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Chloé Godeau

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**THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE
FRANCHE-COMTE**

Préparée au Laboratoire Chrono-environnement – UMR 6249 CNRS

Ecole doctorale n°554

Environnements – Santé

Doctorat « Médecine, santé publique, environnement et société »

Par

Mme. Godeau Chloé

**Evaluation of the impact of the dissemination of
triazole fungicides in agriculture on *Aspergillus
fumigatus* and proposal of solutions**

Thèse présentée et soutenue à Besançon le 30 novembre 2022

Composition du jury :

Dr. Martin-Laurent Fabrice	INRAE Bourgogne-Franche-Comté	Président
Pr. Botterel-Chartier Françoise	Université de Paris-Est Créteil	Rapportrice
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Dr. Rousseau Jolanta	UCCS Artois UMR 8181	Examinatrice
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Dr. Crini Nadia	Université de Bourgogne-Franche-Comté	Directrice
Dr. Rocchi Steffi	Université de Bourgogne-Franche-Comté	Co-directrice

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Liste des publications et communications

Publications dans des revues scientifiques internationales

Godeau, C., Morin-Crini, N., Fenyvesi, E., Haghghi, F., Fourmentin, M., Millon, L., Ramseyer, C., Landy, D., Crini, G., Rocchi, S. (2021). Reducing the unintentional impact of triazole fungicides on *Aspergillus fumigatus*: Are cyclodextrins a solution? *Journal of Environmental Science and Public Health* **5**, 114–136. (IF = 3,6)

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Godeau, C., Rocchi, S., Morin-Crini, N. Pollution environnementale causée par les fongicides triazolés, impact et remédiation. *Journée Des Doctorants*, 18/01/2021, Besançon, France. Communication orale.

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Liste des abréviations

ADN : acide désoxyribonucléique

AI : aspergillose invasive

Anses : Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail

aR-Af : *Aspergillus fumigatus* résistant aux azolés

CD : cyclodextrine

CDC : *Center for Disease Control and prevention*

CEC : capacité d'échange cationique

CHU : centre hospitalier universitaire

CMI : concentration minimale inhibitrice

DIMEB : Heptakis(2,6-di-O-méthyl)- β -cyclodextrine

DG18 : dichloran-glycérol

DMI : inhibiteur de la déméthylation

DOSY : *diffusion ordered spectroscopy*

ECDC : *European Center for Disease prevention and Control*

EFSA : *European Food Safety Authority*

EUCAST : *European Committee on Antimicrobial Susceptibility Testing*

FRAC : *Fungicide Resistance Action Committee*

GC-MS-MS : chromatographie gazeuse triple quadripôle

GUS : indice d'ubiquité dans l'eau souterraine de Gustafson

HP- β -CD : 2-hydroxypropyl- β -cyclodextrine

ICP-AES : *inductively coupled plasma-atomic emission spectroscopy*

Mitra : malt-itraconazole

Mvori : malt-voriconazole

MO : matière organique

M€ : million d'euros

ONU : Organisation des Nations Unies

PCR : *polymerase chain reaction*

qPCR : *quantitative- polymerase chain reaction*

RMN : spectroscopie de résonance magnétique nucléaire

RPMI : milieu de culture *Roswell Park Memorial Institute*

STRAf : *short tandem repeat for A. fumigatus*

TB : tuberculose

TRIMEB : heptakis-(2,3,6-tri-O-méthyl)- β -cyclodextrine

USGS : *United States geological survey*

Résumé

L'impact des activités anthropiques sur les services écosystémiques est avéré. Les études tournées vers l'environnement dans des approches pluridisciplinaires sont nécessaires pour mieux comprendre et limiter cet impact. L'une des problématiques majeures est le développement de la résistance aux antimicrobiens, dont un des exemples est la résistance d'*Aspergillus fumigatus* aux triazolés. Cette espèce de moisissure ubiquiste est un pathogène opportuniste pour l'Homme et les animaux. Les alternatives thérapeutiques pour combattre cette moisissure sont minces et sont essentiellement basées sur une famille de molécules, les triazolés. Des mécanismes de résistance à ces traitements sont décrits sur tous les continents et sont liés, en partie, à l'utilisation, dans l'environnement, de molécules triazolées pour combattre les moisissures phytopathogènes, dont les structures chimiques sont très proches des triazolés à usage médical. Cet impact, qualifié de non intentionnel, est une source d'émergence et de diffusion de souches fongiques résistantes. Ce travail de recherche vise à comparer différents domaines d'application des molécules triazolées, allant des champs de cultures céréalières aux exploitations maraîchères et aux parterres de plantes ornementales, afin de mieux comprendre les conditions d'émergence et/ou de diffusion de cette résistance. Cette approche multi-échelle a permis de mettre en évidence un gradient de résistance en fonction des domaines étudiés. Ainsi, alors que les sols des cultures céréalières ne présentent aucun isolat résistant, 71% des isolats retrouvés dans les plantes ornementales entourant le centre hospitalier de Besançon se sont avérés résistants aux triazolés, classifiant les bulbes de ces plantes traitées aux triazolés comme des « hotspots » potentiels de résistance. La transition vers l'utilisation de plantes à bulbes issues de l'agriculture biologique est nécessaire pour éviter l'exposition de populations à risque autour de l'hôpital notamment. Dans d'autres types d'agriculture où ces fongicides garantissent de bons rendements de production, des approches innovantes doivent être développées pour limiter l'émergence de ce problème de santé publique. Les cyclodextrines, molécules cages issues de la dégradation de l'amidon, semblent être une piste intéressante.

Mots clés : triazolés, *Aspergillus fumigatus*, pollution environnementale, résistance aux antifongiques, cyclodextrine, agriculture

Abstract

The negative impact of human activities on the ecosystem is significant. Environmentally oriented studies in multidisciplinary approaches are needed to better understand and limit this impact. One of the major issues is the development of antimicrobial resistance, of which one example is the resistance of *Aspergillus fumigatus* to triazoles. This ubiquitous mould species is an opportunistic pathogen for humans and animals. The therapeutic alternatives to combat this fungus are few and essentially based on triazoles. However, mechanisms of resistance to these treatments have been described worldwide and are partly linked to the excessive use of triazole molecules in the environment to fight against phytopathogenic moulds, whose chemical structures are very close to those of triazoles in medical use. This impact, described as unintended, is a source of emergence and spread of resistant fungal strains. The aim of this research was to compare different areas of application of triazole molecules, ranging from cereal crop fields to vegetable farms and ornamental plant beds, in order to better understand the conditions in which this resistance develops. This multiscale approach enabled us to highlight a gradient of resistance according to the areas studied. Thus, while the soils of cereal crops did not present any resistant isolates, 71% of the isolates found in the ornamental plants surrounding Besancon hospital centre proved to be resistant to triazoles, classifying the bulbs of these triazole-treated plants as potential resistance “hotspot”. Therefore, the transition to the use of organically grown bulbous plants is necessary to avoid exposure of populations at risk, especially around the hospital. While in certain agricultural types where these fungicides guarantee good production yields, innovative approaches must be developed to limit the emergence of this public health problem. Cyclodextrins, cage molecules resulting from the degradation of starch, seem to be an interesting avenue.

Keywords: triazoles, *Aspergillus fumigatus*, environmental pollution, antifungal resistance, cyclodextrin, agriculture

1. Introduction générale

La pression écologique causée par l'explosion démographique est sans précédent. Nourrir la population mondiale devient un défi de santé publique. La sécurité alimentaire était d'ailleurs l'un des premiers objectifs mondiaux du programme de développement durable de 2015 des Nations Unies. Mais en 2020, plus de 45 millions d'enfants de moins de 5 ans souffraient encore de malnutrition dans le monde.

Les prédictions de l'ONU montrent que la population mondiale sera multipliée par 3 en moins de 100 ans, entre 1960 et 2050, atteignant ainsi les 9 milliards d'individus. Parallèlement, selon l'Union des Industries de la Protection des Plantes (<https://www.uipp.org/>), la surface agricole pourrait passer, sur la même période, de 4300 m² à 1900 m² par individu, principalement dû à la transformation des zones agricoles en zones artificialisées au profit des activités humaines. Les pressions anthropiques qui s'appliquent sur l'agriculture sont donc amenées à augmenter puisque, pour subvenir au besoin global, les productions de denrées alimentaires devraient être augmentées de 70%. A défaut d'un changement dans nos pratiques de consommation, les pertes induites par des bioagresseurs, estimées aujourd'hui à plus de 30% de la production mondiale, devront diminuer.

Sur les cultures céréalières, les bioagresseurs majeurs sont les ravageurs (*e.g.* oiseaux, insectes, rongeurs, acariens), les adventices ou bien encore les maladies, principalement causées par des moisissures. Afin de combattre ces bioagresseurs, de tout temps les hommes ont utilisé des procédés chimiques et développé des composés pesticides. En parallèle, la mécanisation et entre autres les nouveaux procédés d'application des pesticides par pulvérisation, ont permis de grandement faciliter l'usage de ces substances. Depuis les années 1960, l'usage des pesticides a été multiplié par 20, tandis que la production alimentaire mondiale a doublé (Oerke, 2006). L'usage des pesticides a été déterminant dans le développement agricole du siècle dernier. Ils ont notamment joué un rôle important dans la mise en place des productions dites « intensives ». Au sein de ces substances biocides, on retrouve les herbicides contre les adventices, les fongicides agissant contre les moisissures phytopathogènes et pour finir les insecticides utilisés contre les ravageurs. En France, les herbicides et les fongicides sont les deux catégories de pesticides les plus largement utilisées (Ministère de l'agriculture, 2018.)

Les fongicides représentent la sous-catégorie de pesticides qui nous intéresse particulièrement dans ce travail. D'abord utilisés sous l'état de soufre ou de sels de cuivre fin du XIX^{ème} et début du XX^{ème} siècle, d'autres composés de synthèse sont apparus dans les années 1950, avec par

exemple les dithiocarbamates et les phtalimides. L'arrivée sur le marché de composés de la famille chimique des azolés dans les années 1950 a représenté une réelle avancée dans la lutte contre les moisissures phytopathogènes.

Reconnus comme d'intérêt majeur pour la limite des pertes agricoles, et représentant un enjeu économique important, les azolés sont aujourd'hui considérés comme indispensables à l'indépendance de production céréalière de certains pays, notamment la France (Price *et al.*, 2015). En revanche, l'usage massif de ces molécules au cours des dernières décennies est source d'impacts négatifs majeurs, associant pollution environnementale importante et effets sur des organismes non cibles.

Cette problématique actuelle s'intensifiera dans le futur et consistera à allier augmentation de la production nécessaire à la population, réduction drastique des surfaces agricoles et changement climatique. Les facteurs agricoles (*e.g.* l'expansion des zones agricoles sur des écosystèmes naturels et l'intensification des méthodes de production : irrigation, utilisation d'engrais et biocides) sont déjà étroitement liés à l'émergence des maladies infectieuses (Foley *et al.*, 2011 ; Rohr *et al.*, 2019) et aux phénomènes de résistance aux antimicrobiens (Azevedo *et al.*, 2015 ; Ter Kuile *et al.*, 2016 ; Dalhoff, 2018). Le changement climatique entraîne d'une part un stress accru sur les plantes et les animaux, les rendant plus sensibles aux infections fongiques (American Academy of Microbiology, 2019), et il réduit d'autre part de plus en plus la zone d'exclusion thermique qui protège normalement les animaux (notamment mammifères) des champignons. A cela s'ajoute une modification de la diffusion des pesticides dans l'environnement et donc de la contamination des écosystèmes (Figure 1).

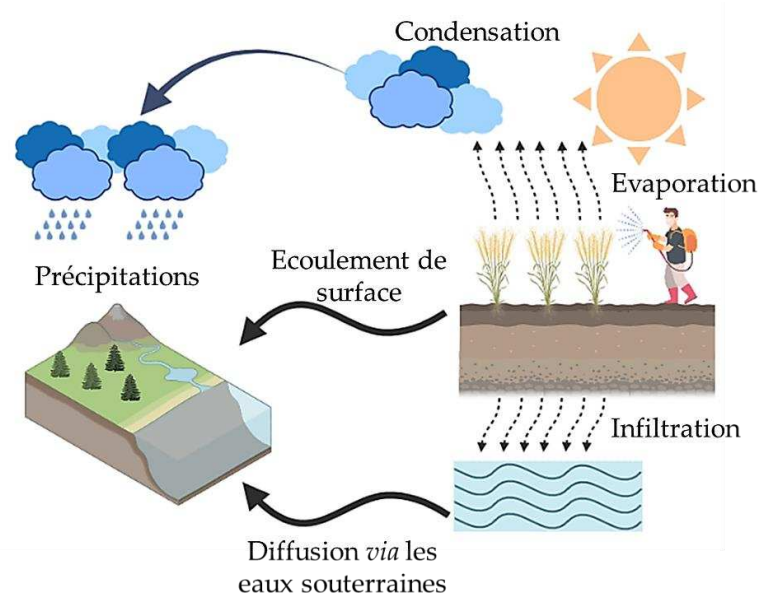


Figure 1 : Diffusion des pesticides dans l'environnement suite aux traitements des cultures.

Ces molécules non désirables sont présentes dans différents compartiments environnementaux. En premier lieu, les sols, qui sont largement exposés de par les techniques modernes de pulvérisation des fongicides sur les cultures. Puis les eaux de surface et les eaux souterraines qui sont impactées par des mécanismes de ruissellement ou d'infiltration. Enfin l'air qui se voit contaminé par des phénomènes de volatilisation. Les pesticides peuvent persister et être transportés *via* ces différents compartiments.

Depuis le début des années 2000, le concept « One health » (une seule santé) est largement mis en avant et souligne l'existence de liens étroits entre les santés humaines, animales et l'état des écosystèmes dans lesquels les êtres vivants évoluent. Ce concept encourage les recherches transdisciplinaires qui permettent de répondre aux problématiques liées à l'émergence de problèmes de santé publique. L'usage des triazolés dans l'environnement et leurs impacts sur *Aspergillus fumigatus*, une espèce pathogène opportuniste pour l'Homme et les animaux, est décrit comme un problème de santé publique émergent, ayant pour cause différents mécanismes de résistance (Macedo *et al.*, 2021). Mieux comprendre la diffusion de ces fongicides dans l'environnement et les conditions de développement de ces résistances est un enjeu important afin d'en limiter les conséquences.

2. Synthèse bibliographique

2.1. Fongicides (tri)azolés

2.1.1. Historique de l'utilisation des (tri)azolés en agriculture

Les fongicides azolés sont des composés organiques hétérocycliques constitués de 5 atomes avec au moins un atome d'azote. Cette famille chimique regroupe différentes sous-familles dont les triazolés qui se caractérisent par la présence de 3 atomes d'azote et de 2 atomes de carbone sur leur cycle insaturé (Figure 2).

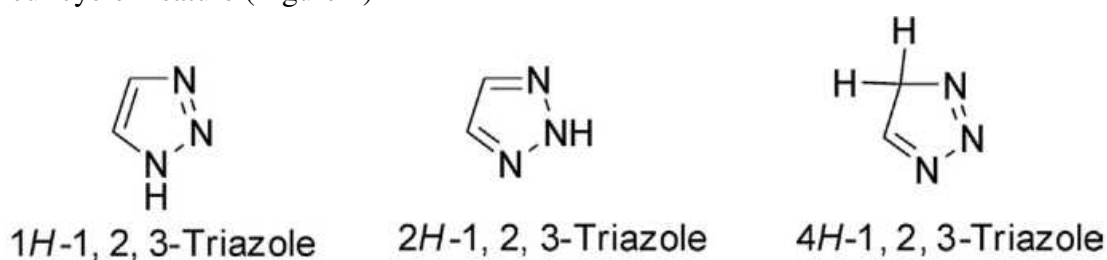


Figure 2 : Les cycles triazolés (Ji Ram et al., 2019)

Le groupe Bayer a été le premier à développer des produits chimiques à base de molécules triazolées pour combattre les moisissures phytopathogènes en 1973 (Schwinn, 1984). De nombreuses autres formulations ont par la suite été synthétisées à base de tébuconazole en 1986, de difénoconazole en 1988 ou bien encore de propiconazole en 2004 (Russell, 2005). Près de 30 molécules triazolées sont actuellement autorisées pour l'usage environnemental (Barber *et al.*, 2020). Elles sont exploitées seules ou en mélange dans des centaines de formulations (Tableau 1).

