R) measured as the mean number of alleles in a population over the eight loci; private allelic richness (PA_R) is the mean number of alleles detected in one population and not in the other over the eight loci; clonal richness is measured as the proportion of unique genotypes compared to the number of strains; multilocus linkage disequilibrium is measured with the $\bar{r}_D$ index; the proportion of pairs of SSR loci being linked was compared to the total number of loci pairs. For each index, statistical difference between treated and untreated plots was calculated using a Mann-Whitney test, with the exception of the proportion of linked pairs index for which we used a χ^2 test (df=3). *P*-value marked with an asterisk * are significant at the 5% confidence level. Situations were population size was not sufficient to produce accurate figures are not presented.

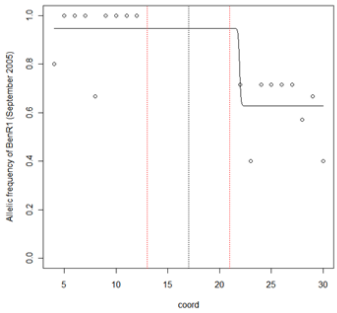
CLINE FITTING AND SELECTION DETECTION

1-D clines were fitted for each collection date, location and selected marker (47 out of 48 possible situations, since ImiR1 frequency was equal in treated and untreated plots in June 2007 in Courteron). For each cline, we recorded the four parameters of the fitted scaled logit function (Table S3) and performed likelihood-ratio tests to detect significant cline patterns (*i.e.,* with a slope significantly different from 0) (Figure 7).

A



B



C

	September 2005	June 2006	September 2006	June 2007
Courteron				
BenR1	1.42	0.30	1.08	4.21 $^{\sigma}$
ImiR1	**12.36**	7.17	0.61	-
MDR1	4.63	**9.21**	**13.69**	0.28
MDR2	1.94	1.15	**9.22**	1.54
Hautvillers				
BenR1	**18.58**	2.43	7.18	0.76
ImiR1	5.17	0.12	**59.26**	0.63
MDR1	**29.23** $^{\sigma}$	1.44	**35.99** $^{\sigma}$	0.56
MDR2	**15.91**	2.28	1.16	4.08
Vandières				
BenR1	**10.14**	0.56	-8.32E-10	0.07
ImiR1	**24.43** $^{\sigma}$	4.77	3.51	0.79
MDR1	**8.19**	4.69	**7.93**	2.43
MDR2	**8.37**	**9.80**	**9.39** $^{\sigma}$	4.06

Figure 7: Success of cline fitting analysis on four fungicide resistance markers

(A) and (B) Examples of clines fitted in Hautvillers (September 2005) for the MDR1 and BenR1 phenotypes, respectively. The clines represent in 1D the frequency of the resistant alleles plotted against the geographic distance (number of grapevine rows between the collection row and the left border row of the plot). In this example, the border between the treated (left) and untreated (right) plot is at row 17. Each cline was fitted using a scaled logit function (see Material and Method section).

Deviance values (twice the difference between the observed likelihood (CFIT) and the expected likelihood) for the situations where the cline fitting analysis was performed. Significant values of likelihood-ratio tests (5% level) are highlighted in bold. $^{\sigma}$ indicates that this cline had the typical sigmoid shape. Other clines were step-shaped.

Clines were significantly fitted in half of the situations, but were more frequently observed in autumn collection dates (67% and 50% of the situations, for September 2005 and September 2006, respectively) compared to spring collection dates (20% and 0% of the situations, for June 2006 and June 2007, respectively). Success in cline fitting was also more frequent for MDR phenotypes (50% and 42% of the situations for MDR1 and MDR2, respectively) compared to BenR1 and ImiR1 phenotypes (17% and 25% of the situations, respectively).

Clines were more often step-shaped but some of them displayed a classical sigmoid shape (Figure 4). In step clines, the center and the slope of the clines were not statistically supported, possibly because too few strains were collected in the center area. Therefore, parameters estimation through migration-selection cline models would not have been possible. Moreover, the observation of such step cline patterns suggests that migration is not gradual between untreated and treated plots in our dataset and that a patch model would be more appropriate to conduct any parameter estimation.

ESTIMATION OF FITNESS COST USING A PATCH MODEL

Fitness cost for each of the four loci was estimated on the three sampling sites according to three methods: (1) by directly averaging yearly costs, (2) using a non-linear regression method, (2) and (3) using a maximum likelihood method, which also gives support limits (Table 3). For each method, the fitness cost was estimated globally over the winter period, or instantaneously, per day. We also estimated fitness costs using the mean frequencies of BenR1 and ImiR1 calculated in long-term surveys over the whole vineyard with a linear regression method. The estimated fitness costs values are indicators of how resistant strains survival would be affected in absence of fungicide treatments. Therefore, the more the total fitness cost rises towards 1, the less are the resistant isolates able to compete.

Instantaneous fitness costs were all very low (range 0.0010, 0.0078 day^{-1}) and may therefore be undetectable in laboratory experiments aiming to compare the fitness of isolates. Nevertheless, when cumulated over a long period of time, they may be sufficient to decrease the frequency of resistant strains in untreated populations. Total fitness cost estimated on the tree sampling sites for the BenR1 locus was in the same order of magnitude for the three methods (0.5245, 0.5820) but this was not the case for the other loci (ImiR1: 0.2532, 0.6822; MDR1: 0.3339, 0.7576; MDR2: -1.1548 , 0.5475), probably due to greater variability in these datasets, also confirmed by the large confidence

Cost of resistance	On three sampling sites data						On vineyard data
	Direct average method		Regression method		Maximum likelihood method		Regression method
	Total	Instantaneous[a]	Total	Instantaneous	Total	Instantaneous	Instantaneous
BenR1	0.5245	0.0053 day-1	0.5820	0.0048 day-1	0.5690	0.0058 day^{-1}	0.00074 day^{-1}
	[0.3367, 0.7123]	[0.0021, 0.0084]	[0.3628, 0.8013]	[0.0025, 0.0089]	[0.3002,0.6963]	[0.0046, 0.0070]	[0.00064, 0.00083]
ImiR1	0.2532	0.0227 day-1	0.6822	0.0063 day-1	0.5285	0.0041 day^{-1}	0.00045 day^{-1}
	[-0.5833, 1.0897]	[-0.0285, 0.0739]	[0.4246, 0.9398]	[0.0030, 0.0154]	[0.3002,0.6963]	[0.0020, 0.0065]	[0.00033, 0.00057]
MDR1	0.3339	0.0039 day-1	0.7576	0.0078 day-1	0.5416	0.0043 day^{-1}	-
	[-0.0997, 0.7675]	[-0.0001, 0.0078]	[0.5857, 0.9296]	[0.0048, 0.0146]	[0.3872,0.6631]	[0.0027, 0.0060]	
MDR2	-1.1548	0.0010 day-1	0.5475	0.0043 day-1	0.3272	0.0022 day^{-1}	-
	[-3.4795, 1.1699]	[-0.0051, 0.0071]	[-0.0042, 1.0993]	[-0.0001, 0.0127]	[0.0380,0.5458]	[0.0002, 0.0043]	

Table 3: Estimation of fitness cost for four fungicide resistance loci, with three possible methods and using two datasets.

Fitness costs and support limits calculated with the direct average method, the regression method or the maximum likelihood method. For each resistance locus, the cost is calculated globally of the winter period (182 days) and instantaneously for one day, using resistance frequency data observed on the three sampling sites. Regression estimation was also undertaken using mean resistance frequencies calculated over the whole vineyard, as reported in survey studies (Walker et al., 2013a). These estimates are only provided for the BenR1 (time span 2002-2011) and ImiR1 phenotypes (time span 1999-2011), for which the use of benzimidazoles and dicarboximides is close to 0 during these periods.

[a] For this method, some null data could not be used (three for the ImiR1 estimate and one for the MDR1 estimate) leading to biased calculations.

intervals calculated. Nevertheless, two out of the three methods (direct average and maximum likelihood methods) gave identical ranking for the fitness alteration: BenR1 > MDR1 > ImiR1 > MDR2, the BenR1 phenotype supporting the greatest fitness cost.

Fitness cost calculated after whole vineyard data were smaller, of a factor 6-7 for BenR1 and 9-14 for ImiR1. In this analysis, the ImiR1 phenotype support a greater fitness cost (x 1.6), compared to BenR1.

DISCUSSION AND CONCLUSION

FUNGICIDES DO NOT SHAPE NEUTRAL GENETIC STRUCTURE OF *B. CINEREA* POPULATIONS

In this study, we hypothesized that fungicides may affect population differentiation and diversity for three main reasons. (1) First, fungicides exert a drastic constraint on treated populations and contribute to decrease their size in summer. This reduction of population size may also favor the fixation of private alleles in treated areas, due to more intense genetic drift (Hoffmann and Willi, 2008). (2) The also creates a temporal and spatial heterogeneity that may prevent the production and dissemination of propagules, therefore reducing gene flow between treated and untreated plots. (3) *B. cinerea* reproduces asexually during summer, *i.e.* when fungicide selective pressure is applied. Therefore, clonality should link, at least temporarily, neutral and selected markers. For all these reasons, allele and genotype frequencies, as well as association between alleles, may differ in treated *vs* untreated plots.

Analyzing population structure (AMOVA, pairwise F_{ST} and clustering analysis) in each sampling site, we found that fungicides sprays had no or only occasional weak impact on population subdivision, as also observed for another fungal pathogen (Rieux et al., 2013). Nevertheless, we observed that populations were partitioned into three genetic clusters that coexist on *Vitis*, as previously described (Figure S1)(Walker et al., 2013b). We also demonstrated that fungicide application had no significant effect on gene diversity, clonal richness and on linkage disequilibrium (Figure 2). This suggests that recombination intensity was similar in treated and untreated plots, probably due to sexual reproduction and/or migration. Thus, contrarily to our expectations, fungicide pressure did seem to affect neither population differentiation, nor associations between loci. Nevertheless, we observed that allelic richness and private allelic richness tend to be lower in treated

than untreated plots (the difference being significant in one location for A_r, and in two locations for PA_r). These last results are in accordance with a greater magnitude of genetic drift in some treated plots compared to the untreated ones, which is consistent with a regular and drastic reduction of population size due to fungicide applications and variation in host availability over time. However, the reduction of gene diversity in treated plots remained limited. The contrary was observed for some other oomycete plant pathogens, whose recombination would be smaller and less effective to counterbalance selection (Grunwald et al., 2006; Matasci et al., 2008). Hence, in *B. cinerea,* positive selection effect is likely limited because treated populations may stay large enough to keep constant genic diversity despite fungicide selective pressure. Moreover, the regular occurrence of sexual recombination, preferentially in winter, as demonstrated several times (Beever and Weeds, 2004; Fournier and Giraud, 2008; Walker et al., 2013b) and also the intense gene flow between treated and untreated plots, but also with some other demes, hosted on *Vitis* or on wild plants should strongly counter positive selection. This is in agreement with the previous observation of intense spillover individuals flows emitted by *Vitis* in June and by *Rubus*, in September (Walker et al., 2013b).

FUNGICIDE RESISTANCE FREQUENCY VARIES OVER TIME, SPACE AND AMONG SELECTED LOCI

Fungicide resistance was observed in this dataset for four selected loci (BenR1, ImiR1, MDR1 and MDR2). In a heterogeneous environment constituted of treated and untreated areas, one may expect (1) important resistance frequencies in treated plots due to positive selective pressure all the more strong since gene flow is weak between plots, (2) decreasing or null resistance frequencies in untreated plots due to the absence of positive selective pressure and the expression of a potential fitness cost in resistant strains. These spatial gradients of resistance frequency between treated and untreated plots may lead to a cline pattern, which shape may evolve according to the balance between the various operating forces (*i.e.* positive selection, negative selection and migration). Cline fitting analysis allows estimating cline parameters and following their variation over time.

In our dataset, we observed two kinds of situations, according to the selected locus considered. For the MDR1 and MDR2 resistances, we observed significantly higher proportions of resistance in the treated areas, for the three locations. Significant cline patterns were observed for these loci, mostly in September collection dates (9 out of 12 possible situations) and rarely in June (2 out of 12 possible situations). These results are in agreement with the occurrence of positive

selection operating during summer. Indeed, MDR phenotypes are known to be selected by fungicides of contemporary use, namely hydroxyanilides (fenhexamid), phenylpyrroles (fludioxonil), carboxamides (boscalid), anilinopyrimidines (cyprodinil, pyrimethanil) or pyridinamines (fluazinam)(Leroux and Walker, 2013a). Therefore, MDR strains exhibit a wide spectrum of cross-resistance towards several of these modes of action (Leroux and Walker, 2013b), still largely used in French vineyards and especially in these plots (Walker et al., 2013a). MDR selection may be more parcimonious than the selection of multiple resistances, because a single event leads to a similar wide-spectrum phenotype without the drawback to accumulate possibly deleterious mutations in the same strain. We observed significant cline patterns at MDR loci in most but not all September situations. However, most clines were not of the typical sigmoid shape but rather step-shaped. The cline theoretical framework supposes a progressive diffusion of resistant alleles from one area to the other; such step-shaped clines are clearly not in accordance with this hypothesis. Finally, MDR1 and MDR2 resistance frequencies were low, but not null in untreated plots. If we consider that the non-intentional spraying of untreated plots is negligible — which is reasonable in the high-value Champagne vineyard were most vine-growers are well equipped — the fact that cline patterns were not observed systematically in September might be due to intense migration of resistant isolates between treated and untreated plots, counterbalancing the effect of positive selection by fungicides. Our results differ in this point from what was observed in *M. fijiensis* (Rieux et al.), for which full susceptibility was shortly observed after the border between treated and untreated populations.

The situation we observed at the BenR1 and ImiR1 loci was different from the previous one. We found similar frequencies of resistance, whatever the collection date and the location. Moreover, we observed significant patterns of clines for only 5 out of 24 possible situations, all in September. This situation is consistent with little or no positive selection exerted on these loci. Indeed, benzimidazoles and dicarboximides were not used on these plots during the study, and more generally, are “old” modes of action intensively used in the 80s but abandoned a decade later because of the generalization of resistance, leading to important efficacy losses, and of the availability of new modes of action with greater intrinsic activity (Walker et al., 2013a) . As a consequence, we still observe nowadays important frequencies of these resistances, despite the negligible use of these fungicides, which suggests over the year a balance between negative selection and indirect positive selection (via multiple resistance, for example). Their similar frequency values in treated and untreated plots is also an evidence intense between-plots migration which homogenized resistance in the three locations, and maybe also in the whole vineyard. Moreover, indirect positive selection may also occur for these loci, because many isolates of our dataset cumulated resistance to

contemporary fungicides and to "old" fungicides, especially to benzimidazoles (pattern of multiple resistances). Therefore, it is possible that the selection exerted by the contemporary fungicides also leads to resistance increase for benzimidazoles, and to a lesser extent, to dicarboximides. This may also explain why positive cline patterns were scarcely observed for BenR1 and ImiR1, despite selective pressure with the concerned fungicides.

To our knowledge, this study is the first case study illustrating such contrast between selective pressures (contemporary *vs* ancient in fungal pathogens.

CONTRASTED RESISTANCE COSTS OPERATE IN VINEYARD

Negative selective pressure, *i.e.* fitness cost, is supposed to occur during winter for treated and untreated areas and to reduce progeny of resistant strains in populations. Along with migration, it is expected that fitness cost accentuated the subsidence of clines during winter. The estimation of the magnitude of this process is therefore deciding to predict the speed of resistance decay in populations and from a practical point of view, to adjust time between two sprays of a single mode of action (rotation).

Through the modeling of the evolution of resistance during the winter phase with three calculation methods, we estimated fitness cost for the four selected loci in the three locations. These costs were highly variable among dates. Variance may be due to sampling (limited numbers of strains were collected in some locations/dates) but also maybe because of more intense genetic drift in treated plots and of heterogeneity in cost expression in stressing environments. For the direct average and the maximum likelihood methods, we found that BenR1 was the locus most affected by negative selection. This is not totally in agreement with the long-term persistence of this resistance at high frequency in natural populations (Walker et al., 2013a). Indeed, estimates calculated from long-term survey, *i.e.* estimated during winter and summer, over the whole vineyard were 6-13 times lower. We also found that BenR1 was more affected than ImiR1, which is again not consistent with vineyard field data and with the rapid decrease of ImiR1 resistance after dicarboximides stop in many agronomic situations over the world. Altogether, this suggests than evolutionary forces at work during summer tend to be favorable to resistant strains. Therefore modeling summer evolution of allelic frequencies, including the possibility of migration between treated and untreated areas and asexual reproduction, will be a very important complement to the present analysis. Finally, it should be stressed that BenR1 fitness cost calculated for the banana pathogen *M. fijiensis,* bearing the same mutation on the β-tubulin gene responsible for a similar phenotype, was 2-3 times smaller (*c*=0.020

in total) (Rieux et al.). Such a difference may hold to the differences in biology of these two fungi (e.g., number of sexual generations per year, continuous host availability…) and it will be interesting in the future to dissect how differences in pathosystem biology translate in more or less pronounced fitness cost of resistance.

On the opposite, the MDR2 locus was found to be the less affected by negative selection, with the direct average and the maximum likelihood estimations. Our estimates of fitness costs were always lower for MDR2 than for MDR1. It was recently demonstrated that this phenotype colonized vineyards, first in France and later in Germany, after a single or limited number of mutation events that occurred in Champagne (Mernke et al., 2011). On the contrary, the MDR1 phenotype is supposed to have emerged several times in France but also in distant countries such as Chile. However, field surveys show that the observed frequency of MDR2 is greater than MDR1 (+ 10-15%) (Walker et al., 2013a). The lower fitness cost associated to MDR2 may explain its maintenance at high frequencies in the field despite its unique origin.