Ces molécules sont particulièrement appréciées pour leur large spectre d'action qui permet de cibler un grand nombre de moisissures phytopathogènes (Hof, 2001 ; Price *et al.*, 2015). Elles sont ainsi utilisées en préventif et en curatif sur de nombreux types de culture (Šelešovská *et al.*, 2021 ; Ayres *et al.*, 2022).

Tableau 1 : Historique de mise sur le marché des molécules triazolées majeures dans la lutte contre les moisissures phytopathogènes et nombre de formulations commerciales associées à chacune d'entre elles. D'après Russell, 2005 ; Jørgensen & Heick, 2021 ; E-phy, Anses

Année de mise sur le marché	Nom du composé	Usages principaux	Nombre de formulations (selon l'Anses)
1969	Triadimefon	céréales, pois, raisins, cucurbitacées, cannes à sucre	19

1973	<i>Triadimenol</i>	céréales, betteraves, choux, raisins	39
1979	<i>Propiconazole</i>	céréale, maïs, riz, cacahuètes, amandes, sorgho, avoine, noix de pécan, abricots, prunes, pruneaux, pêches, nectarines, préservation du bois	115
	<i>Bitertanol</i>	pommes, bananes, céréales, cacahuètes, plantes ornementales, haricots, soja, préservation du bois	13
1983	<i>Flutriafol</i>	céréales, maïs, soja, pommes	30
	<i>Diniconazole</i>	cacahuètes, raisins, céréales, plantes ornementales	11
	<i>Penconazole</i>	raisins, pommes, poires, pêches, prunes, abricots, fraises, plantes ornementales, houblon, concombres, tomates	17
1986	<i>Hexaconazole</i>	raisins, pommes, poires, bananes, légumes, céréales à petits grains, préservation du bois	27
	<i>Cyproconazole</i>	céréales, légumes, pois, haricots, asperges, colza, betteraves, pommes, pêches, amandes	76
	<i>Myclobutanil</i>	cultures pérennes et annuelles, gazon, plantes ornementales, arbres fruitiers, raisins	60
	<i>Tebuconazole</i>	céréales, raisins, cacahuètes, oignons, pois, poivrons, bananes, cannes à sucre, préservation du bois	145
1988	<i>Difenoconazole</i>	carottes, asperges, choux, pommes de terre, céréales, maïs, coton, colza	93
	<i>Tetraconazole</i>	betteraves, blé, raisins, pommes	31
	<i>Fenbuconazole</i>	céréales, vignes, fruits, bananes, noix de pécan, riz	28
1990	<i>Epoxicoanazole</i>	céréales, betteraves, maïs	106
1991	<i>Bromuconazole</i>	céréales, fruits, légumes, vignes, gazon, plantes ornementales	17
1992	<i>Metconazole</i>	céréales, colza, fruits, myrtille, cerises, groseilles, nectarines, pêches, prunes, pistachier, gazon	62
	<i>Fluquinconazole</i>	céréales, traitements des semis	14
	<i>Triticonazole</i>	céréales, gazon, plantes ornementales	17
2002	<i>Prothioconazole</i>	céréales, colza	124
2011	<i>Paclobutrazole</i>	régulateur de croissance des plantes, mangues, coton, maïs, piments, raisins	8
2019	<i>Mefentrifluconazole</i>	céréales, gazon	17

Malgré l'intérêt de ces molécules pour éviter les pertes de rendement et leur impact économique important, de nombreuses études ont souligné le risque de pollution environnementale qu'elles pouvaient engendrer (cf. partie 2.2). Ces constats ont conduit à l'interdiction de l'utilisation de plusieurs d'entre elles, remplacées récemment grâce au développement de nouveaux dérivés de structure chimique très proches des molécules existantes (Bryson *et al.*, 2018). Ainsi des substances très largement utilisées dans l'environnement se sont vu retirer leur droit de mise

sur le marché, à l'image du propiconazole et de l'époxiconazole qui sont interdits depuis 2019 (Anses).

2.1.2. Mode d'action des fongicides (tri)azolés

Les fongicides (tri)azolés font partie de la famille des inhibiteurs de la déméthylation (DMI). Ils rentrent par diffusion facilitée dans la cellule fongique (Mansfield *et al.*, 2010 ; Esquivel *et al.*, 2015) et agissent ensuite sur une enzyme clé impliquée dans la biosynthèse d'un des composés majeurs de la membrane des champignons: l'ergostérol (Figure 3).

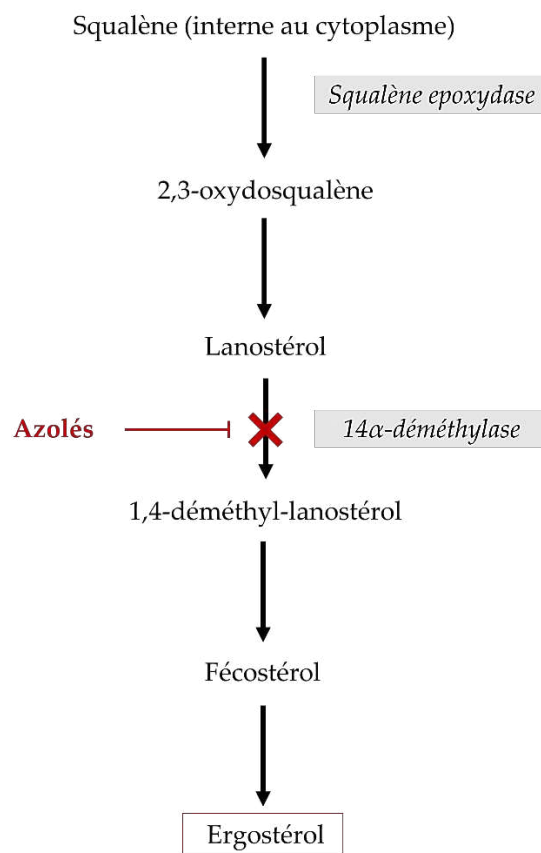


Figure 3 : Voie de synthèse de l'ergostérol et action des antifongiques (tri)azolés.

Les triazolés agissent sur l'enzyme 14 α -déméthylase. Elle est impliquée dans une étape clé de la biosynthèse des stérols des membranes biologiques et fait partie de la superfamille des cytochromes P450 (Lepesheva & Waterman, 2007).

En ciblant spécifiquement cette enzyme, les triazolés bloquent toute la voie des stérols, induisant un dérèglement membranaire qui provoquera un effet fongistatique sur les cellules. (Figure 4). Les cellules fongiques sont ainsi bloquées dans leur développement.

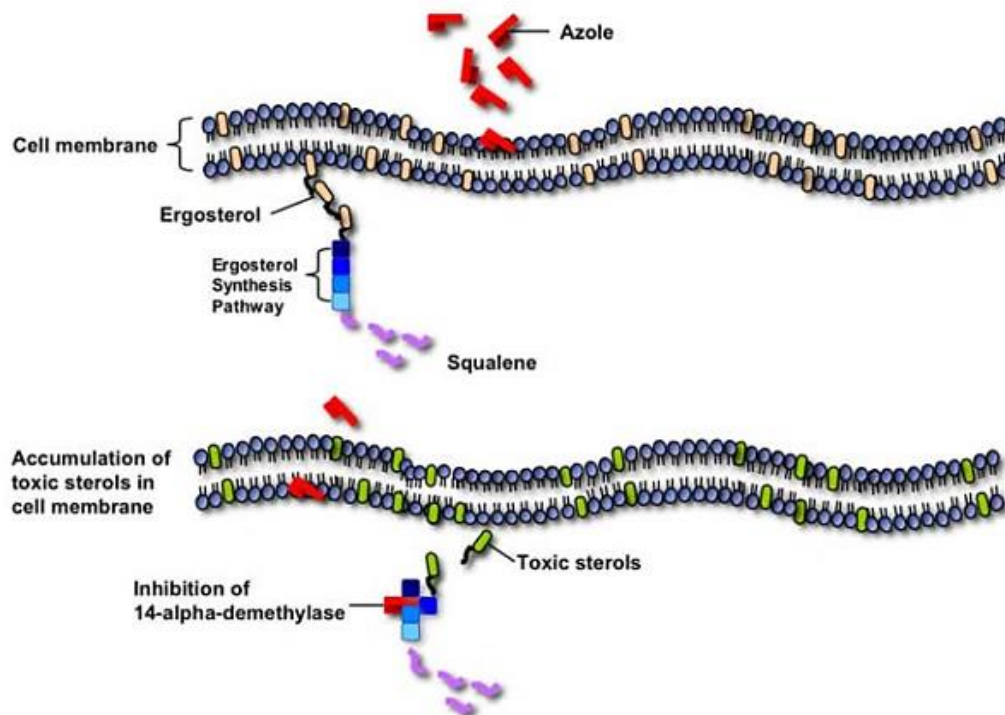


Figure 4 : Action des molécules azolées sur la membrane fongique. Une accumulation de stérols toxiques dans la membrane induit un effet fongistatique (Mycoses Study Group Education and Research Consortium, 2014).

Utilisés spécifiquement pour leur action contre les moisissures, ces traitements sont susceptibles, dans certains cas, de provoquer des effets non-intentionnels. Leur mode d'action les rend capables d'agir sur des organismes non ciblés en bloquant par exemple la synthèse de composés métabolisés par des cytochromes. Ces impacts peuvent toucher d'autres cellules eucaryotes de mammifères (Yang *et al.*, 2018). Les triazolés, le tébuconazole notamment, sont également décrits comme inhibiteurs de certains cytochromes hépatiques humains (Habenschus *et al.*, 2019 ; Knebel *et al.*, 2019). Malgré le risque potentiel d'utilisation de ces composés (*cf.* partie 2.4 et 2.5) leur usage environnemental est largement répandu dans le monde.

2.1.3. Utilisation environnementale des molécules (tri)azolées

Les molécules (tri)azolées, bien que controversées dans leur usage ces dernières années, restent considérées comme d'intérêt majeur dans la lutte contre les moisissures phytopathogènes. Introduites dans les années 70 comme des molécules révolutionnaires capables de remplacer les fongicides organiques au mercure ou au cuivre utilisés depuis plus de 200 ans (Eastmond & Balakrishnan, 2010 ; Gupta, 2017), elles ont rapidement été autorisées sur tous les continents. Leur usage environnemental global n'a fait qu'augmenter depuis les années 1990 (Zhang, 2018). Les (tri)azolés ont en effet la spécificité d'être efficaces sur un très grand nombre de

moisissures. Leur large spectre d'action et leur facilité d'usage en ont donc fait des molécules très utilisées encore de nos jours. Ces substances représentent à elles seules une très grande part de marché des fongicides utilisés sur les cultures. En combattant les agents responsables de la fusariose, de la septoriose ou encore de la rouille (*e.g. Fusarium spp, Microdochium spp, Septoria spp, Puccinia recondita, Drechslera teres*) ces molécules ont pu permettre de limiter les impacts de ces maladies sur les rendements quantitatifs et qualitatifs des cultures. Leur flexibilité d'usage les rend particulièrement avantageuses : ces molécules peuvent être pulvérisées sur les cultures, ou bien directement appliquées sur les graines avant semis ce qui limite le risque d'exposition professionnelle au moment du traitement (Montfort *et al.*, 1996 ; Rothrock *et al.*, 2012 ; Meena *et al.*, 2021).

La quantité moyenne de fongicides appliqués dans les pays d'Europe est de 1,26 kg.ha⁻¹ pour une année. Les molécules (tri)azolées y sont particulièrement représentées notamment avec le prothioconazole et le tébuconazole. Les pratiques varient selon l'activité agricole, avec des quantités appliquées qui peuvent aller de 0,07 à 5,59 kg.ha⁻¹ au sein des différents pays européens et un nombre d'applications variable. En France, jusqu'à 4 traitements à base de triazolés peuvent être appliqués par saison, notamment sur les cultures de fruits ou de légumes (Jørgensen *et al.*, 2008 ; Hayes *et al.*, 2016 ; Lamichhane *et al.*, 2016 ; Abolghasemi *et al.*, 2018).

Les continents les plus consommateurs de ces molécules restent l'Asie et l'Europe, qui se partagent deux tiers des ventes mondiales de produits à base d'azolés (Jørgensen & Heick, 2021). Une étude de l'European Crop Protection parue en 2016 sur les différentes pratiques agricoles européennes montre que ces molécules sont utilisées sur un grand nombre de cultures pour chaque pays (Figure 5, Kim *et al.* 2016; European Crop Protection). Elles sont particulièrement représentées dans le traitement des céréales, principalement le blé, l'orge et le maïs, cultures qui à elles seules, en France, représentent une surface d'exploitation de près de 10 millions d'hectares (Ricroch *et al.*, 2016 ; Rabbat *et al.*, 2022).

Concernant leur utilisation en Asie, entre les années 2013 et 2016, la Chine aurait consommé plus de 80 000 tonnes de fongicides (tri)azolés. Sur la même période, la consommation de tébuconazole a doublé, faisant de cette molécule l'une des plus utilisées du pays pour l'usage agricole (Chen *et al.*, 2020). En plus du tébuconazole, le difénoconazole fait également partie des 20 principaux pesticides autorisés en Chine (Zhang, 2018).

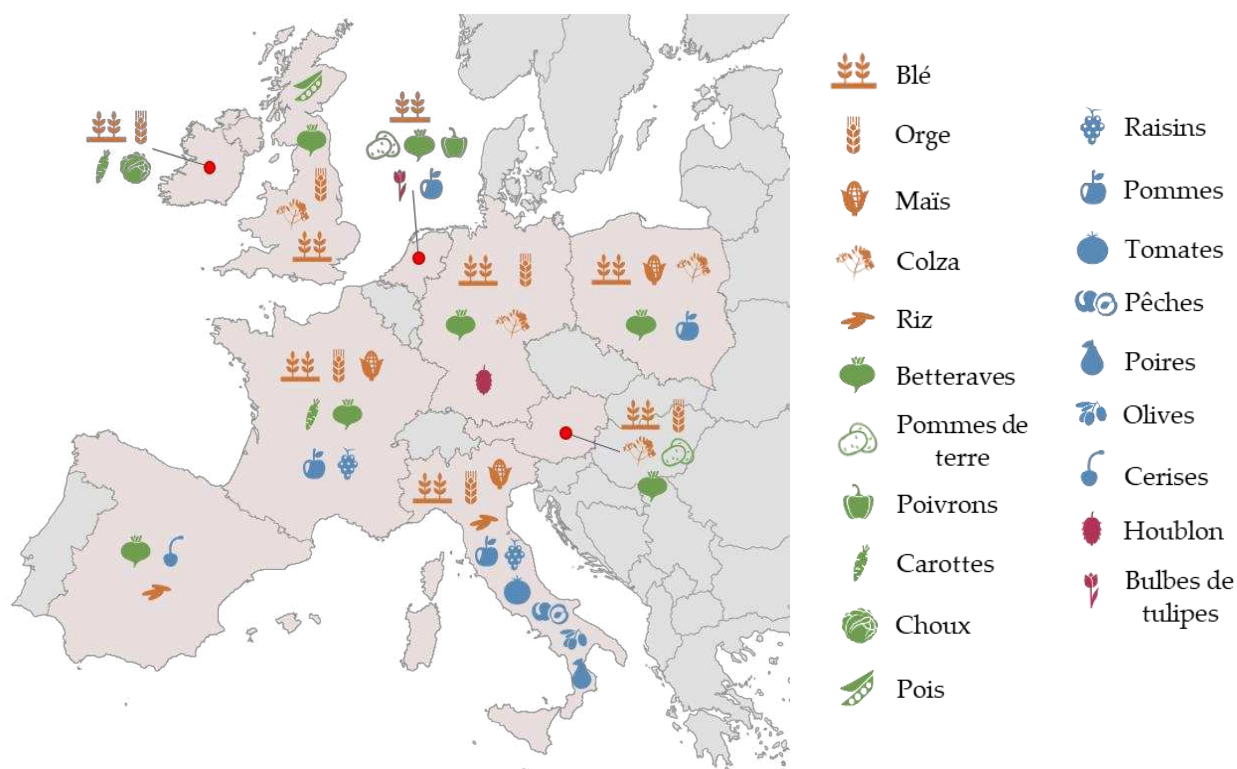


Figure 5: Principaux types de cultures traitées avec des molécules triazolées dans les pays d'Europe. D'après les données de l'European Crop Protection (2016)

Les Etats-Unis, quant à eux, ont également largement augmenté leur consommation de molécules triazolées ces dernières années. En dix ans, la consommation de certaines molécules a été multipliée par 4, surtout en raison de leur utilisation sur des cultures telles que le maïs, le blé ou encore le soja (Toda *et al.*, 2021 ; Figure 6). Le tébuconazole est d'ailleurs l'une des molécules fongicides la plus largement utilisée en Géorgie du sud, où son application varie entre 7 à 8 fois par saison (Glinski *et al.*, 2018).