PARAMETERS ESTIMATION AND PERSPECTIVES

The first fitness cost estimates were produced assuming no gene flow from an external compartment with low resistance frequencies. We saw above that in nature, this hypothesis is likely violated. If such gene flow is suspected to occur, *e.g.*, just before June sampling, it would mean that the observed decay in resistance frequencies is not only attributable to the cost of resistance. As a consequence, the estimates above must be interpreted as upper bounds of the 'real' cost of resistance. For ImiR1, MDR1 and MDR2, these estimates are highly variable between temporal transitions. We even sometimes obtained negative values (data not shown), reflecting that the frequency of resistant strains may even increase during 'winter'. This observation has two consequences. First, some extreme cost values were obtained using frequencies computed on a very small sample size. Some of the variance observed may therefore hold to sampling variance. Because maximum likelihood allows taking into account the variability associated with sampling, it is likely better adapted to the present data than the direct and regression approaches. Second, it is possible that some processes at work during 'winter' have been underestimated. If the observed increases in resistance frequencies during 'winter' cannot be attributed to sampling variance, then we should account for the processes that produce such increase.

As an extension, it is possible to account for migration from an untreated area with low resistance frequency (Figure 2). The model becomes:

$$p_{June} = \frac{\left[(1-m)p_{T,Sept}N_T + mp_{U,Sept}N_U\right](1-c)}{\left[(1-m)p_{T,Sept}N_T + mp_{U,Sept}N_U\right](1-c) + \left[(1-m)(1-p_{T,Sept})N_T + m(1-p_{U,Sept})N_U\right]},$$

where m is migration rate between the local patches and the untreated compartment, N_T and N_U are the effective sizes of local and untreated populations. The equation can be rewritten:

$$p_{June} = \frac{\left[(1-m)p_{T,Sept} + mp_{U,Sept}r\right](1-c)}{\left[(1-m)p_{T,Sept} + mp_{U,Sept}r\right](1-c) + \left[(1-m)(1-p_{T,Sept}) + m(1-p_{U,Sept})r\right]}$$ with *r*

the ratio of effective population size between the two areas. Assuming the resistance frequency is negligible in the untreated area:

$$p_{June} = \frac{(1-m)p_{T,Sept}(1-c)}{(1-m)(1-cp_{T,Sept}) + mr}.$$

This equation shows that migration will indeed decrease the expected frequency in June, all the more so as the migration rate and the ratio in population sizes are important.

Moreover, other parameters than fitness cost could be inferred from further modeling of the summer phase. In addition to specific climatic conditions, 'summer' is, in the present context, characterized by maximal host availability, and by the application of three fungicide treatments in treated fields only. Asexual reproduction occurs during this period, leading to the production of numerous spores and likely provoking exchanges between adjacent treated and untreated areas as well as with other more distant patches (Figures 2 and 3). Formalizing a scenario of evolution of allelic frequencies is possible but requires accounting for asexuality that generates linkage between loci during the 'summer', leading to possible selective sweeps between selected loci and neutral markers. It will also impose to precisely determine the migration regime that takes places, *i.e.* what are the compartments and which exchanges take place between them? What are the resistance frequencies in these compartments? From the data already available, one may suspect that there are 3 compartments in the studied agricultural landscape: treated patch, the adjacent untreated patch and the rest of the world. Besides, migration does not seem affected by distance and might be approximated through an island model. Once formalized, the transition between June and September could be used in a maximum likelihood framework to exploit the whole dataset at once and estimate positive selection and migration rates.

From a practical point of view, the establishment of selection estimates for the various fungicides may lead to better balance and management of fungicide programs, which may, in term,

lead to a better durability of the molecules. Similarly, estimating the intensity and the timing of gene flow between treated, untreated and the other demes is promising if one wish to improve the timing of fungicide applications or to implement efficient prophylactic measures.

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	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value	Fixation indices
September 2005						
Among locations	2	5.3	-0.003	-0.2	0.763	-0.002
Among treatments within locations	3	8.7	0.027	1.4	**0.020**	0.014
Within treatments	203	396.9	1.955	98.8	**0.009**	0.012
Total	208	410.8	1.979			
June 2006						
Among locations	2	12.1	0.030	1.3	0.058	0.013
Among treatments within locations	3	12.2	0.060	2.6	**<0.001**	0.027
Within treatments	187	409.5	2.190	96.1	**<0.001**	0.040
Total	192	433.7	2.280			
September 2006						
Among locations	2	8.0	0.0155	0.8	0.056	0.008
Among treatments within locations	3	7.5	0.012	0.6	0.091	0.006
Within treatments	285	543.0	1.905	98.6	**<0.001**	0.014
Total	290	558.5	1.933			
June 2007						
Among locations	2	7.2	0.079	3.3	0.134	0.032
Among treatments within locations	3	5.8	-0.057	-2.4	0.849	-0.024
Within treatments	100	239.9	2.400	99.1	0.228	0.009
Total	105	253.0	2.420			
September 2005						
Among treatments	1	4.4	0.019	0.9	0.113	0.009
Among locations within treatments	4	9.6	0.012	0.7	0.103	0.007
Within locations	203	396.9	1.955	98.4	**0.008**	0.016
Total	208	410.9				
June 2006						
Among treatments	1	4.4	-0.009	-0.4	0.899	-0.004
Among locations within treatments	4	19.8	0.089	3.9	**<0.001**	0.039
Within locations	187	409.5	2.190	96.5	**<0.001**	0.035

Total	192	433.7	2.270			
September 2006						
Among treatments	1	2.5	-0.005	-0.3	0.800	-0.003
Among locations within treatments	4	13.0	0.028	1.4	**<0.001**	0.014
Within locations	285	543.0	1.905	98.8	**<0.001**	0.011
Total	290	558.5				
June 2007						
Among treatments	1	2.0	-0.025	-1.0	0.693	-0.010
Among locations within treatments	4	11.1	0.023	1.0	0.115	0.009
Within locations	100	239.9	2.400	100	0.275	-0.001
Total	105	253.0	2.397			

Table S1: Molecular analysis of the variance (Amova) with geography (plot localization) and treatment (with or without botryticides) as a grouping factor, for four collection dates

P-values in bold are significant at the 5% confidence level.

NB: low numbers of strains were collected in two treated locations in June 2007 (Table 1), which may explain non-significant results.

K	September 2005		June 2006		September 2006		June 2007	
	|Ln''(K)|	Delta K	|Ln''(K)|	Delta K	|Ln''(K)|	Delta K	|Ln''(K)|	Delta K
1	-	-	-	-	-	-	-	-
2	136.64	2.64	26.28	20.91	17.77	8.27	**94.68**	**115.80**
3	50.05	8.84	53.01	15.58	13.11	6.74	54.74	48.64
4	22.72	2.25	**88.66**	**23.35**	105.85	30.22	75.89	2.13
5	30.75	8.11	2.76	0.05	13.07	3.08	81.68	2.83
6	**474.59**	**107.12**	157.32	4.66	24.27	4.01	63.03	2.74
7	724.45	3.10	129.50	2.75	64.45	10.30	152.32	7.58
8	2273.42	28.90	41.26	0.21	**274.46**	**36.90**	75.03	1.77
9	3605.82	0.66	25.56	0.41	217.89	2.32	54.90	3.87
10	-	-	-	-	-	-	-	-

Table S2: Results of individual-based Bayesian clustering analyses computed from K=1 to K=10 (STRUCTURE).

All analyses were run 10 times. Optimal K values (as determined by the Evanno method (Evanno et al., 2005)) are in bold for each collection date.

	Locus BenR1				Locus ImiR1				Locus MDR1				Locus MDR2			
	Sept. 2005	June 2005	Sept. 2006	June 2007	Sept. 2005	June 2005	Sept. 2006	June 2007	Sept. 2005	June 2005	Sept. 2006	June 2007	Sept. 2005	June 2005	Sept. 2006	June 2007
Courteron																
c	26.988	10.000	23.616	32.719	10.663	16.471	11.514	-	9.876	16.504	21.343	10.000	15.485	24.503	16.152	32.410
b	84.090	0.417	91.680	1.501	99.990	82.778	77.935	-	30.672	68.671	21.792	1.000	60.583	72.055	90.732	93.094
h_1	0.455	0.427	0.571	0.641	0.056	0.814	0.926	-	0.708	0.023	0.163	0.873	0.053	0.000	0.061	0.950
h_p	0.307	1.000	0.247	1.000	0.395	1.000	1.000	-	1.000	0.255	0.415	1.000	0.082	0.041	0.239	1.000
Hautvillers																
c	21.943	9.372	26.917	8.310	25.521	24.513	7.580	23.804	22.969	25.492	21.607	9.691	21.998	23.112	10.763	9.683
b	17.340	99.895	67.852	50.024	84.980	68.402	78.066	99.960	0.484	59.936	0.475	98.324	24.897	41.954	41.797	99.999
h_1	0.627	0.509	0.423	0.480	0.813	0.968	0.047	0.958	0.000	0.074	0.000	0.163	0.020	0.861	0.125	0.102
h_p	0.859	0.373	0.628	0.359	0.749	0.380	0.274	1.000	0.558	0.100	0.601	0.146	0.257	0.680	0.086	0.364
Vandières																
c	20.458	11.320	4.272	11.442	22.224	18.403	3.547	18.482	14.625	19.086	6.121	19.337	20.512	6.458	22.000	4.659
b	91.983	11.144	95.549	24.424	0.236	95.775	69.685	73.966	28.171	20.870	15.624	55.990	75.429	70.257	0.640	99.901
h_1	0.000	0.538	0.725	0.286	0.000	0.000	0.814	0.824	0.653	0.000	0.612	0.600	0.737	0.029	0.237	0.083
h_p	0.272	0.160	0.000	0.067	1.000	0.120	1.000	0.595	0.674	0.167	0.687	0.643	0.863	0.199	0.912	0.377

Table S3: Cline parameters estimated by CFIT when fitting clines for the four resistance loci in three grapevine locations from Champagne.

c: center of the cline; *b*: slope of the cline; h_1: lower asymptotic frequency, h_p determines h_2 (the higher asymptotic frequency) as $h_2 = h_1 + h_p (1-h_1)$.

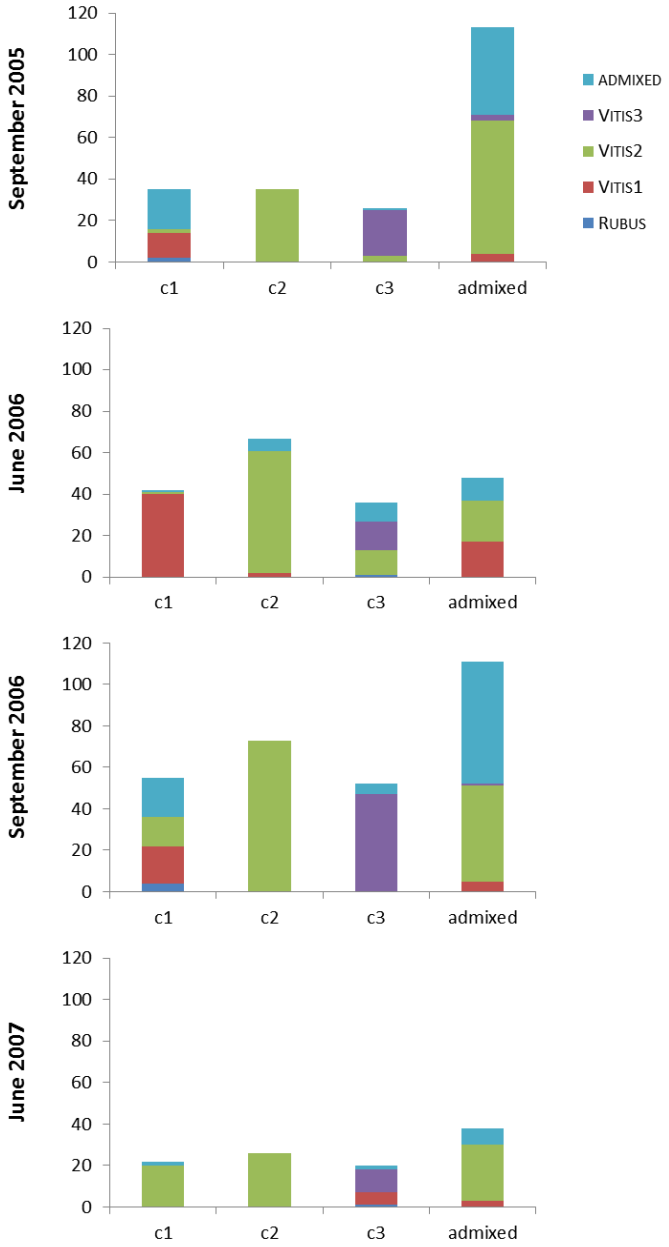

Figure S1: Structure observed in populations from three locations in Champagne

Population structure for the four collection dates was visualized for K=3 clusters (c1, c2 and c3; STRUCTURE analysis) and compared to the structure previously found in a larger dataset including these Champagne locations (Walker et al., 2013b). This previous study showed that grapevine populations were mostly differentiated into three clusters adapted on *Vitis* (named *VITIS1*, *VITIS2* and *VITIS3*) and that some strains, adapted on blackberry and belonging to the *RUBUS* cluster could also be collected on grapevine. This host plant differentiation was found again, at least partially, on this reduced dataset and was independent from the sample site or the disease control strategy. Admixed individuals could not be assigned to any cluster.

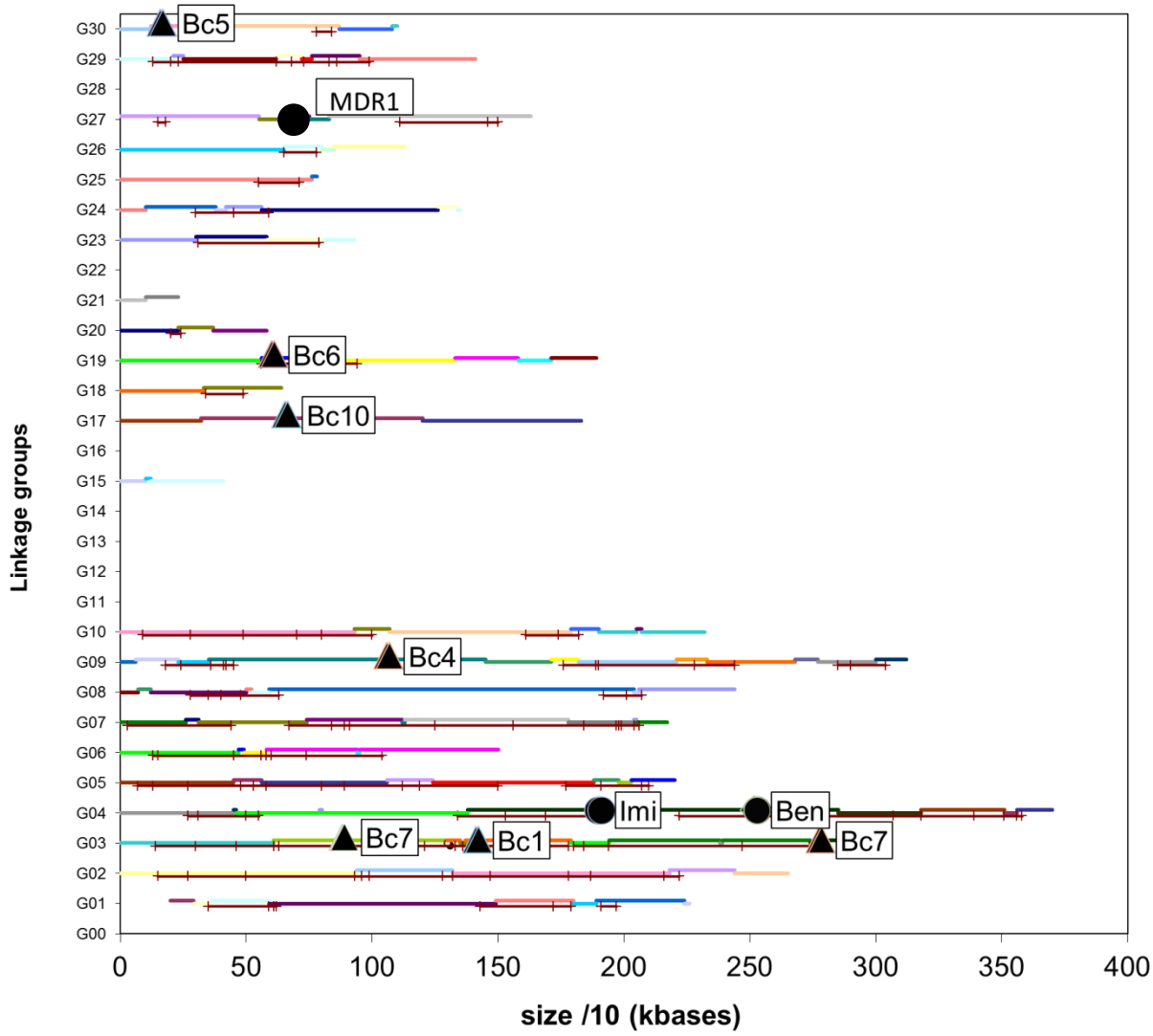

Figure S2: Mapping of neutral and selected markers on genome of *Botrytis cinerea*

Markers used in this study were localized after blast analysis on the contigs assembly from the recently sequenced *B. cinerea* genome (Amselem et al., 2011). Each coloured line represent a genome metacontig.

Conclusions

- Les fongicides ne modifient pas la structure neutre des populations analysées dans cette étude.

- La diversité génotypique des populations traitées et non traitées par des fongicides n'est pas modifiée, de même que le mode de reproduction. Par contre, la richesse allélique et la richesse en allèles privés diminuent dans certaines populations traitées, indiquant un effet plus intense de la dérive, vraisemblablement consécutif aux traitements par les fongicides, qui entraînent des goulots d'étranglement drastiques et réguliers dans ces parcelles.