Avec le mode d'agriculture actuel, l'arrêt complet de l'utilisation des molécules azolées sur les cultures pourrait conduire à de grandes pertes en termes de rendement et donc en termes financiers. Les pertes estimées seraient de 5%, 6,8% et 8,7% pour le Danemark, l'Angleterre et la France respectivement. Pour chacun de ces pays, la perte économique associée estimée serait donc de 34 M€, 157 M€ et enfin 408 M€ par an respectivement (Blake *et al.*, 2011).

Ces chiffres témoignent de l'intérêt de ces molécules dans l'agriculture, notamment pour les pays d'Europe, qui, dans le cas d'un arrêt de leur utilisation pourraient ne plus être en mesure d'assurer une production suffisante à leur indépendance (Price *et al.*, 2015).

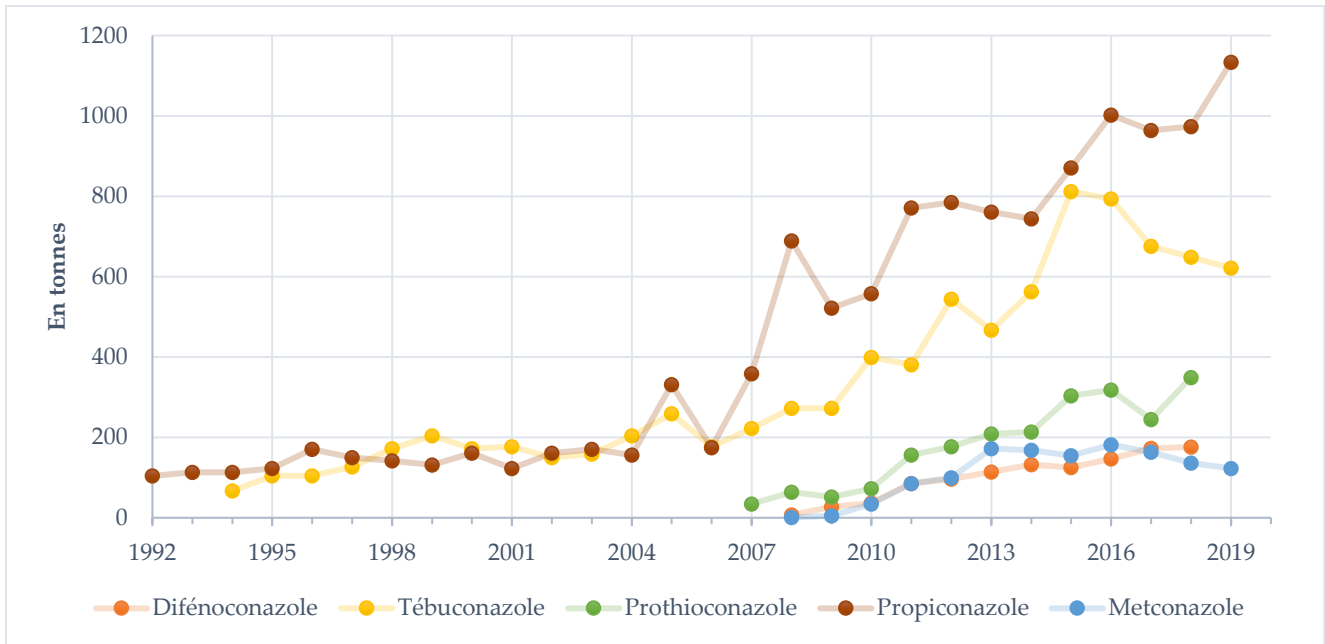


Figure 6 : Augmentation de l'usage estimé de 5 molécules triazolées dans le domaine agricole aux Etats-Unis entre 1992 et 2019. D'après les valeurs de l'U.S.G.S.

L'usage de ces molécules ne se limite pas aux cultures céréalières, maraîchères ou viticoles elles sont également largement répandues dans le traitement des plantes ornementales, du bois ou bien encore des gazons sur les terrains de sport (Jeanvoine *et al.*, 2017 ; Godeau *et al.*, 2020 ; Fisher *et al.*, 2021). Leurs nombreux domaines d'application les rendent omniprésentes dans l'environnement.

2.2. Risque de contamination environnementale

2.2.1. Contamination atmosphérique aux (tri)azolés

La pollution atmosphérique par les pesticides trouve son origine principale dans les pratiques agricoles (Lichiheb *et al.*, 2015). L'air est exposé aux risques de contamination par les triazolés notamment quand les fongicides sont pulvérisés, ce qui en fait un vecteur important de la diffusion des résidus de ces molécules. Les études menées sur la qualité de l'air sont souvent ciblées sur des pesticides organochlorés et organophosphorés (Coscollà *et al.*, 2013 ; Estellano *et al.*, 2015 ; Zivan *et al.*, 2016) du fait de leur toxicité. Néanmoins, les triazolés font également l'objet d'études, principalement du fait de leur persistance (Socorro *et al.*, 2016). Mattei *et al.* (2018) ont par exemple montré que la demi-vie par ozonolyse du difénoconazole et du tétraconazole était supérieure à 800 jours. Des recherches ont mis en évidence la présence de tébuconazole et de penconazole dans l'atmosphère en Espagne à raison de 32 pg.m^{-3} et 3,54 pg.m^{-3} respectivement (Coscollà *et al.*, 2014).

En France, la contamination se fait principalement dans le domaine rural. Une étude réalisée par Désert *et al.* (2018) sur 6 ans en Provence-Alpes-Côte-d'Azur et en Corse, a révélé que le tébuconazole était la deuxième molécule la plus fréquemment mesurée sur 45 substances considérées à risque de pollution atmosphérique. Le difénoconazole quant à lui faisait partie des molécules retrouvées dans les concentrations les plus importantes avec 10% des échantillons présentant des concentrations supérieures à 1 ng.m^{-3} (Figure 7). Bien que les molécules triazolées ne soient pas celles retrouvées dans les quantités les plus importantes dans l'air, cette pollution est soulignée dans différentes études à travers le monde (Pivato *et al.*, 2015 ; Schummer *et al.*, 2010 ; Villiot *et al.*, 2018 ; Climent *et al.*, 2019a).

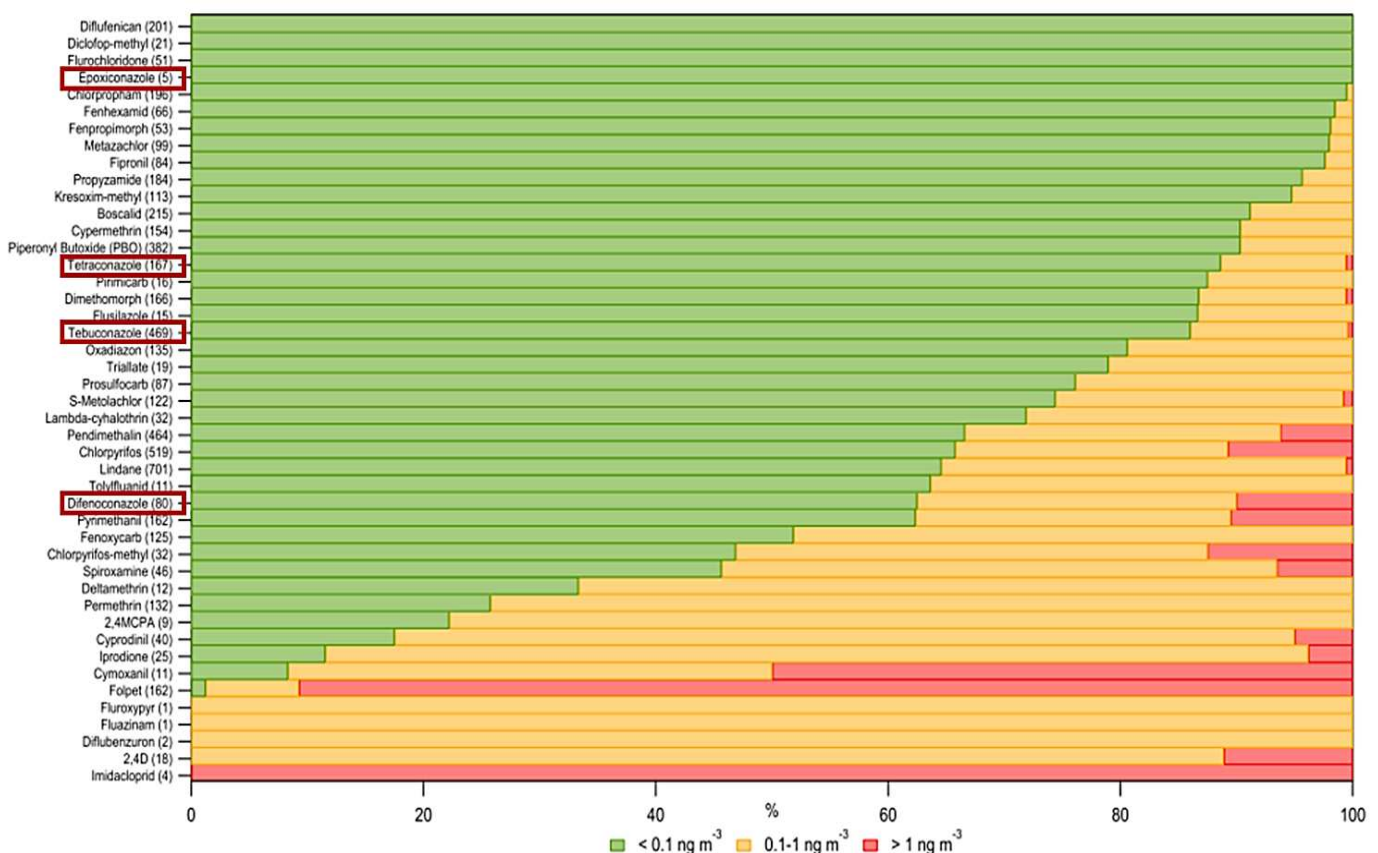


Figure 7 : Distribution des différentes substances actives retrouvées dans l'atmosphère avec des concentrations inférieures à 1 ng.m^{-3} , comprises entre $0,1$ et 1 ng.m^{-3} et supérieures à 1 ng.m^{-3} . Les nombres entre parenthèses correspondent aux nombres totaux d'échantillons dans lesquels la substance active concernée a été retrouvée. Encadrées en rouge et de haut en bas, les molécules triazolées époxiconazole, tétraconazole, tébuconazole et difénoconazole (Désert *et al.*, 2018).

Du fait du risque important de voir ces molécules se diffuser aussi facilement dans l'air, à l'échelle locale et globale, le contrôle de la qualité atmosphérique représente un enjeu important (Aktar *et al.*, 2009 ; Kim *et al.*, 2016).

2.2.2. Contamination de l'environnement aquatique aux (tri)azolés

La présence de résidus de pesticides dans l'eau de surface ou dans les eaux souterraines est particulièrement problématique. Elle fait d'ailleurs l'objet d'un suivi développé en lien avec la Directive-Cadre Européenne sur l'eau. Cette pollution met en péril la santé publique en nuisant à l'approvisionnement des populations en eau potable de qualité (Mekonen *et al.*, 2016 ; Pazikowska-Sapota *et al.*, 2020). Les principales causes de contamination des eaux par les pesticides sont le ruissellement, la lixiviation, le drainage de surface ou bien encore la dérive lors de la pulvérisation (Mojiri *et al.*, 2020).

Les triazolés font partie des molécules fongicides les plus fréquemment retrouvées dans les eaux de surface (de Souza *et al.*, 2020 ; Tableau 2). Des études menées sur les continents les plus consommateurs révèlent leur présence dans de nombreuses rivières et de nombreux lacs.

Tableau 2 : Synthèse non exhaustive des études montrant une contamination des eaux par les molécules triazolées.

Molécule	Concentration (ng.l ⁻¹)	Pays	Références
Difénoconazole	807	Grèce	Papadakis <i>et al.</i> , 2018
Tetraconazole	47	Etats-Unis	Battaglin <i>et al.</i> , 2011
Epoconazole	55	Danemark	McKnight <i>et al.</i> , 2015
	5,14	Tunisie	Necibi <i>et al.</i> , 2021
Myclobutanil	51	Chili	Climent, Herrero-Hernández, <i>et al.</i> , 2019
	63	Grèce	Kapsi <i>et al.</i> , 2019
	230	Etats-Unis	Glinski <i>et al.</i> , 2018
Metconazole	12	Canada	Metcalfe <i>et al.</i> , 2019
	6	Etats-Unis	Battaglin <i>et al.</i> , 2011
	72	Argentine	De Gerónimo <i>et al.</i> , 2014
Propiconazole	178,3	Belgique	Van De Steene <i>et al.</i> , 2010
	82	Danemark	McKnight <i>et al.</i> , 2015
	0,60	Portugal	Gonzalez-Rey <i>et al.</i> , 2015
	307	Grèce	Papadakis <i>et al.</i> , 2018
	810,38	Chine	Peng <i>et al.</i> , 2018
	16	Canada	Metcalfe <i>et al.</i> , 2019
Tébuconazole	0,83	Tunisie	Necibi <i>et al.</i> , 2021
	35	Argentine	De Gerónimo <i>et al.</i> , 2014
	38	Danemark	McKnight <i>et al.</i> , 2015
	15,38	Espagne	Ccancapa <i>et al.</i> , 2016
	300	Brésil	Caldas <i>et al.</i> , 2019
	480	Etats-Unis	Glinski <i>et al.</i> , 2018
	53	Etats-Unis	Battaglin <i>et al.</i> , 2011

	133,43	Chine	Peng <i>et al.</i> , 2018
	50,04	Chine	Xu <i>et al.</i> , 2019
	19	Canada	Metcalfe <i>et al.</i> , 2019
	67	Chili	Climent <i>et al.</i> , 2019b
	9,83	Tunisie	Necibi <i>et al.</i> , 2021

La persistance des triazolés dans l'environnement les rend particulièrement sujets au lessivage, ce qui peut toucher également les eaux souterraines qui sont une source essentielle dans la production d'eau potable (Aliste *et al.*, 2021). Chau *et al.* (2015) ont montré, suite à une étude réalisée au Vietnam, que plus d'un tiers des bouteilles d'eau analysées contenaient des résidus d'hexaconazole présentant un risque pour la santé humaine.

2.2.3. Contamination des sols aux (tri)azolés

Le sol est un compartiment directement exposé au risque de pollution par les triazolés, principalement quand ils sont appliqués par pulvérisation. Non seulement il constitue le plus grand réservoir de pesticides environnementaux, mais il représente également un vecteur de diffusion vers les autres compartiments, notamment l'eau (Al-Wabel *et al.*, 2016).

Certaines études démontrent la présence des molécules triazolées dans les sols au Brésil ou bien en Jordanie par exemple (Fernandes *et al.*, 2020 ; Kailani *et al.*, 2021). Le difénoconazole et le tébuconazole ont aussi été mis en évidence par Pan *et al.* (2019) comme étant parmi les molécules les plus fréquemment retrouvées dans les sols agricoles du nord de la Chine (Figure 8). Mais la plupart des données proviennent d'études européennes. Hvězdová *et al.* (2018) ont par exemple montré que plus de 70% des sols de République Tchèque qu'ils ont étudiés contenaient des triazolés. L'époxiconazole, le tébuconazole, le flusilazole et le propiconazole étaient les molécules les plus retrouvées avec 48%, 36%, 23% et 13% des sols contaminés respectivement. Une étude menée par Silva *et al.* (2019) sur 317 sols d'Europe a montré que l'époxiconazole et le tébuconazole faisaient partie des 7 composés les plus fréquemment retrouvés dans les sols sur les 76 molécules dont les résidus ont été dosés, avec respectivement les 3^e et 7^e position (Figure 9 A). Les résultats observés pour certains pays d'Europe sont présentés (Figure 9 B).