- Pour deux des quatre loci impliqués dans la résistance aux fongicides étudiés ici, des clines génétiques, résultant de l'équilibre migration-sélection, sont détectés en particulier à l'automne, notamment pour des pressions de sélection contemporaines (phénotypes MDR). Dans le cas de cette étude, ces clines ne permettent pas d'inférer les paramètres de sélection.

- Pour pallier l'utilisation des clines pour estimer la sélection, un modèle en patch a été développé pour modéliser l'évolution de la résistance pendant la période hivernale. Les premiers estimateurs de fitness produits pour quatre phénotypes de résistance sont partiellement cohérents avec les attendus du terrain. Ces estimations pourront être améliorées en tenant compte des flux migratoires intenses entre les patchs.

Perspectives

- Différentes pistes ont déjà été envisagées et discutées avec les co-auteurs de l'étude pour améliorer la modélisation de la phase hivernale afin de prendre en compte les flux migratoires intenses entre les patchs. Faute de temps, ces développements n'ont pas pu être réalisés avant la soumission du manuscrit de thèse, mais seront intégrés à l'étude avant soumission de l'article.

- La phase estivale sera modélisée dès que possible, permettant ainsi d'estimer la sélection positive exercée sur les quatre phénotypes et le taux de migration entre les deux patchs.

- Nous tirerons parti des estimateurs générés par cette étude pour réfléchir à l'optimisation des stratégies de lutte chimique (voir discussion générale).

- Ces données pourraient également être utilisées pour détecter et quantifier les goulots d'étranglement exercés par les fongicides sur les parcelles traitées.

DISCUSSION GENERALE ET CONCLUSIONS

Stellenbosch, Afrique du Sud

DISCUSSION GÉNÉRALE

Cette thèse avait pour objectifs d'identifier les facteurs et les mécanismes qui structurent et diversifient les populations de *B. cinerea*, mais également, connaissant la structure des populations et leur dynamique spatio-temporelle, de comprendre comment ces populations répondent à la sélection par les fongicides et comment les résistances vont évoluer dans les populations. L'objectif de cette dernière section de la thèse est de discuter de la cohérence des différents résultats, des nouvelles pistes de recherche qu'ils peuvent générer et des implications pour la protection des plantes.

1. DE GRANDES POPULATIONS, DIVERSES MAIS STRUCTURÉES

Depuis la fin des années 90, la mesure de la diversité et de la différenciation des populations de *B. cinerea* fait l'objet d'un effort de recherche intense, dans de nombreux pays et sur des hôtes variés (voir revue dans la section 4.2 de l'introduction). Ces recherches se basent au minimum sur les fréquences de deux éléments transposables, conduisant aux génotypes *vacuma* et *transposa*, et aux mieux sur différents types de marqueurs neutres polymorphes. Ces études ont régulièrement conclu que les populations de *B. cinerea* étaient diverses, différenciées par leur plante d'origine ou parfois par la géographie. Ce travail de thèse rejoint globalement ces conclusions, mais il permet également de les nuancer et de constater que la structuration des populations de *B. cinerea* est bien plus complexe, sous l'effet de plusieurs facteurs.

Premièrement, cette diversité observée est en particulier liée à un complexe d'espèces cryptiques, présentes en sympatrie sur les mêmes hôtes. Le succès et la facilité d'usage des marqueurs *vacuma* et *transposa*, démontrés ici comme non pertinents pour distinguer ces deux espèces, a sans doute largement participé à mésestimer la diversité des populations de *B. cinerea* dans deux nombreuses études et à donner une vision imparfaite (parce que positionnant mal la barrière aux flux de gènes) de la structuration en deux espèces cryptiques. La publication de ces résultats, en proposant des outils plus adaptés, devrait permettre un cadre de travail commun aux études de génétique des populations de pourriture grise. Elle ouvre également de nouvelles perspectives quant à la description de l'aire de distribution de la nouvelle espèce *B. pseudocinerea* : pour le moment principalement détectée en France, et dans une moindre mesure dans d'autres pays européens, elle n'a encore jamais été trouvée dans l'hémisphère Sud, ce qui résulte soit d'un manque de prospection, faute d'outils, soit d'une adaptation aux cultures et climats européens, d'une dispersion faible, éventuellement limitée par des barrières physiques, d'une discontinuité de la répartition des hôtes ou d'une histoire de recolonisation post-glaciaire complexe.

Deuxièmement, toutes les populations de *B. cinerea sensu stricto* ne sont pas aussi diverses que décrites dans la littérature. Nous avons mis en évidence l'importante clonalité des populations collectées sous serre, soumises à un mode de reproduction apparemment exclusivement asexué et échangeant des migrants de manière occasionnelle avec les populations extérieures. Le système de culture est dans notre étude le facteur qui différencie le plus fortement les populations. Quelques articles antérieurs ont étudié les populations de *B. cinerea* sous serre uniquement (Alfonso *et al.* 2000 ; Calpas *et al.* 2006 ; Decognet *et al.* 2009), et la comparaison entre des populations « indoor » et « outdoor » n'a, à notre connaissance, été évoquée que dans une seule étude (Karchani-Balma et al. 2008). Notre travail constitue donc la première description précise de la différenciation entre ces deux systèmes de culture (notamment, démonstration des flux de génotypes entre les différents compartiments ; voir également annexe de l'article 2), même s'il semble trivial que des environnements aussi contrastés puissent conduire à des structures de populations différentes. Sous serre, cette clonalité favorise la généralisation d'individus, adaptés aux conditions confinées, et éventuellement porteurs de résistance. La rapidité des épidémies justifie une alternance stricte des modes d'action utilisés. La prophylaxie constitue sans doute la méthode de lutte la plus durable, sur le long terme.

Troisièmement, notre étude montre que plusieurs mécanismes interagissent pour générer l'importante diversité observée dans les populations « outdoor » grâce à différents indices. Nous démontrons ici que la recombinaison, probablement due à l'existence d'une reproduction sexuée, a lieu de manière régulière, probablement pendant l'hiver. Elle joue un rôle important en cassant les déséquilibres de liaison entre loci, maintenant ainsi un pool de génotypes variés sur lequel la sélection naturelle peut opérer. Alors que la reproduction asexuée semble particulièrement efficace (elle suffit aux populations sous serre pour maintenir des épidémies efficaces année après année ; en milieu ouvert, les cycles asexués, particulièrement courts en conditions optimales, suffisent à entretenir et amplifier l'épidémie), la reproduction sexuée est maintenue chez *B. cinerea*, malgré son double coût évolutif (Williams 1975 ; Maynard-Smith 1978). Le cas particulier de *B. cinerea* confirme une fois de plus le rôle moteur de la reproduction sexuée pour la production de génotypes rares, dont certains peuvent être à forte valeur sélective, comme cela a été suggéré pour les parasites (Gandon *et al.* 2007). Selon la théorie de la Reine Rouge (Van Valen 1973), *B. cinerea* est engagé dans une course aux armements avec ses différentes plantes-hôtes. Le champignon peut être lui-même parasité (par des mycoparasites, des transposons). Le maintien d'une recombinaison régulière, *via* la reproduction sexuée, lui procurerait ainsi un double avantage évolutif. Un des paradoxes de l'étude des populations de *B. cinerea* reste l'abondance des preuves indirectes de la reproduction sexuée, comparativement aux observations directes extrêmement rares des organes sexués *in natura* (Beever et al. 2004). Ce hiatus constitue probablement un verrou majeur à la compréhension définitive du cycle de vie de ce pathogène. Des prospections au champ systématiques, à plusieurs périodes de l'année, semblent la seule stratégie efficace, quoique laborieuse, pour lever ce paradoxe. Il

est possible également que la reproduction sexuée ait lieu partiellement sur d'autres hôtes que ceux qui sont cultivés par l'homme et qui sont le plus fréquemment prospectés. En effet, *B. cinerea* est un champignon polyphage, également capable d'infecter des plantes sauvages, omniprésentes dans les paysages cultivés. Ces hôtes alternatifs sont par définition non traités par les fongicides et ne subissent pas de prophylaxie (par exemple, taille des organes porteurs de sclérotes, comme en vigne) et constitueraient de bons supports pour la reproduction sexuée, en complément de celle qui se déroule sur les hôtes cultivés. Enfin, une piste à explorer serait d'estimer l'importance du phénomène de parasexualité *in natura*, qui pourrait également contribuer à l'importante diversité observée et expliquer la rareté des observations d'apothécies (Beever *et al.* 2003 ; Roca *et al.* 2012).