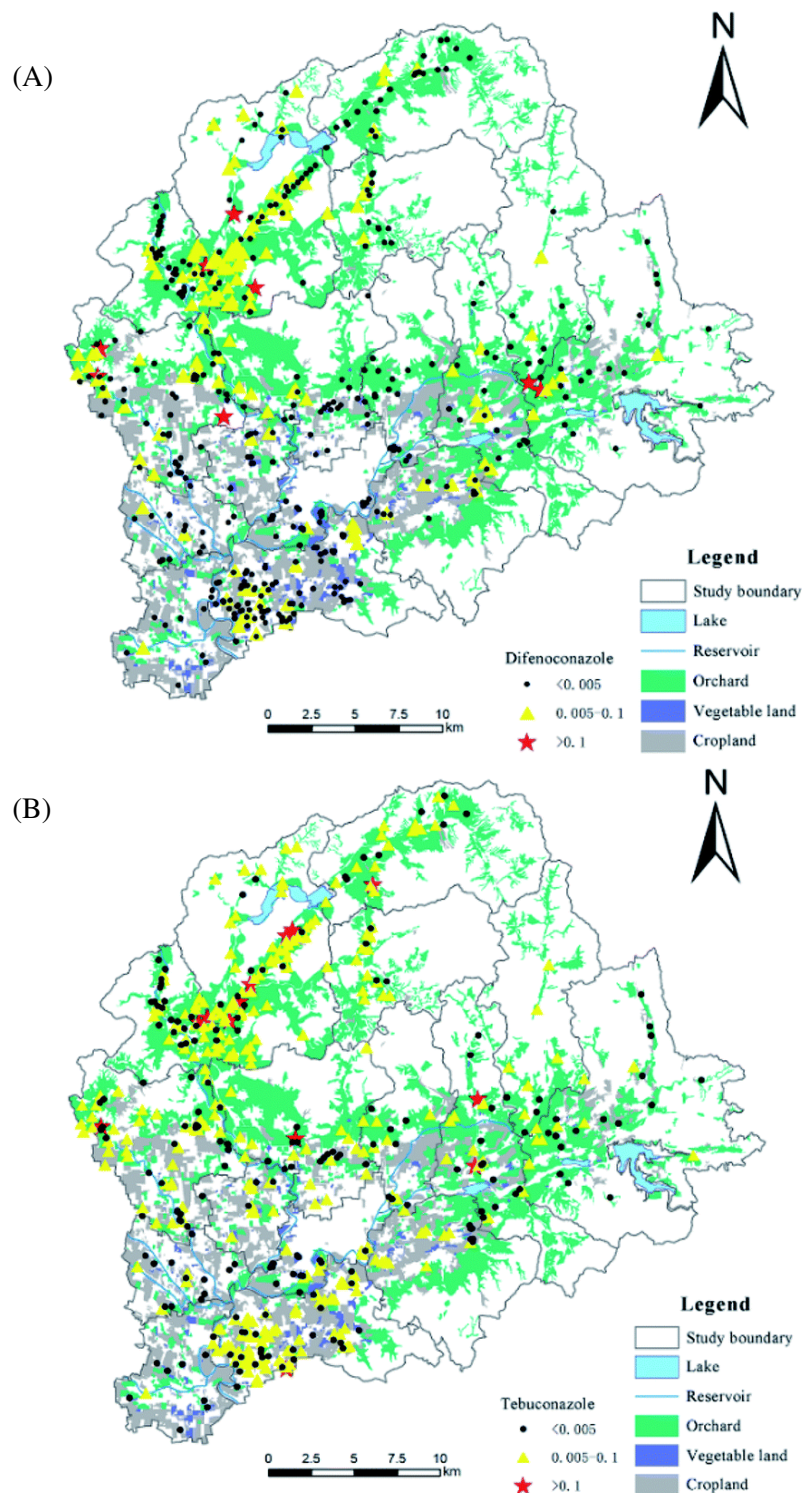
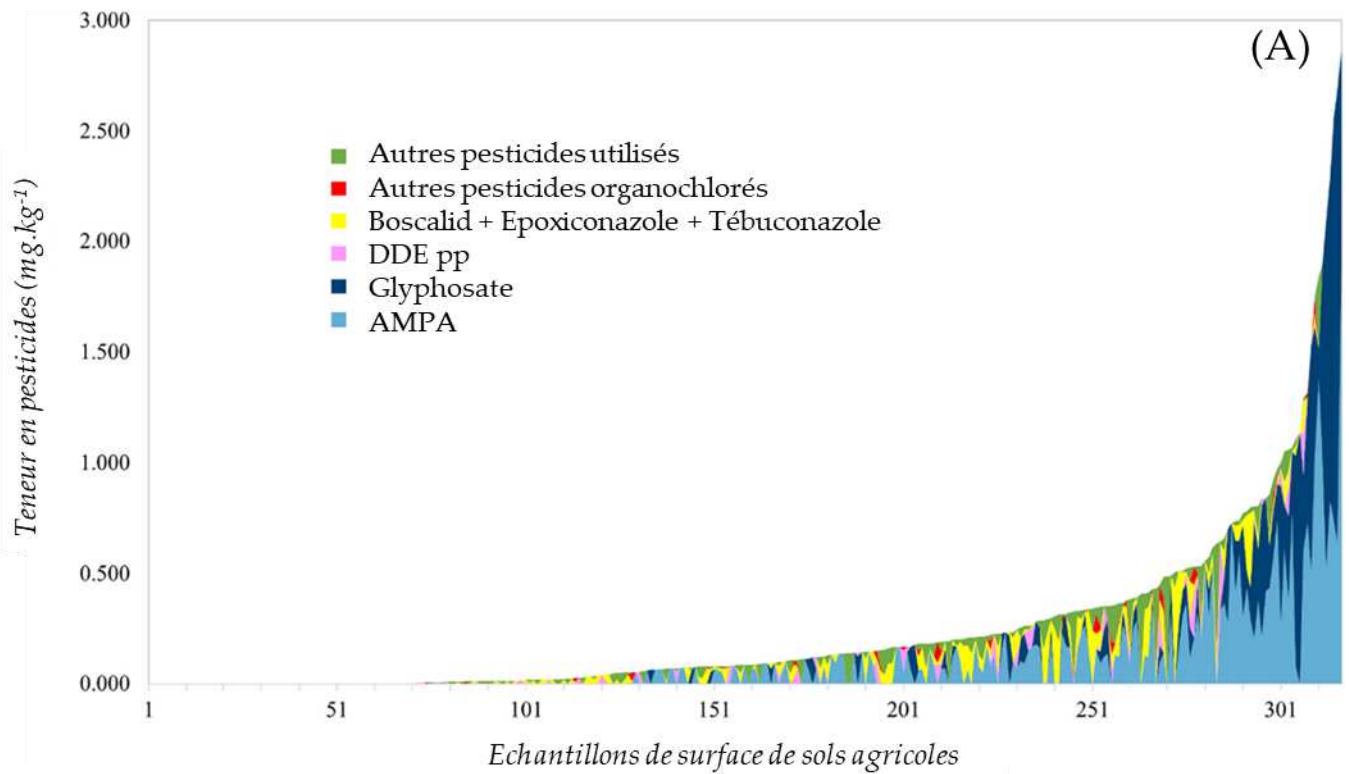


Figure 8 : Distribution des résidus de difénoconazole (A) et de tébuconazole (B) dans des sols agricoles de Chine en mg.kg⁻¹ (Pan et al., 2019).

Les triazolés sont fréquemment retrouvés dans les sols en mélange avec d'autres familles de pesticides, notamment les carboxamides comme le boscalid, ou bien les organophosphorés comme le glyphosate. Plusieurs molécules triazolées peuvent également être retrouvées ensemble dans le même prélèvement de sol : c'est souvent le cas de l'époxiconazole et du tébuconazole.



Pays	Nombre de sols contaminés aux triazolés	Molécule la plus souvent retrouvée
Pologne	20/30	Epoxiconazole
Royaume-Uni	19/30	Epoxiconazole
Pays-Bas	13/30	Epoxiconazole
Espagne	13/30	Epoxiconazole
Allemagne	12/30	Epoxiconazole
Hongrie	9/30	Tébuconazole
Danemark	7/30	Epoxiconazole
Italie	5/30	Prothioconazole
France	5/30	Epoxiconazole/Tébuconazole

(B)

Figure 9 : (A) Evaluation de la contamination en pesticides de 317 prélèvements de sols de surface (0-15 cm) dans l'Union Européenne. (B) Focus sur les molécules triazolées les plus fréquemment retrouvées dans les 270 prélèvements de sol de 9 pays d'Europe. D'après Silva et al., 2019.

L'effet de ces « cocktails » de fongicides associés ou non avec d'autres pesticides est mal connu même si leur coexistence influence probablement leur persistance ou bien encore leurs effets sur les organismes vivants par exemple (Clasen *et al.*, 2018 ; Rossi *et al.*, 2020 ; Jakl *et al.*, 2021).

2.3. Paramètres abiotiques des sols et contaminations aux triazolés

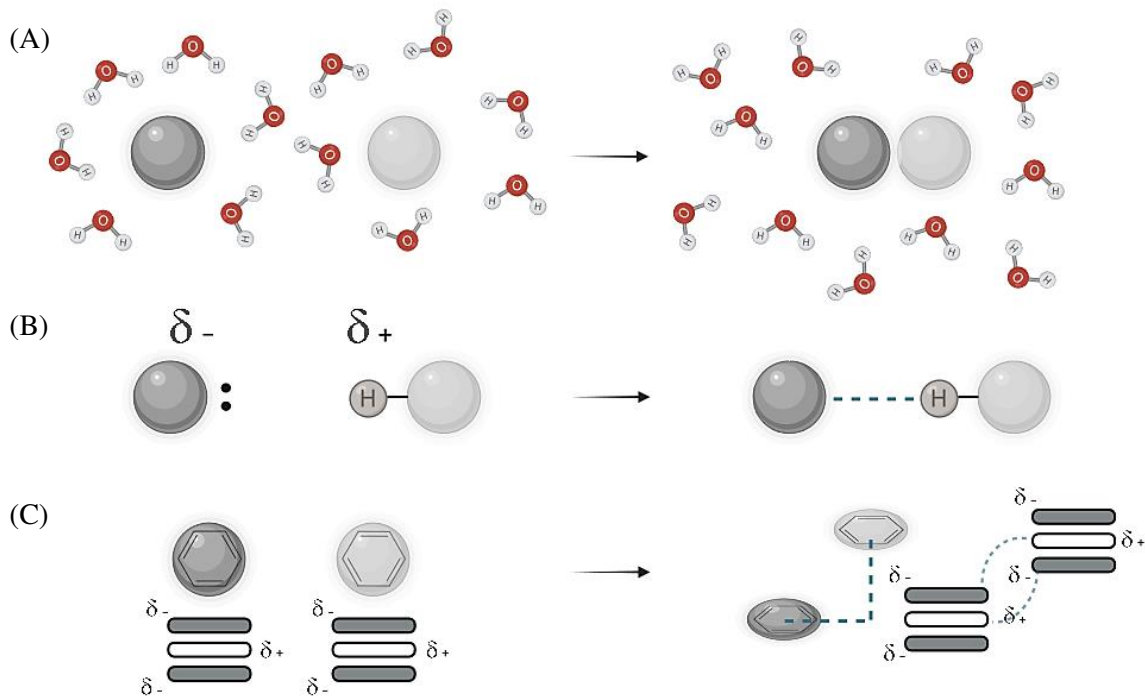
Différents paramètres peuvent influencer sur la dissipation, le devenir et la persistance des pesticides dont les (tri)azolés dans les sols. Les facteurs environnementaux globaux tel que le climat par exemple peuvent conditionner la vaporisation ou bien le lessivage des fongicides (tri)azolés dans les sols. Les propriétés physicochimiques des sols ainsi que les caractéristiques propres aux (tri)azolés ou au sol vont aussi jouer un rôle.

2.3.1. Lien avec la matière organique des sols

La matière organique (MO) du sol joue un rôle important dans de nombreux processus et fonctions du sol. Dans les sols agricoles notamment, la MO est considérée comme un élément indispensable à une production alimentaire durable (Johnston *et al.*, 2009 ; Schjønning *et al.*, 2018). Elle impacte entre autres la structuration des sols, constitue un réservoir d'éléments nutritifs indispensables aux plantes et à d'autres organismes présents dans le sol et permet d'y augmenter la réserve utile en eau (Diacono & Montemurro, 2015 ; Obour *et al.*, 2018 ; Wood *et al.*, 2018).

La MO est également décrite comme un des principaux facteurs qui affecte la persistance des pesticides dans les sols. Quand le taux de matière organique est supérieur à 5%, elle est considérée comme site d'adsorption préférentiel des pesticides (Đurović-Pejčev *et al.*, 2009 ; Čadková *et al.*, 2013).

Concernant les triazolés, Conde-Cid *et al.* (2019) ont montré que la MO augmentait les capacités d'adsorption du propiconazole dans les sols. Ces résultats ont également été observés par Gondar *et al.* (2013) qui ont souligné l'importance des groupes carboxyliques présents dans la MO pour l'adsorption du penconazole. Des tests d'adsorption *in vitro* sur cette même molécule dans des sols amendés en MO ont montré que ce paramètre jouait un rôle dominant dans l'adsorption du penconazole dans les sols (Aït Hammi *et al.*, 2019). Sánchez-Martín *et al.* (2000) ont décrit l'importance de la MO dans l'adsorption des triazolés, soulignant le risque d'accumulation en surface des sols. Siek *et al.* (2021) ont caractérisé cette adsorption : le tébuconazole interagit avec la MO par la formation d'interactions hydrophobe, de liaisons hydrogènes et d'interactions π - π (Figure 10).



(A) : attraction mutuelle de 2 molécules ayant peu d'affinité pour l'eau par des forces de van der Waals (forces de dispersion, forces de London...)

(B) : liaison intermoléculaire entre un atome d'hydrogène lié de façon covalente à un atome très électronégatif et un atome électronégatif porteur d'un doublet non liant

(C) : interactions non covalentes entre des cycles aromatiques contenant des liaisons π

Figure 10 : Représentations schématiques de (A) l'interaction hydrophobe (B) la liaison hydrogène et (C) les interactions π - π . Réalisé avec Biorender

La teneur en MO du sol est décrite comme étant le paramètre le plus important qui permet d'expliquer les variations de sorption des molécules triazolées. Les argiles semblent être également impliqués, mais dans une moindre mesure (Singh, 2002 ; Badawi *et al.*, 2016).

2.3.2. Structure du sol et pH

La structure du sol (et notamment la présence des argiles) est décrite comme un des facteurs conditionnant l'adsorption des pesticides.

Copaja & Gatica, (2021) ont montré qu'un amendement du sol en argile permettait d'augmenter la rétention de deux pesticides organophosphorés, le clorpiryfos et le diazinon. La quantité d'argile présente dans le sol conditionne en effet la formation de complexes argilo-humiques avec la matière organique. Ces complexes, riches en charges négatives, vont déterminer la capacité d'échange cationique (CEC) du sol en attirant des éléments nutritifs. Cette caractéristique du sol peut en partie expliquer la rétention de certains pesticides. Sund, (1956) a par exemple montré que la CEC jouait un rôle déterminant dans la rétention dans les sols de l'aminotriazole, un herbicide de la famille des triazolés, en lien avec son groupement amine.

Parmi les minéraux argileux, certains sont décrits pour leur capacité d'adsorption des triazolés. La kaolinite a été soulignée comme jouant un rôle dans l'adsorption de l'époxiconazole et du tébuconazole surtout due à des interactions hydrophobes (Bošković *et al.*, 2020). Les argiles de type montmorillonite ont également été décrites comme déterminantes dans l'adsorption du triadimefon dans les sols. Ces argiles présentent de très grandes capacités de sorption pour cette molécule, et ce particulièrement quand le sol est riche en argile et pauvre en MO (Celis *et al.*, 1999). Des argiles modifiées ont été étudiées afin d'évaluer leur capacité à dépolluer les sols. Andrades *et al.*, (2004), ont montré que des argiles modifiées avec des cations organiques présentaient des capacités d'adsorption 24 fois plus importantes pour le penconazole que l'argile naturel. L'adsorption du penconazole est également plus importante dans le cas de la modification de la montmorillonite avec des micelles cationiques comparativement au contrôle (Suciu & Capri, 2009).