Un autre moteur au maintien de la diversité chez *B. cinerea* semble être la grande taille des populations. Dans une population moins sujette à la dérive génétique, il est plus probable qu'une mutation apparaisse et se maintienne à une fréquence non négligeable, sans être éliminée aléatoirement. Le nombre de mutations générées dans une population est donc dépendant de la taille de celle-ci et du taux de mutation associé à l'allèle considéré. De grandes tailles de population maximisent donc le nombre d'allèles nouveaux introduits dans les populations, la sélection naturelle faisant par la suite le tri entre les allèles délétères et les plus adaptés localement. Par ailleurs, nous avons également observé que lorsque la taille des populations est régulée par l'application de fongicides, un effet plus important de la dérive pouvait être observé *via* la diminution de la richesse allélique et de la richesse allélique privée. Malheureusement, aucune étude n'a produit jusqu'à ce jour une évaluation de la taille efficace des populations (*i.e.* la taille d'une population qui subirait la même intensité de dérive génétique qu'une population « idéale » de Wright-Fisher) de *B. cinerea* en milieu naturel, pour valider cette hypothèse. Ceci pourrait être entrepris ultérieurement à partir des jeux de données fournis par l'article 5. Par ailleurs, l'estimation du taux de mutation pour un locus donné (par exemple, associé à une résistance à un fongicide) n'a également jamais été proposée chez ce pathogène, bien que formalisé mathématiquement dans des modèles (Skylakakis 1982 ; Shaw 1989), et que son importance pour l'évolution de la résistance et sa gestion fasse l'objet de nombreux développements théoriques (Birch *et al.* 1997 ; Shaw 2000 ; Hall *et al.* 2004).

Enfin, la migration semble également jouer un rôle primordial dans le maintien de la diversité au loci neutres. En effet, la migration peut apporter régulièrement de nouveaux allèles dans les populations. Les paramètres de dispersion des spores de cette espèce ne sont pas connus et pourraient faire l'objet d'un projet de recherche en soi. Cependant, nous n'avons pas observé (ou faiblement) de différentiation géographique, aux échelles régionales et inter-régionales, confortant ainsi les observations des auteurs précédents. Ce patron peut s'expliquer par l'action combinée d'une migration opérant sur de longues distances, voire à l'échelle d'un continent (Isenegger *et al.* 2008a), et par des tailles de populations très grandes qui peuvent également masquer tout signal de différenciation. A

l'échelle de la France, nous avons effectivement mesuré des flux de génotypes entre les cinq clusters différenciés par l'hôte d'origine dans son système de culture, démontrant ainsi des flux à plus courte distance. Notre travail montre de manière originale que les flux inter-clusters n'ont pas tous la même intensité, et qu'ils sont asynchrones, conséquence probable de la mise en œuvre de différents filtres sélectifs (voir ci-dessous). En cela nos résultats contredisent les travaux précédents faisant l'hypothèse d'une migration continue et équivalente entre tous les dèmes. Dans le cas de notre étude, la migration participe donc bien au maintien de la diversité neutre au sein des différents dèmes. A l'échelle supérieure, la balance entre migration et sélection maintient ces dèmes de manière stable dans l'espace et dans le temps. Par ailleurs, l'influence de la migration a également été détectée dans notre étude aux loci sélectionnés (résistance aux fongicides) mais plutôt pour son effet homogénéisant, comme précédemment mis en évidence dans d'autres systèmes (Lenormand 2002). Nous avons montré que les fréquences des allèles de résistance aux fongicides varient au cours du temps, la résistance étant observée en fréquence plus faible en hiver ; ceci est probablement le résultat de l'effet conjoint de la sélection négative et de la migration, en provenance de réservoirs non traités (vigne en agriculture biologique, ou plantes sauvages). En conséquence, les clines aux loci sélectionnés (qui résulteraient de l'équilibre entre sélection et migration) ne sont visibles que dans certaines situations où la sélection directionnelle opère de manière suffisamment forte pour contrebalancer la migration pendant la période estivale. Pendant la période hivernale, cette importante migration est probablement une des raisons de la surestimation de nos paramètres de coût de la résistance. Là encore, si ces travaux amènent des preuves indirectes de la migration, il reste à quantifier globalement les flux de gènes entre populations. Cet objectif sera approché prochainement par l'estimation des taux de migration à courte distance entre patchs traités et non traités.

Le dernier moteur de la diversité des populations de *B. cinerea* est clairement la sélection naturelle. D'une part, nos résultats, montrant l'existence d'une barrière incomplète aux flux de gènes entre populations prélevées sur des hôtes différents, sont compatibles avec l'existence d'une adaptation partielle à l'hôte, due à une divergence écologique et/ou temporelle. D'autre part, l'adaptation au système de culture et aux fongicides pourraient résulter d'une sélection directionnelle. Enfin, la spéciation entre *B. cinerea* et *B. pseudocinerea* résulte d'une divergence dont les mécanismes restent à identifier. L'impact de la sélection naturelle sur la diversité des populations de *B. cinerea* fait l'objet du paragraphe suivant de cette discussion.

2. *BOTRYTIS* CINEREA, UN MODÈLE D'ADAPTATION

L'ensemble de ces travaux de thèse met en évidence plusieurs adaptations de *B. cinerea*.

La fréquence des souches résistantes dans les populations résulte clairement de la balance entre la sélection directionnelle positive, qui favorise les mutants résistants dans les zones traitées, pendant la saison de culture, et la sélection directionnelle négative (ou purifiante), qui s'exprime seule et élimine les allèles résistants trop coûteux lorsque les fongicides ne sont plus appliqués (Wright 1969 ; Lande 1983; Carriere *et al.* 1994 ; Otto 2004). De tels coûts adaptatifs ont été mis en évidence pour des insecticides (Roush *et al.* 1987 ; Chevillon *et al.* 1997 ; Coustau *et al.* 2000 ; Bourguet *et al.* 2004), et plus rarement pour des fongicides (Rieux *et al.*). Le contraste entre ces deux forces est particulièrement visible dans le cas des résistances MDR1 et MDR2, sélectionnées spécifiquement par les fongicides utilisés dans les programmes appliqués sur nos sites d'essais, puisque leur fréquence oscille au cours de l'année et qu'elles sont supérieures dans les zones traitées. A l'inverse, la sélection négative homogénise la fréquence des résistances anciennes BenR1 et ImiR1 dans les zones traitées et non traitées, en l'absence de sélection positive contemporaine. Le cas de l'acquisition d'un phénotype MDR est également intéressant. En effet, une résistance aux différents modes d'actions pourrait également être déterminée par une résistance multiple, *i.e.* l'acquisition simultanée de plusieurs allèles déterminants la résistance aux différentes molécules. La MDR est, dans les deux cas étudiés ici, monogénique, ce qui est sans doute plus parcimonieux que la résistance multiple, car évitant l'accumulation d'allèles délétères tout en produisant un phénotype d'intérêt similaire. Pendant longtemps, les programmes de traitement champenois sont restés très monolithiques, utilisant les mêmes modes d'action appliqués aux mêmes stades de culture, d'année en année (en général, fenhexamid à la fin floraison, fludioxonil à la fermeture de la grappe et fluazinam ou pyriméthanil à la véraison). Il est donc probable que cette stratégie, certes d'alternance, mais répétée dans le temps, ait facilité l'adaptation vers les souches MDR, résistantes simultanément à tous ces modes d'action. En ce sens, on s'attend à ce que le coût associé aux allèles MDR soit faible, ce qui semble être le cas, comparativement aux loci BenR1 et ImiR1, dans nos premiers calculs de coût sélectif. Par ailleurs, comme discuté précédemment, le coût sélectif des quatre allèles résistants étudiés a sans doute été surestimé, du fait de la non prise en compte de la migration. Cela semble particulièrement criant pour le locus BenR1, pour lequel nous attendions un faible coût. En effet, à la fin des années 80, lorsque cette résistance fut généralisée en Champagne, le diéthofencarbe (autre classe d'anti-microtubule) fut utilisé en mélange avec le carbendazime (ayant sélectionné les souches BenR1), conduisant à un remplacement d'allèle (Fisher 1928 ; Haldane 1932) permettant d'améliorer la fitness (mutant BenR2) dans ces nouvelles conditions environnementales. Avec l'abandon de ce

mélange, la fréquence du mutant BenR1 est restée stable dans les populations, preuve que ce coût de résistance était faible voire nul, alors que le mutant BenR2 n'est quasiment plus détecté.

De la même manière, l'adaptation de *B. cinerea* aux conditions de culture sous serre semble être la conséquence d'une sélection directionnelle, favorisée par le mode de reproduction asexuée, qui permet de fixer rapidement, grâce à l'absence de recombinaison, un allèle (ou une combinaison d'allèles) adapté(e) à la culture sous serre. Notre étude montre que dans les serres particulièrement bien isolées des populations extérieures (Provence en particulier), un clone devient dominant en une saison dans la population. Des travaux en serre ont montré que ces clones dominants étaient particulièrement bien adaptés à leur environnement, grâce à une agressivité optimisée sur leur hôte et une sporulation accrue, comparativement à des clones présents à plus faibles fréquences (Decognet *et al.* 2009).