Le pH joue également un rôle déterminant dans la capacité de sorption des pesticides dans les sols. Il affecte notamment les fonctions du sol dont les activités microbiennes qui peuvent permettre de dégrader les molécules (Buerge *et al.*, 2006 ; Sun *et al.*, 2020). Le pH intervient également dans l'hydrolyse et la solubilité des pesticides et peut impacter leur réactivité (Gao *et al.*, 2012).

La nature des molécules elles-mêmes représente également une variable importante dans leur capacité à s'adsorber sur les sols.

2.3.3. *Caractéristiques propres aux molécules*

Plusieurs propriétés propres à chaque molécule peuvent caractériser leur capacité à persister dans les sols. La structure et la taille moléculaire dans un premier temps déterminent le potentiel d'ionisation de la molécule. Une taille non adaptée à l'espace disponible sur les sites d'adsorption abaissera la capacité de la molécule à se fixer sur les structures internes des argiles. La solubilité du composé déterminera son comportement entre les phases liquides et solides des sols. Ainsi, des molécules à faible solubilité seraient plus susceptibles d'être adsorbées dans les sols (Guimont, 2005). Les principales caractéristiques chimiques de 3 molécules triazolées fréquemment retrouvées dans l'environnement sont présentées dans le Tableau 3.

Tableau 3 : Caractéristiques chimiques des molécules triazolées époxiconazole, tébuconazole et difénoconazole.

	Formule chimique	Poids moléculaire (g.mol ⁻¹)	Log Kow (à 25°C)	pKa	Solubilité à 20°C (mg.L ⁻¹)
Epoxiconazole	C ₁₇ H ₁₃ ClFN ₃ O	329,7	3,3	5	29
Tébuconazole	C ₁₆ H ₂₂ ClN ₃ O	307,8	3,7	NR	6,63
Difénoconazole	C ₁₉ H ₁₇ Cl ₂ N ₃ O ₃	406,2	4,36	1,07	15

NR : Non renseigné dans la littérature

La nature des molécules définit leur « affinité » pour les différents types de sol. L'adsorption d'une molécule plutôt hydrophobe et non ionisable sera influencée par la teneur en carbone organique total du sol (Sadegh-Zadeh *et al.*, 2017) tandis que l'adsorption d'une molécule plutôt ionisable sera plus sensible aux variations de pH et de CEC (Kah & Brown, 2006 ; Bošković *et al.*, 2020).

La demi-vie dans le sol est également un paramètre à prendre en compte dans le potentiel contaminant d'un composé pour l'environnement. Pour les triazolés, ces valeurs sont très variables dans la littérature (de quelques jours à plusieurs mois ou années pour une même molécule). Ces variations s'expliquent notamment par des conditions de mesures différentes selon les études considérées (*e.g.* nature des sols, concentration appliquée, technique de mesure). Certaines d'entre elles sont présentées dans le Tableau 4.

Tableau 4 : Exemples de demi-vies décrites dans la littérature pour les molécules triazolées d'époxiconazole, de tébuconazole et de difénoconazole.

Molécule	Demi-vie dans le sol (en jours)	Références
Epoxiconazole	178	Du <i>et al.</i> , 2022
	78 à 184	Buerge <i>et al.</i> , 2006
	52 à 354	Kaziem <i>et al.</i> , 2020
	120 à 354	Nélieu <i>et al.</i> , 2016
	353,5	Pesticide Properties Database
	737 à 1004	Bromilow <i>et al.</i> , 1999
Tébuconazole	8,2 à 12,4	Herrero-Hernández <i>et al.</i> , 2011
	11,2 à 14,4	Dong & Hu, 2014
	26,8 à 36,9	Matadha <i>et al.</i> , 2019
	40,76 à 43,86	Ye <i>et al.</i> , 2013
	63	Pesticide Properties Database
	32,2 à 216,6	Cui <i>et al.</i> , 2018

	289 à 610	Strickland <i>et al.</i> , 2004
<i>Difénoconazole</i>	4,9 à 5,8	Zhang <i>et al.</i> , 2015
	4,68 à 8,09	Mukhopadhyay <i>et al.</i> , 2011
	15,5 à 23	Huan <i>et al.</i> , 2013
	2,82 à 23,26	Wang <i>et al.</i> , 2012
	21 à 27,7	He <i>et al.</i> , 2016
	54,2 à 55	Wang <i>et al.</i> , 2008
	9,6 à 68,4	Guo <i>et al.</i> , 2010
	130	Pesticide Properties Database
	142 à 1600	Health Canada's Pest Management Regulatory Agency, 2016

Fantke *et al.* (2014) ont travaillé sur 333 pesticides dans le but de créer un modèle de prédiction des risques associés à ces molécules. Sur ces 333 pesticides, l'époxiconazole, le tébuconazole et le difénoconazole ont été considérés comme les 18^e, 72^e et 136^e molécules avec la demi-vie la plus importante dans les sols respectivement. Ces valeurs témoignent de leur potentiel de persistance dans les sols en comparaison avec d'autres molécules.

Les molécules triazolées, de par leur utilisation et leurs caractéristiques, en particulier leur persistance, présentent un risque important de pollution environnementale aux conséquences multiples.
















2.4. Impacts associés à l'utilisation des (tri)azolés

La pollution environnementale causée par l'usage massif des molécules triazolées à travers le monde est une problématique qui prend de plus en plus d'ampleur, notamment du fait des impacts néfastes qui peuvent être constatés sur les organismes. Les conséquences de cette exposition sont un sujet préoccupant qui nécessite un suivi constant (Lv *et al.*, 2017).

2.4.1. Impacts sur la santé animale

L'évaluation des différents risques que représentent les triazolés pour la santé est complexe. Les notions de dose et de durée d'exposition, entre autres, sont des variables qui vont déterminer les effets observés. Selon l'European Commission of Food Safety, la plupart de ces molécules ont des effets nocifs et/ou toxiques. Certaines d'entre elles ont d'ailleurs déjà fait l'objet d'une interdiction de mise sur le marché en Europe, en raison de ces effets indésirables (Tableau 5).

Tableau 5 : Date de réévaluation de l'agrément de molécules triazolées et leurs classifications en Europe, en rouge les molécules retirées du marché, en vert les molécules dont l'agrément peut encore être renouvelé.
D'après l'European Commission of Food Safety, Règlement (CE) 1272/2008, 2008

Molécules	Date d'expiration/renouvellement de l'agrément	Classification des risques connus
Tébuconazole	08/2022	   H410 ; H400 ; H302 ; H361d
Propiconazole	12/2018	  H360D ; H317 ; H400 ; H410 ; H302
Metconazole	04/2023	  H302 ; H361d ; H411
Myclobutanil	05/2021	   H302 ; H319 ; H361d ; H411
Epoxiconazole	04/2020	  H351 ; H360Df ; H411
Tétraconazole	12/2022	 H302 ; H332 ; H411
Difénoconazole	12/2022	  H302 ; H332 ; H319 ; H400 ; H410

H302 : nocif en cas d'ingestion

H317 : peut provoquer une réaction allergique de la peau

H319 : provoque une grave irritation des yeux

H332 : nocif en cas d'inhalation

H351 : susceptible de provoquer le cancer

H360D : peut nuire à l'enfant à naître

H360Df : peut nuire à l'enfant à naître, susceptible de nuire à la fertilité

H361d : suspecté de nuire à l'enfant à naître

H400 : très toxique pour la vie aquatique

H410 : très toxique pour la vie aquatique avec des effets à long terme

H411 : toxique pour la vie aquatique avec des effets à long terme

La présence des triazolés dans tous les compartiments eau, air et sol intensifie les risques d'exposition : ces molécules peuvent ainsi avoir des effets néfastes sur les animaux aquatiques, les oiseaux ou bien encore les mammifères. Le risque de bioaccumulation de ces résidus dans les tissus de ces organismes peut impacter l'ensemble de la chaîne alimentaire et à terme représenter un risque potentiel pour la santé humaine (Konwick *et al.*, 2006 ; Bhagat *et al.*, 2021).

Dans la littérature on retrouve plusieurs effets toxiques liés à l'exposition aux triazolés. De façon non exhaustive on peut citer l'hépatotoxicité, la neurotoxicité, la reprotoxicité. Les triazolés peuvent également être décrits comme cancérigènes et perturbateurs endocriniens (Currie *et al.*, 2014 ; Zhu *et al.*, 2014 ; Chu *et al.*, 2016 ; Zhou *et al.*, 2016 ; De la Paz *et al.*, 2017 ; Ortiz-Santaliestra *et al.*, 2020 ; Pereira *et al.*, 2019 ; Ku *et al.*, 2021 ; Kabakci *et al.*, 2021). Des effets délétères sur le système immunitaire, les activités cardiaques et le métabolisme des lipides sont également décrits (Hinfray *et al.*, 2006 ; Sancho *et al.*, 2010 ; Li *et al.*, 2011 ; Mu *et al.*, 2013 ; Mu *et al.*, 2015, 2016 ; Souders *et al.*, 2019 ; Teng *et al.*, 2019). Ces impacts dépendent de la nature et de la durée de l'exposition.

Les triazoles sont considérés comme une classe de pesticides pouvant également induire des symptômes aigus. La présence de résidus dans l'environnement et l'ingestion de nourriture contaminée peuvent avoir des conséquences néfastes sur la santé humaine. Un rapport de l'EFSA publié en 2018 montre en effet que sur 12 168 échantillons de nourriture provenant de plus de 30 pays d'Europe, la moitié présentait des quantités détectables pour certains des 157 pesticides étudiés. Le tébuconazole et le difénoconazole faisaient partie des pesticides les plus fréquemment retrouvés dans les choux, les poireaux, les pêches ou bien encore les fraises (EFSA authority, 2018). De nombreux modèles visant à évaluer le risque toxique lié à l'exposition aux triazolés ont été établis (van der Ven *et al.*, 2020). Ces études soulignent le nombre important de fonctions biologiques qui peuvent être perturbées chez l'Homme et entraîner différentes pathologies et/ou symptômes (Figure 11).

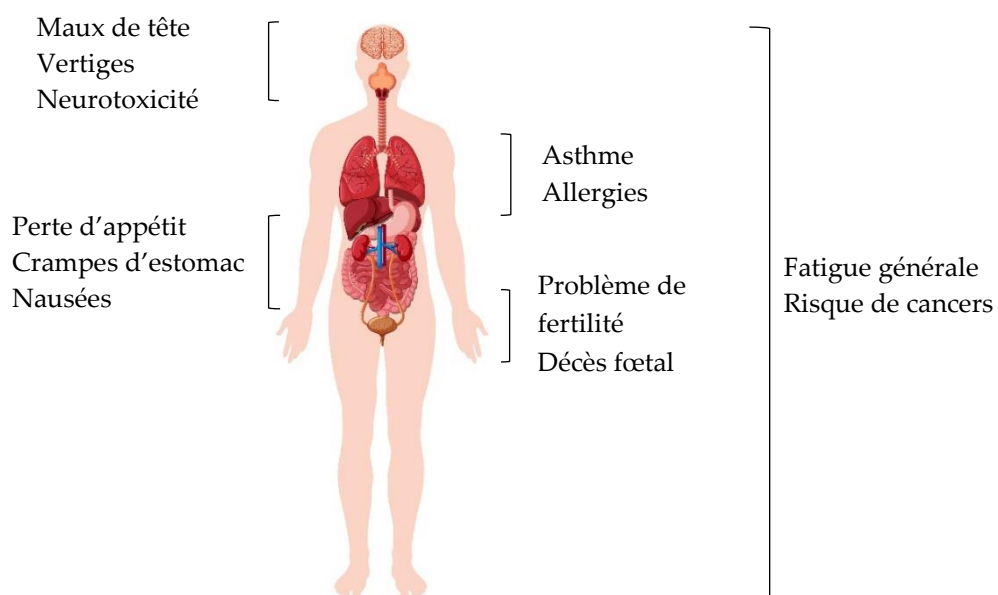


Figure 11 : Exemples de risques liés à une exposition aux pesticides, dont les triazolés, chez l'Homme. D'après Yura *et al.* 2021. Réalisé avec Biorender

Le dosage du tébuconazole et de ses métabolites secondaires (*e.g.* TEB-OH ; TEB-COOH) a été effectué dans l'urine de travailleurs viticoles montrant que les valeurs médianes étaient de 4 à 249 $\mu\text{g.L}^{-1}$, soulignant le risque d'exposition professionnelle (Fustinoni *et al.*, 2014). Le propiconazole est le 27^e pesticide sur 140, retrouvé le plus fréquemment dans les cheveux de 311 femmes enceintes d'une cohorte française, avec 71% de détection (Béranger *et al.*, 2018). Les cheveux sont le signe d'une exposition à long terme. Çelik *et al.*, (2021) ont montré que sur 132 personnes, l'époxiconazole, le tébuconazole, le difénoconazole et le triadimefon étaient retrouvés dans 14%, 13%, 11% et 16% des échantillons de cheveux respectivement. Ces résultats ont démontré qu'outre les professionnels agricoles, le reste de la population subit aussi cette exposition probablement due à l'environnement et aux habitudes alimentaires.

2.4.2. *Emergence de résistance chez les moisissures phytopathogènes*

L'application massive et répétée des fongicides triazolés sur les cultures peut conduire à l'émergence et/ou la dominance de moisissures phytopathogènes résistantes aux traitements. Certaines espèces sont intrinsèquement résistantes à cette catégorie de molécules. Pour d'autres, l'usage répété des mêmes traitements conduit à la sélection de gènes leur conférant une résistance. Les applications foliaires, les traitements des graines ou bien la présence des composés dans les sols peuvent favoriser cette émergence (van Toor *et al.*, 2012).

Cet avantage permet aux populations résistantes d'être plus compétitives que les souches sensibles dans la survie et dans la reproduction suite à une exposition. La présence de mutations génétiques conférant une résistance entraîne en contrepartie un coût métabolique qui impacte d'autres aspects (*e.g.* croissance). En présence d'une pression de sélection dans l'environnement, la balance coût/bénéfice induite par les mutations conférant la résistance reste favorable aux populations mutées (Billard *et al.*, 2011 ; Hawkins & Fraaije, 2018).

La plasticité génomique, la diversité génétique au sein d'une population, les modes de reproduction possibles et la capacité de la moisissure phytopathogène à se disperser sont autant de paramètres qu'il est important de prendre en compte dans l'évaluation d'une potentielle acquisition de résistance (Zhan & McDonald, 2004 ; Hahn, 2014 ; Garnault *et al.*, 2021). Bien que l'utilisation de variétés commerciales moins sensibles à certaines maladies soit envisageable, l'usage des fongicides en prévention ou en traitement reste actuellement la norme pour le contrôle des maladies en agriculture conventionnelle (Kim & Diers, 2000 ; Spolti *et al.*, 2014). Le Fungicide Resistance Action Committee (FRAC) a pour rôle d'identifier les

problèmes liés aux résistances aux fongicides dans les cultures et de faire des recommandations pour éviter leur émergence. A ce titre les moisissures phytopathogènes majeures sont référencées en fonction du risque associé de voir leur sensibilité diminuer face aux triazolés (Tableau 6).

Par exemple, l'espèce *Blumeria graminis* responsable de l'oïdium dans les cultures céréalières, les vergers ou bien les cultures maraichères est considérée comme à risque de développer des résistances. Meyers *et al.*, (2019) ont montré une diminution de la sensibilité de cette espèce pour le tébuconazole et le prothioconazole sur 400 isolats prélevés aux Etats-Unis, soulignant l'existence potentielle d'une résistance croisée. Ces phénomènes sont décrits de longue date en Europe, au Pays-Bas (de Waard *et al.*, 1986) pour le triadimefon, en République Tchèque (Švec *et al.*, 1995) et en France (Godet & Limpert, 1998) pour le triadimenol.

Tableau 6 : Diagramme d'évaluation des risques de développement de résistance des moisissures phytopathogènes pour les (tri)azolés (inhibiteur de déméthylase). Le risque combiné est obtenu en multipliant le risque lié au pathogène par le risque lié au fongicide. Adapté de Brent & Hollomon, 2007.

		<u>Risque faible</u> <i>Rhizoctonia sp.</i> Rouilles Maladies telluriques Maladie du charbon	<u>Risque modéré</u> Piétin-verse <i>Mycosphaerella graminicola</i> <i>Rhynchosporium</i>	<u>Risque important</u> <i>Botrytis cinerea</i> <i>Blumeria graminis</i> <i>Magnaporthe grisea</i> <i>Venturia inaequalis</i> <i>Plasmopara viticola</i> <i>Penicillium sp.</i> <i>Mycosphaerella fijiensis</i> <i>Phytophthora infestans</i>
		1	2	3
<u>Risque modéré</u> Inhibiteur de déméthylase – (Tri)azolés	2	2	4	6
Risque combiné				

Risque combiné : 0,5-1,5 = risque faible ; 2-6 = risque modéré ; 9 = risque élevé

Cook *et al.* (2021) ont montré la présence de mutations dans le gène *Cyp51* (codant pour l'enzyme 14- α -déméthylase, une enzyme clé de la synthèse de l'ergostérol) d'isolats issus de Chine et de Nouvelle Zélande de *Puccinia striiformis* responsables de la rouille jaune. Connues comme causant une diminution de la sensibilité aux triazolés, ces mutations peuvent être le point de départ de l'émergence de phénotypes de résistance.

La maladie la plus décrite sur le blé est la septoriose. Elle est causée par *Zymoseptoria tritici* et peut induire des pertes de rendement jusqu'à 50% (Torriani *et al.*, 2015). Cette espèce a développé une résistance complète contre les inhibiteurs externes de quinones (Garnault *et al.*, 2021) d'où l'importance des triazolés dans la lutte contre cette maladie, pour laquelle les options sont limitées. Birr *et al.* (2021) ont cependant montré que la sensibilité de cette espèce pour le tébuconazole et le propiconazole avait diminué entre 1999 et 2009 (Figure 12) avant de se stabiliser, soulignant le risque de voir émerger des résistances.

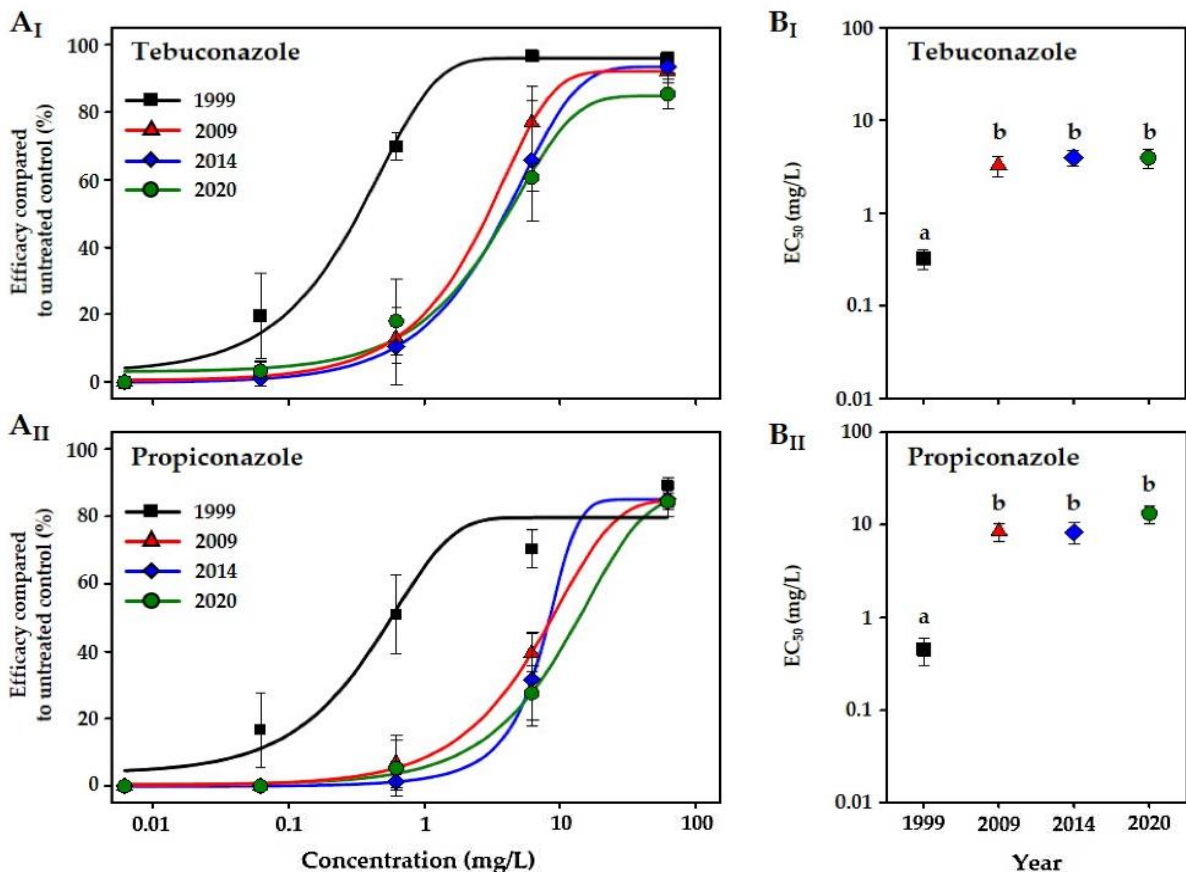


Figure 12 : Modification de la sensibilité des populations de *Zymoseptoria tritici* isolées dans le nord de l'Allemagne entre 1999 et 2020, aux triazolés, tébuconazole (A_I ; B_I) et propiconazole (A_{II} ; B_{II}). Les données sur l'efficacité des différentes concentrations de fongicides sont exprimées en pourcentage de réduction de la croissance fongique par rapport au témoin non traité. (B_I-B_{II}) Comparaison des valeurs de la EC₅₀ (mg.L⁻¹). Les valeurs de la EC₅₀ ont été déterminées comme étant la concentration de fongicide réduisant la croissance fongique de 50 % ($p \leq 0,05$). (Birr *et al.*, 2021).

L'émergence de résistances dans des espèces connues comme étant des moisissures phytopathogènes peut mettre en péril la production alimentaire. L'apparition de ce phénomène en lien avec les molécules triazolées représenterait un risque important de pertes de rendement de culture. Un usage plus raisonné et moins systématique de ces molécules est nécessaire pour éviter ce phénomène.

2.4.3. Impact non intentionnel sur la microbiologie du sol

La persistance des fongicides triazolés peut induire de fortes perturbations dans la microbiologie des sols, non seulement sur les espèces fongiques mais également sur les populations bactériennes. En provoquant un dérèglement des communautés microbiennes des sols, ces molécules peuvent altérer les fonctions de dégradation qu'assurent ces micro-organismes (Tableau 7, Roman *et al.*, 2021).

Tableau 7 : Effet d'une forte dose de fongicides triazolés sur les micro-organismes du sol (Roman *et al.* 2021).

Fongicide triazolé	Activité microbienne du sol	Biomasse microbienne	Population microbienne totale	Population de moisissures	Population de bactéries	Structure des communautés microbiennes
Difenoconazole	Cellules rouges	Cellules rouges	Cellules blanches	Cellules blanches	Cellules blanches	Cellules blanches
Epoxiconazole	Cellules rouges	Cellule verte	Cellules blanches	Cellules blanches	Cellules blanches	Cellules blanches
Flutriafol	Cellules blanches	Cellules blanches	Cellules blanches	Cellules rouges	Cellules bleues	Cellules blanches
Hexaconazole	Cellules blanches	Cellules blanches	Cellules rouges	Cellules blanches	Cellules rouges	Cellules blanches
Myclobutanil	Cellules blanches	Cellules rouges	Cellules blanches	Cellules blanches	Cellules blanches	Cellules blanches
Pacloutrazole	Cellules blanches	Cellules blanches	Cellules blanches	Cellules rouges	Cellules rouges	Cellules jaunes
Propiconazole	Cellules blanches	Cellules blanches	Cellules rouges	Cellules rouges	Cellules rouges	Cellules jaunes
Tebuconazole	Cellules rouges	Cellules rouges	Cellules blanches	Cellules rouges	Cellules blanches	Cellules jaunes
Tetraconazole	Cellules blanches	Cellules rouges	Cellules blanches	Cellules blanches	Cellules blanches	Cellules blanches
Triadimefon	Cellules blanches	Cellules rouges	Cellules blanches	Cellules rouges	Cellules bleues	Cellules jaunes
Triticonazole	Cellules blanches	Cellules blanches	Cellules blanches	Cellules rouges	Cellules bleues	Cellules jaunes

Cellules rouges : diminution par rapport au contrôle

Cellules bleues : augmentation par rapport au contrôle

Cellule verte : pas d'effet du fongicide sur le paramètre concerné

Cellules jaunes : modification de la structure des communautés microbiennes

Cellules blanches : paramètres non mesurés

Muñoz-Leoz *et al.* (2013) ont montré qu'une exposition des sols à de fortes concentrations de difénoconazole (500 mg.kg⁻¹) provoque un stress qui conduit à une diminution de l'activité microbienne totale. Le stress induit se reflète notamment par un quotient respiratoire doublé en présence du fongicide. Ces résultats ont été confirmés par da Rocha *et al.* (2020) qui ont montré qu'une exposition à cette même molécule aux doses recommandées pour une application environnementale pourrait être responsable d'une diminution de la biomasse microbienne globale de 30% après 84 jours de contact. Lors de l'exposition, les micro-organismes dépensent plus d'énergie dans la dégradation des composés que dans leur développement ce qui se traduit par une augmentation du quotient métabolique. Satapute *et al.* (2019), quant à eux, ont établi que le propiconazole, à hautes doses, peut également induire une diminution de la communauté microbienne.

De nombreuses études ont mis en évidence la toxicité du tébuconazole sur la diversité et la quantité de micro-organismes du sol, bien que cet effet soit dose-dépendant. Des doses élevées de cette molécule ont des effets sur les communautés bactériennes et la biodiversité des micro-

organismes, au moins transitoirement. Les sous-produits du tébuconazole semblent également avoir un impact (Storck *et al.*, 2016). Des effets d'une application de tébuconazole sur les sols sont visibles à des concentrations de 10 mg.kg⁻¹, à des doses de 100 mg.kg⁻¹ la toxicité du tébuconazole pour les sols est avérée. Il impacte notamment les activités enzymatiques et les fonctions de respiration du sol (Wang *et al.*, 2016). Le triadimefon, appliqué à la même concentration, diminue également la population bactérienne (Yen *et al.*, 2009). Dans des concentrations plus faibles, de l'ordre de 1 mg.kg⁻¹, le triadimefon induit déjà une diminution significative de l'activité déshydrogénase des sols après 30 jours, signe d'une diminution potentielle de l'activité microbienne (Singh, 2005). Le risque d'accumulation des fongicides triazolés dans les sols peut avoir des effets indésirables sous-estimés sur la microbiologie de ce compartiment et impacter leur propre biodégradation (Fernández-Calviño *et al.*, 2017).

L'impact non-intentionnel des fongicides triazolés dans les sols le plus largement décrit sur les micro-organismes est la pression de sélection qu'ils peuvent induire sur une moisissure ubiquiste d'intérêt médical pour l'Homme, appelée *Aspergillus fumigatus*.

2.5. Impacts sur *Aspergillus fumigatus*

Bien qu'utilisées en vue de combattre les moisissures phytopathogènes responsables du développement de pathologies en particulier sur les feuilles (Weiland & Koch, 2004 ; Shcherbakova *et al.*, 2021), les molécules triazolées peuvent également diffuser dans d'autres matrices, comme les sols, en fonction de la méthode utilisée pour leur application. Lors d'une pulvérisation sur grande culture par exemple, une très faible proportion de la molécule active atteint sa cible, le reste diffuse dans l'environnement dans lequel elle rencontre de nombreuses autres moisissures (Pimentel & Burgess, 2012 ; Pose-Juan *et al.*, 2015).

Une des moisissures ubiquistes que l'on peut y retrouver est *Aspergillus fumigatus*.

2.5.1. Présentation de l'espèce

Cette moisissure saprophyte se nourrit de la matière organique en décomposition. Elle est une moisissure filamenteuse du phylum des Ascomycètes. Du point de vue morphologique cette espèce est reconnaissable en culture par un aspect en colonies de couleur vertes/bleues avec une texture poudreuse (Figure 13). Dans certains cas, les spores présentent toutefois une absence de pigment et apparaissent blanches. En microscopie, cette moisissure présente des têtes aspergillaires unisériées, ainsi que des spores rondes de surface lisse ou en épi, portées par une seule rangée de phialides (Mokobi, 2021).

Du fait de la structure très résistante de ces spores, de leur hydrophobicité et de leur petite taille (de l'ordre de 2 à 3 μm), cette espèce est particulièrement résistante à la dispersion qui se fait principalement par les vents (Haiduven, 2009 ; O'Gorman, 2011). On peut donc facilement la retrouver dans l'air, que ce soit en milieu extérieur ou en milieu intérieur (Arné *et al.*, 2011 ; Aguiar *et al.*, 2014 ; Pilmis *et al.*, 2018 ; Godeau *et al.*, 2020).

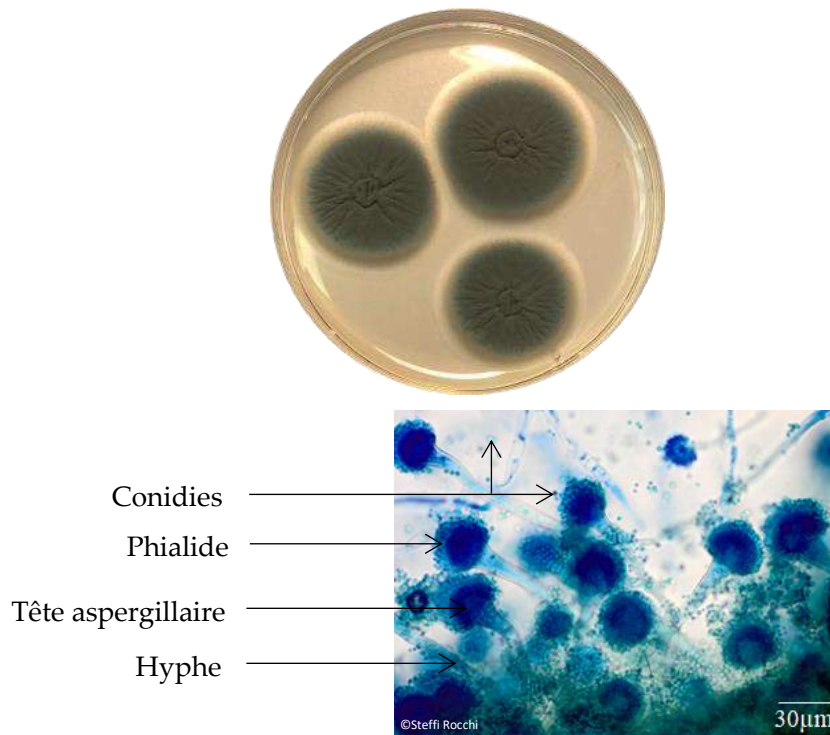


Figure 13 : Observations macroscopique (après culture sur gélose DG18 pendant 4 jours à 37°C) et microscopique (coloration au bleu de lactophénol) de colonies d'*Aspergillus fumigatus*.

Cette espèce n'est pas décrite comme phytopathogène, mais sa thermo-tolérance, qui lui permet de se développer à des températures allant jusqu'à 70°C, en fait un pathogène opportuniste pour de nombreux animaux (J. C. Rhodes, 2006). Des cas de pathologies à *A. fumigatus* ont par exemple été décrits chez des chevaux, des chiens mais aussi des dauphins ou des oiseaux (Ricardo *et al.*, 2018 ; Groch *et al.*, 2018 ; Talbot *et al.*, 2018 ; Valdes *et al.*, 2018 ; Arné *et al.*, 2021). Cette moisissure est également un pathogène opportuniste pour l'Homme qui respire une centaine de ses conidies par jour (Beisswenger *et al.*, 2012). Des pathologies chroniques provoquées par cette espèce peuvent être retrouvées chez des patients atteints de mucoviscidose. La virulence de cette espèce réside également dans sa capacité de production de mycotoxines, telles que la fumagilline, la pseurotine ou encore la fumigatoxine (Iwata, 1969 ; Fallon *et al.*, 2010 ; Yu *et al.*, 2018).