Le cas de l'adaptation à la plante-hôte chez *B. cinerea* est plus complexe. Les quatre clusters génétiques mis en évidence en milieu ouvert contiennent majoritairement des individus collectés sur une même plante, mais pas exclusivement. Cette coexistence en sympatrie de populations différenciées sur des hôtes distincts pourrait être le résultat d'une spécialisation à l'hôte, générée par de la sélection disruptive (l'adaptation à l'hôte), et conduisant à une divergence écologique (incomplète) grâce à ce « trait magique » qui suffit à lui seul à limiter le flux de gènes en sympatrie (Gavrilets 2004). Ainsi les individus adaptés à un hôte donné se reproduisent entre eux sur cet hôte, ce qui les isole des populations adaptées aux autres hôtes. Cette spécialisation semble cependant incomplète car nous avons identifié des flux non négligeables de migrants de première génération (« spillover individuals »), parvenus de manière aléatoire sur des hôtes différents de leurs hôtes d'origine. L'intensité de ces flux semble rythmée par les saisons et la mise en œuvre décalée de filtres sélectifs par les hôtes (au printemps pour la vigne et en autonne pour la ronce). La détection d'individus « admixed » montre qu'une partie de ces migrants est capable de se croiser avec les individus adaptés à l'hôte « puits » ; mais ces cas semblent cependant rester rares, comme le montrent les plus faibles intensités (deux à trois fois) des flux d'admixture. La migration entre dèmes, accompagnée de flux de gènes par recombinaison, pourrait expliquer que la divergence entre ces dèmes, observée aux loci neutres, n'est que partielle. Cette spécialisation écologique incomplète s'explique peut-être à cause de l'arsenal biochimique diversifié dont dispose *B. cinerea* (Amselem *et al.* 2011), et qui permet une croissance, même non optimale, sur une grande variété d'hôtes. Dans le cas particulier de *B. cinerea*, le trait d'adaptation à l'hôte ne serait donc pas complètement magique, en ce sens qu'il ne serait pas à lui seul suffisant pour empêcher l'appariement non aléatoire entre individus adaptés à un même hôte, donc pour entrainer un isolement reproducteur complet. Cette hypothèse sera vérifiée par des expérimentations de mesure d'agressivité *in planta*, comme indicateur

de valeur sélective, en croisant les facteurs « cluster d'appartenance des souches » et « hôtes » (hôte d'origine ou non). En effet, selon notre hypothèse d'une adaptation, même incomplète, la différenciation en fonction d'un hôte devrait s'accompagner d'une augmentation de la fitness sur cet hôte (Anderson *et al.* 2004). Enfin, la différenciation de trois clusters génétiques sur le même hôte est également surprenante. Il est possible qu'elle soit dûe au fait que ces clusters répondent à des exigences écologiques différentes, que nous n'avons pas identifiées. En d'autres termes, la divergence écologique pourrait jouer à des échelles extrèmement fines (l'organe, l'état physiologique de la plante). Mais l'isolement temporel peut sans doute également jouer un rôle dans le maintien de ces trois clusters sur la même plante. Ainsi, la différenciation du cluster *VITIS3* semble s'accompagner d'un isolement temporel (reproduction sexuée estivale) et probablement également d'un isolement écologique (état physiologique de l'hôte à cette période). Les causes de l'asynchronie de la période de reproduction dans ce cluster restent à explorer (infection des souches de *B. cinerea* par des mycoparasites dans ce cluster ? Recombinaison par d'autres mécanismes que la reproduction sexuée, par exemple par parasexualité ?).

Enfin, la divergence entre les deux espèces sympatriques *B. cinerea* et *B. pseudocinerea* a eu lieu entre 7 et 18 millions d'années (estimation par une horloge moléculaire). Les causes de cette divergence ne sont pas connues. Dans l'article 1, nous supposons des exigences écologiques distinctes mais extrêment fines entre ces deux espèces (préférences printanières de *B. pseudocinerea* ?), restant à décrire. La niche écologique de *B. pseudocinerea* serait plus rare, ou plus fugace que celle de *B. cinerea,* ce qui expliquerait pourquoi cette espèce est moins abondante et possède une aire de répartition moins étendue (espèce relique ?). En dépit de ces premières observations, nous n'avons actuellement aucun indice étayant le mode de spéciation (sympatrie ? Allopatrie suivi d'une mise en contact secondaire ?). Des travaux de phylogéographie seraient probablement informatifs pour répondre à cette question. Plus généralement, la radiation évolutive au sein du genre *Botrytis* n'est pas complètement caractérisée, car malgré la multiplicité des gènes utilisés pour établir sa phylogénie (Staats *et al.* 2005 ; Walker *et al.* 2011 ; Leroch *et al.* 2013), la topologie du clade contenant les espèces *B. cinerea, B. pseudocinerea, B. pelargonii, B. fabae, B. calthae* ainsi que la nouvelle entité *B. cinerea* groupe *s* (Leroch et al. 2013) reste mal résolue. Le séquençage en cours des génomes de ces différentes espèces (projets J. Van Kan et M. Hahn) devrait permettre de résoudre complètement la phylogénie de ce genre, en offrant l'accès à un plus grand nombre de marqueurs, et permettre *in fine* de renseigner sur l'histoire et le mécanisme de divergence.

Au final, *B. cinerea* est donc une espèce modèle pour étudier la sélection naturelle, offrant de manière pédagogique des scenarios contrastés de divergence. Cette diversité des patrons d'adaptation est permise par les traits de vie de l'espèce, déjà discutés plusieurs fois dans ce travail, mais également par l'équipement de son génome. Son séquençage récent a en effet mis en évidence l'importante diversité de son métabolisme secondaire

(déterminants de pathogénie notamment ; Amselem *et al.* 2011). *Botrytis cinerea* constitue donc un bon modèle pour les études de *genome scan,* beaucoup plus puissantes pour localiser les parties du génome sous sélection et identifier des loci d'intérêt (par exemple, mutations conférant la résistance aux fongicides dont nous ne connaissons pas la cible, comme pour les anilinopyrimidines ou les souches résistantes aux SDHIs non altérées pour la Sdh ou traits d'adaptations aux hôtes).

3. De l'évolution à la protection des plantes : implications en pratique

Ce travail de thèse ouvre également de nouvelles perspectives en matière de lutte contre la pourriture grise, par plusieurs aspects.

Nous avons identifié des flux de génotypes entre les cinq dèmes cohabitant dans les paysages agricoles. Leur intensité mériterait d'être mesurée par d'autres méthodes, mais nos travaux suggèrent que la migration est suffisante pour contrebalancer en partie l'adaptation partielle à l'hôte et l'adaptation locale aux fongicides. La manipulation de l'adaptation par le contrôle des flux de gènes pourrait constituer une méthode simple et compatible avec les exigences des politiques agro-environnementales actuelles, si elle parvient à être maîtrisée *in natura* (Lenormand 2002). Dans les paysages viticoles que nous avons étudiés, une technique de lutte efficace sur une parcelle de vigne devrait donc viser en priorité à limiter suffisamment les flux de gènes en provenance des autres réservoirs (parcelles de vigne, litière, adventices, hôtes sauvages) mais également à maintenir un flux suffisant de migrants sensibles pour diluer les allèles de résistance dans les populations traitées. En pratique, c'est cet équilibre que cherchent à atteindre les viticulteurs lorsqu'ils pratiquent la prophylaxie (désherbage des parcelles et de leurs abords, tailles de sarments sclérotés, destruction des grappes contaminées et de la litière, par le labour par exemple). De plus, en optimisant (par la modélisation, par exemple) la proportion de parcelles de vigne traitées par rapport aux vignes non traitées (agriculture biologique ou biodynamique, usage d'antifongiques naturels ou de synthèse n'évoluant pas de résistance) et leur agencement dans le paysage, il deviendrait possible de maintenir des fréquences faibles de résistance dans les parcelles traitées, à l'instar de ce qui a été prospecté pour la gestion de la résistance aux insecticides ou aux OGM (Lenormand *et al.* 1998b ; Vacher *et al.* 2003). Les estimations des taux de migration d'allèles résistants que nous prévoyons d'établir par le modèle en patch devraient permettre d'affiner ces stratégies. Par ailleurs, nous avons montré que les populations sous serre étaient principalement contaminées par des migrants provenant des populations extérieures, rejoignant ainsi des observations plus anciennes (Kerssies *et al.* 1997 ; Korolev *et al.* 2006 ; Decognet *et al.* 2009). Cela confirme l'intérêt des prophylaxies strictes (filtration de l'air, étanchéité des structures, désinfections, quarantaines, certification du matériel végétal entrant) mises en place dans les serres les mieux équipées,

en amont de l'épidémie, mais également pendant (gestion quotidienne des litières, qui sont généralement laissées au sol pendant la période de culture).