L'aspergillose invasive (AI), principalement causée par *A. fumigatus*, est une pathologie associée à plus de 4% des décès dans les hôpitaux tertiaires européens (Brookman & Denning,

2000). Cette maladie a récemment été introduite dans l'International Classification of Diseases de l'Organisation Mondiale de la Santé (<https://icd.who.int>). Le nombre de cas à travers le monde est souvent sous-estimé en raison de lacunes dans le diagnostic. Un récent rapport a démontré que l'incidence des aspergilloses en Chine était de 312/100 000 habitants chaque année, dont 77 cas d'AI (Zhou *et al.*, 2020). D'après la fondation « Global Action for Fungal Infection » (<https://gaffi.org/>), chaque année 30 millions de personnes sont à risque de développer une AI, et parmi eux, 300 000 déclarent la maladie.

2.5.2. *Traitements des pathologies causées par Aspergillus fumigatus*

La proximité en termes de structure entre les cellules fongiques et les cellules humaines, qui sont des eucaryotes, complexifie la prise en charge de ces pathologies pour lesquelles le traitement peut avoir des effets indésirables croisés sur des cellules humaines non ciblées.

Avant les années 1940, les pathologies fongiques étaient mal prises en charge, jusqu'à la découverte des premiers traitements à base de polyènes (Maertens, 2004). L'amphotéricine B, un antifongique appartenant à cette famille de molécule, est alors devenu le traitement de choix des maladies systémiques causées par des moisissures en médecine humaine (Burton *et al.*, 1972 ; Jenks & Hoenigl, 2018). Cette molécule permet d'induire une lyse cellulaire en se liant à un composé majeur de la membrane fongique, l'ergostérol (Zotchev, 2003 ; Carolus *et al.*, 2020). Cependant, ces traitements présentent différents inconvénients et peuvent entraîner l'apparition d'effets indésirables liés à leur administration par perfusion ou bien à leur action néphrotoxique (Deray, 2002 ; Tragiannidis *et al.*, 2021). Dans les années 1990, une nouvelle forme de ce traitement a été développée, l'amphotéricine B liposomale, dont l'objectif était d'augmenter la tolérabilité de cette molécule (Groll *et al.*, 2019).

Les azolés ont été introduits dans les années 1980. D'abord les imidazolés avec la mise sur le marché du ketoconazole (Heeres *et al.*, 1979) puis les triazolés, 10 ans plus tard (Lass-Flörl, 2011), venant combler le manque de molécules imidazolées disponibles pour le traitement des pathologies causées par *A. fumigatus*.

L'arrivée des triazolés sur le marché dans les années 1990 (déjà utilisées depuis près de 20 ans en agriculture) a modifié la prise en charge des maladies fongiques.

Particulièrement appréciées pour leur facilité d'utilisation et leur toxicité relativement basse, le spectre d'activité élargi des molécules triazolées de première génération en ont directement fait des traitements de choix pour une action préventive ou curative des infections fongiques.

L'itraconazole a ainsi été approuvé en 1992 et présente un large spectre d'action notamment sur les *Aspergillus spp.* Le voriconazole, triazolé de deuxième génération, a quant à lui été introduit dans les années 2000. Ces 2 molécules ont longtemps constitué la première ligne de défense contre les aspergilloses. Depuis, de nouvelles molécules triazolées, le posaconazole (2006) (Keating, 2005) et l'isavuconazole (2015) (Shirley & Scott, 2016), deux nouveaux dérivés, sont venus compléter la gamme de traitements autorisés.

Ces traitements permettent de faciliter la prise en charge des pathologies aspergillaires (Figure 14).

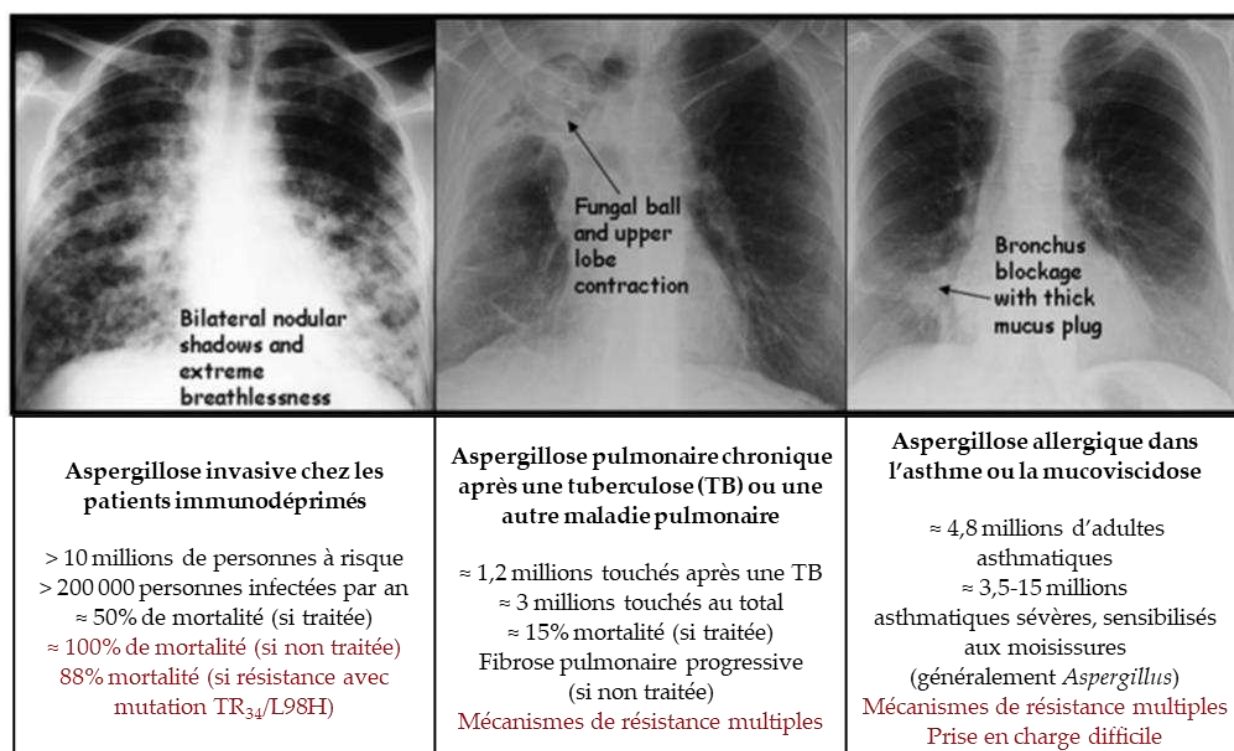


Figure 14 : Principales pathologies causées par *Aspergillus fumigatus* et leurs incidences. D'après Bowyer & Denning, 2014.

Aujourd'hui, un nombre relativement faible de molécules aux modes d'action différents sont disponibles pour le traitement des pathologies fongiques chez l'Homme. Les pathologies causées par *A. fumigatus* sont principalement traitées par des perturbateurs de l'intégrité membranaire. L'arsenal thérapeutique disponible contre *A. fumigatus* est donc restreint (Figure 15).

La découverte de nouvelles molécules antimicrobiennes d'origine naturelle est rare et le développement de dérivés d'origine synthétique est un processus long s'appuyant souvent sur des molécules déjà commercialisées.

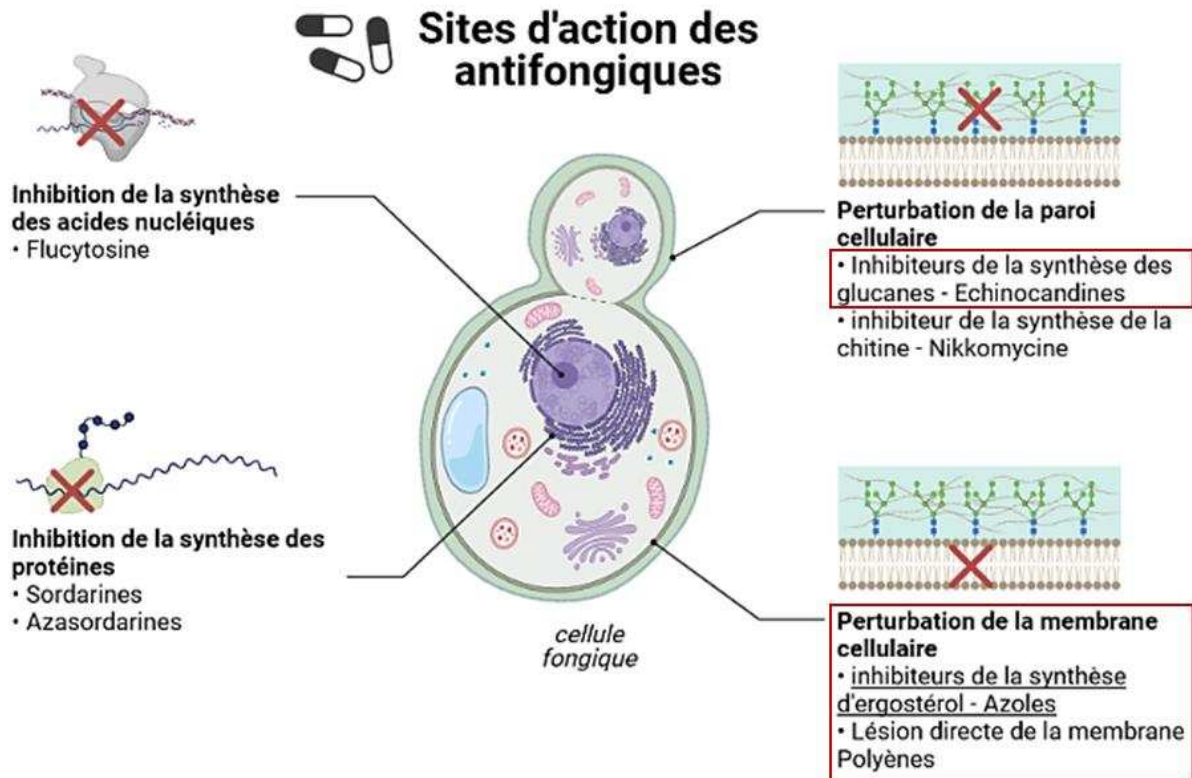


Figure 15 : Les modes d'actions des différentes familles d'antifongiques sur la cellule fongique. Encadrées en rouge les familles de molécules utilisées préférentiellement dans le traitement des pathologies aspergillaires. Réalisé avec Biorender.

Dans ce contexte, préserver l'efficacité des molécules existantes, et particulièrement des triazolés, est indispensable afin d'éviter l'impasse thérapeutique.

2.5.3. Résistance chez *Aspergillus fumigatus* : lien avec l'environnement et impacts sanitaires

L'intérêt que représentent les triazolés pour l'usage médical a rapidement été freiné par la découverte, avant les années 2000, des premiers cas de souches résistantes à ces molécules, moins de 10 ans après le début de leur utilisation (Snelders *et al.*, 2008). Cette problématique de résistance est amplifiée par l'augmentation du nombre de patients à risque de développer des pathologies opportunistes dues à la prise en charge de patients avec les techniques médicales modernes (*e.g.* chimiothérapies, greffes... ; Kim *et al.*, 2016). De nombreuses études ont montré l'existence de ces résistances sur tous les continents (Figure 16 ; Howard *et al.*, 2009 ; Lockhart *et al.*, 2011 ; Chowdhary *et al.*, 2012 ; van der Linden *et al.*, 2015 ; Lestrade *et al.*, 2019a ; Rivelli Zea & Toyotome, 2022). Ce phénomène complexifie la prise en charge des patients (van der Linden *et al.*, 2013).

Les taux de mortalité des patients atteints d'AI sont de 20 à 50% (Nivoix *et al.*, 2008 ; Lestrade *et al.*, 2019b) dans le cas d'un traitement de première intention avec les triazolés. Dans le cas d'une souche résistante, ce taux est compris entre 50 et 100% (Howard *et al.*, 2009 ; Steinmann *et al.*, 2015).

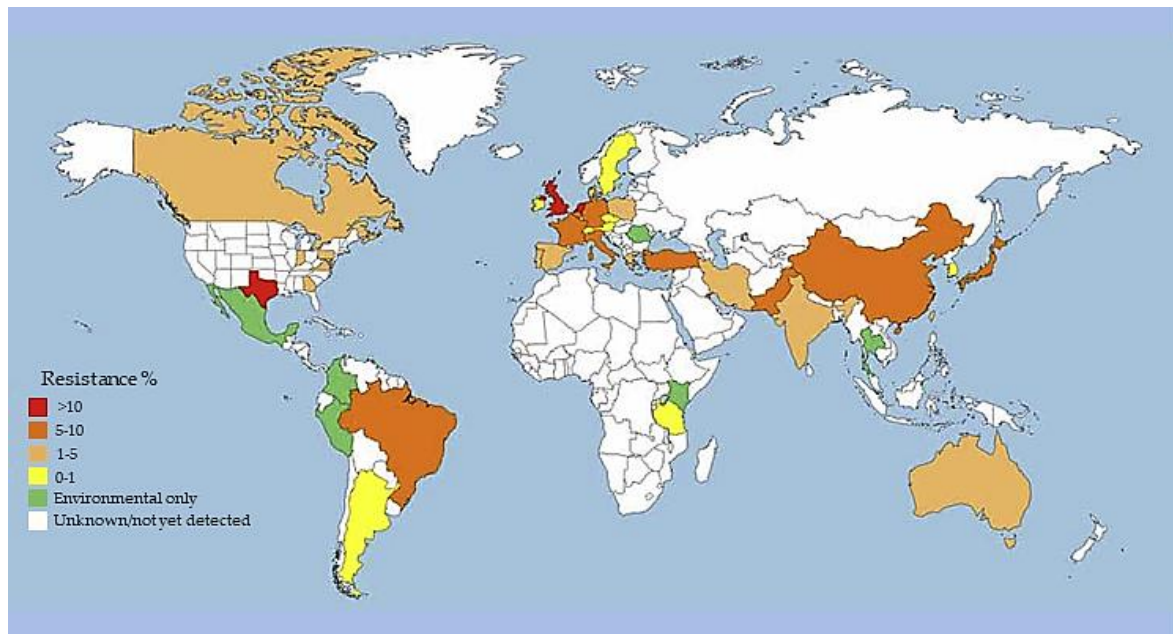


Figure 16 : Épidémiologie mondiale des fréquences de résistance (en %) aux azolés dans les isolats cliniques et environnementaux d'*Aspergillus fumigatus*. (Lestrade *et al.*, 2019b)

De nombreuses mutations ont pu être identifiées sur le gène *Cyp51A* comme principale cause conférant de la résistance à un ou plusieurs triazolés (Rivero-Menendez *et al.*, 2016). Des substitutions d'acides aminés dans la région codante du gène ont été décrites (Tableau 8 ; Diaz-Guerra *et al.*, 2003 ; Mellado *et al.*, 2004 ; Pelaez *et al.*, 2012) issues d'une adaptation suite à un traitement au long cours (Camps *et al.*, 2012). D'autres mutations, plus complexes, associant des substitutions dans la région codante et l'insertion de répétitions en tandem dans le promoteur du gène (*e.g.* TR₃₄/L98H, TR₄₆/Y121F/T289A) ont aussi été retrouvées chez des patients n'ayant jamais reçu de traitement triazolés (Mellado *et al.*, 2007 ; Berger *et al.*, 2017). La mutation TR₃₄/L98H notamment a été décrite comme la mutation la plus fréquemment retrouvée dans des souches d'*A. fumigatus* provenant de l'environnement (Rivero-Menendez *et al.*, 2016) posant la question d'une origine environnementale de cette résistance.

Tableau 8 : Exemple de mutations décrites dans le gène *Cyp51A* et phénotypes de résistance correspondant chez *Aspergillus fumigatus*. D'après (Guegan & Gangneux, 2017).