En matière de lutte chimique, le principe maître de la gestion de la résistance est la maximisation de l'hétérogénéité de la pression sélective exercée par les fongicides. Deux types de stratégies sont généralement pratiqués (van den Bosch *et al.* 2008) et leurs efficacités respectives pour limiter l'évolution de la résistance sont largement explorées par la modélisation et des études empiriques (REX Consortium 2013). La stratégie de mélange (entre deux modes d'actions par exemple) consiste à retarder l'apparition ou l'augmentation en fréquence des gènes de résistance, se basant sur le fait que la probabilité qu'un individu soit résistant à deux modes d'action (résistance multiple) est plus faible que pour une résistance simple et que le pathogène est « contrôlé deux fois » en une génération. La stratégie d'alternance consiste à maintenir la fréquence des gènes de résistance à une fréquence stable et acceptable, en optimisant les délais entre les applications d'un même mode d'action, permettant ainsi à la sélection négative d'agir. Dans cas particulier du contrôle de la pourriture grise au vignoble, la seconde stratégie semble plus adaptée, car d'une part, l'activité intrinsèque des fongicides est souvent plus faible chez *B. cinerea* que chez d'autres champignons, et d'autre part, une fréquence importante de souches présentant des résistances multiples est détectée au champ. Ceci explique pourquoi la stratégie d'alternance (impliquant une limitation annuelle des modes d'action) est recommandée au vignoble (Note nationale Vigne 2013). L'estimation des coefficients de sélection exercés sur les allèles de résistance et de leurs valeurs sélectives constituent des indices précieux pour optimiser cette stratégie (*i.e.* optimiser la balance entre sélection positive et coût de la résistance), notamment par la modélisation. En utilisant ces paramètres calculés sur des situations réelles (tenant compte de la situation agronomique, de la biologie du pathogène, du déterminisme de la résistance), on peut effectivement imaginer d'optimiser par la modélisation une stratégie « sur mesure » (optimisant par exemple le ratio d'un mélange ou la fréquence des alternances), et non plus basée sur l'exemple d'un fongicide théorique appliqué à un pathogène théorique. Ceci semble prometteur et peut-être irréaliste mais mérite une exploration *via* les prochains projets de recherche de notre équipe. Ces modélisations pourraient concerner en particulier l'évolution des résistances MDR (cas original d'un trait monogénique qui induit un pattern de résistance multiple) et à la résistance aux SDHIs (qui risque de devenir préoccupante dans les années à venir). Elles pourraient également remettre en question ou affiner l'analyse du risque de résistance (Tableau 2) et l'organisation des observatoires de résistance, tels qu'ils sont mis en pratique actuellement.

CONCLUSIONS

Un article récent conclut, à propos de l'avenir de l'espèce humaine : « *We must recognize that a sustainable future will ultimately require: (i) negative population growth for a number of generations, followed by zero growth ; (ii) a steady-state economy based on sustainable use of renewable energy and material resources; and (iii) new social norms that favour the welfare and the entire global population over that of specific individuals and groups. It is also essential that we recognize that humanity has not yet resolved the genetic or cultural adaptations needed to accomplish these tasks*" (Nekola *et al.* 2013). Cette vision, pessimiste, et notamment la seconde assertion, rappelle cependant toute la valeur contextuelle de ce travail de thèse: celui de l'amélioration des pratiques de lutte contre les maladies des plantes, dans le souci de la durabilité des ressources naturelles.

Une manière d'atteindre cet objectif, d'actualité, pourrait être d'appréhender le problème particulier de la résistance aux fongicides par une approche pluridisciplinaire, et non pas uniquement phytopharmacienne, approche tentée dans cette thèse. Evoluant dans le domaine de la protection des plantes, je constate régulièrement que les recommandations d'emploi des pesticides ont certes évolué, puisqu'elles n'impliquent plus depuis longtemps leur recours systématique, mais qu'elles restent souvent basées, en pratique, sur l'expertise des prescripteurs acquise par l'expérimentation. Or, le contrôle de la maladie tenté par l'homme n'est qu'une contrainte parmi d'autres que subissent les champignons phytopathogènes. A ce titre, la lutte chimique doit être raisonnée globalement, en intégrant le fonctionnement démo-génétique des populations dans leur paysage. Loin de mettre en défaut ces savoir-faire et expertises, il semble donc que la compréhension fine des mécanismes qui conduisent à l'adaptation des champignons phytopathogènes aux fongicides puisse un jour permettre d'adapter en toute connaissance de cause, voire d'améliorer, les pratiques de la protection chimique, pour augmenter leur efficacité et leur durabilité, tant environnementale qu'économique. En tant qu'ingénieur agronome en poste depuis treize ans à l'INRA, et régulièrement amenée par les prescripteurs à donner des avis sur la gestion de la résistance, il m'a semblé opportun de me former aux sciences de l'évolution pour tenter de raisonner les stratégies anti-résistance en apportant un peu plus de théorie à la pratique, en décloisonnant également deux univers, et en apportant de nouvelles compétences à notre équipe de recherche. Le cas particulier de *Botrytis cinerea* au vignoble m'en a donné l'occasion.

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RÉSUMÉ

La sélection naturelle constitue un processus clé de l'adaptation des populations à leur environnement, favorisant les variants présentant les meilleures valeurs sélectives. Les champignons présentent généralement des traits biologiques (diversité des modes de reproduction, grandes tailles de populations, fortes capacités de dispersion, entre autres) qui favorisent leur adaptation à des environnements variés. La compréhension des mécanismes qui sous-tendent l'évolution de leurs populations sous les contraintes, naturelles et anthropiques, qu'elles subissent constitue donc un enjeu majeur pour la protection des plantes, en particulier dans le contexte actuel de durabilité des méthodes de lutte. Dans cette thèse, nous avons décrit la structure et la diversité des populations Botrytis cinerea à l'aide de marqueurs neutres et sélectionnés et d'un échantillonnage emboîté, et avons proposé des mécanismes pouvant expliquer les résultats observés. Puis nous avons analysé la réponse adaptative des populations de B. cinerea en Champagne, aux applications de fongicides. Premièrement, nous avons montré que la pourriture grise était causée par un complexe de deux espèces cryptiques, vivant en sympatrie sur des hôtes communs. De plus, les populations françaises de B. cinerea sont structurées en cinq dèmes, caractérisés par le système de culture (sélection directionnelle), la plante-hôte (adaptation écologique), et dans une moindre mesure, par la géographie. Sur vigne, nous avons mis en évidence une entité dont l'isolement génétique semble lié à un isolement temporel. Par ailleurs, nous avons montré que l'application de fongicides conduit à la sélection de phénotypes résistants spécifiquement à quasiment tous les modes d'action homologués, selon des proportions variant suivant les vignobles et les usages. Plus particulièrement, la résistance aux fongicides inhibiteurs de la succinate déshydrogénase (SDHI) est causée par au moins sept mutations affectant les gènes encodant la protéine cible de ces fongicides, déterminant ainsi une grande variété de phénotypes. Enfin, nous avons montré que les fongicides ne modifiaient pas la structure neutre des populations mais qu'ils pouvaient conduire à une perte de richesse allélique dans les populations traitées ainsi qu'à un équilibre sélection-migration détectable dans certaines situations sous forme de clines au loci sous pression de sélection contemporaine tels que ceux déterminant la résistance multidrogues. La modélisation de l'évolution des fréquences de résistance hivernale a permis d'estimer le coût de la résistance pour quatre loci déterminant la résistance aux fongicides. Cette thèse a permis d'appréhender le fonctionnement des populations de B. cinerea et de comprendre et quantifier partiellement les mécanismes sélectifs opérant in natura. Ces informations seront utilisées pour raisonner des stratégies anti-résistance adaptées localement et durables.

Mots clé : sélection, valeur sélective, adaptation, cline, spéciation, fongicides, résistance, SDHIs, résistance multidrogues, stratégies de lutte, *Botrytis cinerea, Botrytis pseudocinerea*, vigne, ronce, litière, serres.

ABSTRACT

Natural selection is the most powerful force driving population adaptation to their environment, favoring the variants with the best fitness. Fungi generally exhibit biological traits (diversity of reproduction modes, large population sizes, and intense dispersion) that favor their adaptation to changing environments. Therefore, disentangling the mechanisms that explain their evolution under natural and anthropic constraints constitute a major challenge for plant protection, especially in the actual context of agriculture sustainability. In this thesis, we described *Botrytis cinerea* population structure and diversity, using neutral and selected markers and a hierarchical sampling, and proposed mechanisms that may explain these observations. We then analyzed the adaptive answer of this species towards fungicide applications. First, we showed that grey mold populations were caused by a complex of two cryptic species, living sympatrically on the same hosts. Second, *B. cinerea* populations are divided into five demes, according to the cropping system (directional selection), the host-plant (ecological adaptation), and to a lesser extent, by geography. On grapevine, we identified a specific populations exhibiting temporal isolation, as an evidence of extreme exploration of the viticultural conditions. Moreover, fungicide applications select resistance towards all unisite modes of action, with few exceptions, but at varying proportions according to vineyards and fungicide use. More specifically, resistance to succinate dehydrogenase inhibitors (SDHIs) is caused by at least seven mutations altering the target genes of these fungicides, and determines a large variety of phenotypes in the field. At last, we showed that fungicides did not shape population structure but that they could decrease allele richness in treated areas and lead to migration-selection equilibrium, detectable in some situation and for loci under contemporary selective pressures as clines. Modeling the evolution of resistance during winter allowed estimating fitness cost of four loci involved in contemporary fungicide resistance, such as multidrug resistance. As a conclusion, this thesis helped to understand how *B. cinerea* populations evolve and to detect and quantify selective mechanisms at work *in natura*. This information will be useful to deign sustainable and locally-adapted anti-resistance strategies.

Keywords: selection fitness, adaptation, cline, speciation, fungicides, resistance, SDHIs, multidrug resistance, control strategies, *Botrytis cinerea, Botrytis pseudocinerea*, grapevine, bramble, litter, greenhouses.