Origine supposée de cette mutation	Mutations sur le gène <i>Cyp51A</i>	Phénotype de résistance
Traitement au long court	G54/W/R/E/V/A	Itraconazole/Posaconazole
	M220/V/L/T/I	Itraconazole/Posaconazole
	G138C	Pan-azolé
	G448S	Pan-azolé
	P216L	Itraconazole/Posaconazole
	Y431C	Itraconazole/Posaconazole/Voriconazole
	Y121F	Voriconazole
Environnementale	TR ₃₄ /L98H	Pan-azolé
	TR ₄₆ /Y121F/T289A	Voriconazole/Posaconazole
	TR53	Pan-azolé

La proximité en termes de structure chimique des triazolés à usage médical et à usage environnemental (Figure 17) a été décrite comme source d'une résistance croisée (Snelders *et al.*, 2012 ; Garcia-Rubio *et al.*, 2021). L'utilisation massive des molécules triazolées dans l'environnement a rapidement été mise en cause dans l'émergence de ces résistances.

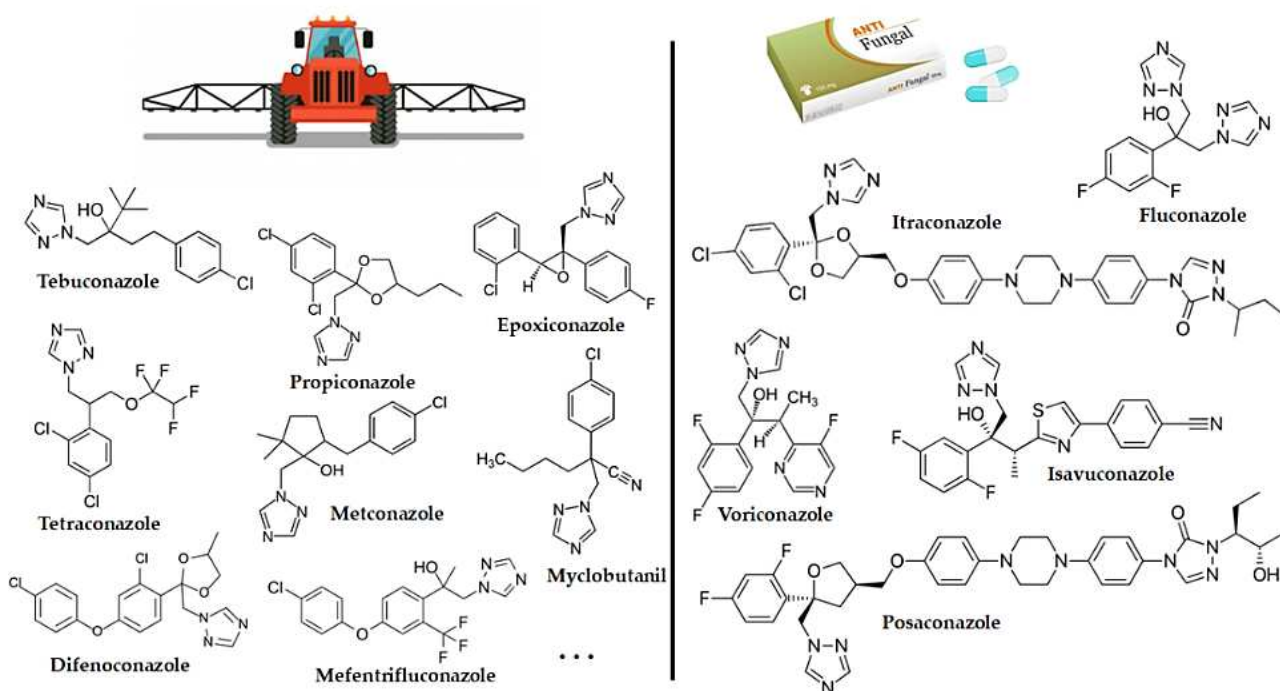


Figure 17 : Structures chimiques des molécules triazolées à usage environnemental (gauche) et à usage médical (droite).

De nombreuses études ont été menées pour comprendre l'apparition de ce phénomène. Bien que ces molécules ne soient pas considérées comme pouvant avoir une action mutagène chez *A. fumigatus*, la pression de sélection qu'elles peuvent induire favorise la population de souches

résistantes (Bastos *et al.*, 2021). Certaines conditions semblent être particulièrement propices au développement de ces résistances et sont considérées comme des « hotspots » potentiels. Les « hotspots » sont décrits comme étant des zones qui regroupent à la fois des conditions favorables au développement et à la reproduction d'*A. fumigatus* et des résidus de triazolés (Verweij *et al.*, 2020).

Ainsi, les déchets de bulbes de fleurs traitées, les déchets verts ou bien encore les copeaux de bois traités peuvent représenter autant de sites d'apparition potentielle de ce phénomène (Schoustra *et al.*, 2019 ; Verweij *et al.*, 2020 ; Burks *et al.*, 2021).

Un autre aspect important à prendre en considération dans l'émergence de cette résistance est la capacité d'*A. fumigatus* à se reproduire de façon sexuée. Il existe 3 modes de reproduction potentiels pour cette moisissure : reproductions asexuée, parasexuée et sexuée (Verweij *et al.*, 2016 ; Centers for Disease Control and Prevention (U.S.), 2019). La reproduction sexuée semble se faire principalement dans l'environnement. Quand elle se produit, la méiose est source d'une grande variabilité génétique chez les souches environnementales (O'Gorman *et al.*, 2009 ; Stensvold *et al.*, 2012). Du fait du grand nombre de générations qui sont synthétisées dans le temps, le risque de voir apparaître des mutations spontanées est relativement élevé. Ces mutations pourraient potentiellement par la suite être favorisées par la pression de sélection induite dans l'environnement.

L'importance de l'utilisation des molécules triazolées, tant sur le plan environnemental que médical, n'est plus à prouver. Cependant, l'apparition globale d'une résistance d'ampleur grandissante complexifie grandement l'utilisation de ces composés. Les alternatives thérapeutiques chez l'Homme sont minces, particulièrement au vu du faible nombre de molécules autorisées. Dès 2013, l'European Centre for Disease prevention and Control (ECDC) souligne l'ampleur de cette résistance pouvant être considérée comme un risque émergent de santé publique (ECDC, 2013). Du fait de la diffusion globale de cette résistance, *A. fumigatus* a également été introduit, en 2019, comme pathogène à surveiller par l'U.S. Centers for Disease Control and Prevention (Centers for Disease Control and Prevention (U.S.), 2019).

Décrire ce phénomène dans l'environnement et mieux comprendre quelles sont les conditions qui semblent le favoriser représente un enjeu majeur pour pouvoir le contrôler et développer des méthodes afin de le limiter. C'est dans ce contexte que se situe ce travail de thèse.

3. Objectifs

Les objectifs de la thèse étaient d'évaluer la diffusion des fongicides triazolés environnementaux dans certains agroécosystèmes et de mesurer leur impact sur des populations fongiques d'*A. fumigatus*. Ce travail pluridisciplinaire allie chimie et mycologie. Pour ce faire, une analyse mycologique et/ou descriptive quantitative de la présence des triazolés a été réalisée dans différents domaines d'application de ces fongicides, que sont les grandes cultures, les cultures maraîchères et des pots de fleurs ornementales, afin de pouvoir intégrer une approche multi-échelle à l'étude. Quatre axes permettront de retranscrire les résultats obtenus. Trois concernent la caractérisation des différents domaines d'étude précédemment cités, le dernier axe permet de discuter de différents leviers d'action potentiels pour limiter l'émergence de populations résistantes d'*Aspergillus fumigatus*. Les objectifs de l'étude sont décrits ci-après, ainsi que les moyens mis en œuvre pour les réaliser (Figure 18).

- ✓ **EVALUER** la diffusion et l'accumulation des fongicides triazolés dans l'environnement

Sur des prélèvements de sol
Au moyen d'analyses en chromatographie gazeuse triple quadripôle

- ✓ **DETECTER** la présence de souches d'*Aspergillus fumigatus* résistantes

Sur des prélèvements de différentes matrices (sol/air)
Au moyen de cultures sur milieux supplémentés en triazolés

- ✓ **ETABLIR** les phénotypes de résistance de ces isolats

Sur des isolats issus des cultures sur milieux supplémentés en triazolés
Au moyen de la technique de référence de test de sensibilité (EUCAST)

- ✓ **CARACTERISER** les mutations spécifiques impliquées dans cette résistance

Sur les isolats déterminés résistants en test de sensibilité
Au moyen du séquençage de leur gène *Cyp51A*

- ✓ **COMPRENDRE** les conditions « privilégiées » de la présence de cette résistance dans les sols

Sur des domaines d'application de ces fongicides à différentes échelles
Au moyen d'analyses structurales des sols et de comparaisons *inter* et *intra* échelles

- ✓ **PROPOSER** des méthodes de remédiation pour limiter ce phénomène

Sur des sols contaminés
Au moyen de supports biodégradables et innovants et/ou en modifiant les pratiques

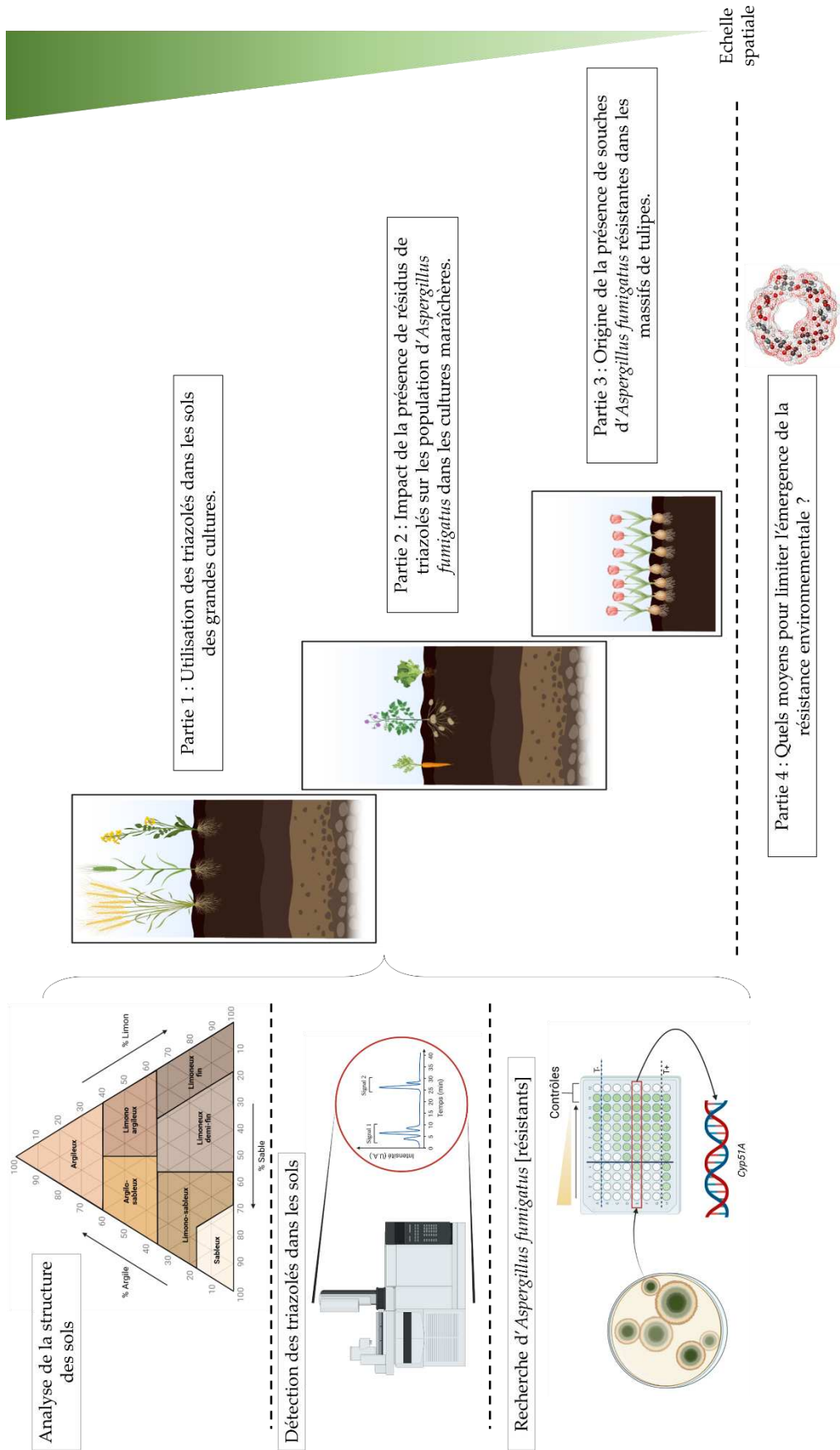


Figure 18 : Organisation du travail de thèse. Réalisé avec Biorender

4. Résultats pour les différents axes de travail

4.1. Utilisation des triazolés dans les sols des grandes cultures

Hypothèses de travail :

- L'utilisation des triazolés dans les cultures de céréales peut favoriser les conditions d'émergence et/ou de sélection de la résistance chez *Aspergillus fumigatus*.
- Les différents types de sol peuvent influencer la persistance des molécules triazolées.

Résumé :

Les impacts de l'utilisation des triazolés en grande culture sur les populations d'*Aspergillus fumigatus* ont été étudiés au moyen de deux campagnes de prélèvements de sols réalisées en Lorraine et en Bourgogne. Les parcelles prélevées ont été sélectionnées en fonction des antécédents de traitement fongicide et des caractéristiques des sols obtenus auprès des agriculteurs. Des parcelles ayant reçu des fongicides triazolés ont été comparées avec des parcelles en agriculture biologique pour chaque campagne. Différentes caractéristiques des sols ont été mesurées (pH, matière organique, granulométrie, capacité d'échange cationique) afin d'évaluer leurs effets sur la persistance des fongicides. Le dosage des résidus de 5 molécules triazolées (bromuconazole ; difénoconazole ; époxiconazole ; propiconazole et tébuconazole) a été réalisé par chromatographie gazeuse triple quadripôle et une analyse mycologique de chaque prélèvement a été effectuée. Des résidus de tébuconazole ont été retrouvés dans tous les sols prélevés (8,2 nanogrammes par gramme de sol en moyenne) alors que seulement quelques sols contenaient de l'époxiconazole. Les niveaux de concentrations de ces résidus n'ont pas pu être mis en relation avec les caractéristiques des sols (*e.g.* pH, taux de matière organique...), moins contrastées qu'attendu, que ce soit entre les régions ou entre les parcelles d'une même région. Aucun isolat d'*Aspergillus fumigatus* résistant aux azolés (aR-Af) n'a pu être mis en évidence. Suite à une analyse globale de communauté fongique par PCR quantitative en temps réel, nous avons pu conclure que cet environnement ne semble pas être une niche de développement de cette moisissure. Il ne peut donc probablement pas constituer un « hotspot » de résistance.

Eléments essentiels :

- Des prélèvements de sols ont été réalisés sur 22 parcelles de cultures céréalières réparties dans 2 régions différentes (Lorraine et Bourgogne) à raison de 10 prélèvements par parcelle.
- Les analyses des paramètres du sol montrent moins de contraste qu'attendu entre les 2 régions, le paramètre le plus contrasté étant le pH. Une majorité de sol était de structure argile limoneuse.
- Le dosage des pesticides a montré que tous les sols étaient contaminés par des triazolés. Le tébuconazole est aussi bien retrouvé dans les sols traités l'année concernée que dans les sols non traités, ou que dans ceux en agriculture biologique. L'époxiconazole est uniquement présent dans 5 parcelles de Lorraine sur 11.
- Des cultures fongiques ont été réalisées à partir des 220 prélèvements des sols : 166 isolats ont été identifiés comme appartenant à la section *Fumigati* et aucun d'entre eux ne s'est avéré résistant aux triazolés.
- Les ADN totaux de chaque sol ont été extraits. Une qPCR panfongique a montré une variabilité entre les échantillons. Une qPCR spécifique à *Aspergillus fumigatus* a également permis de quantifier la présence de cette espèce qui représente une très faible proportion de l'ADN fongique total pour chaque sol.

Démarche d'échantillonnage et analyse structurale des sols.

Les prélèvements de sols ont été réalisés dans des parcelles agricoles faisant partie d'une zone d'étude gérée par l'UMR 1347 Agroécologie de Dijon ou de la Zone Atelier Moselle, dont les agriculteurs ont autorisé ces prélèvements. Dix prélèvements de surface ont été réalisés par parcelle en respectant un transect. Les données des différentes parcelles sélectionnées dans chacune des deux régions sont présentées dans les Tableaux 9 et 10 pour la Lorraine et la Bourgogne respectivement les cartographies de chaque zone sont présentées Figures 19 et 20. Pour rappel, l'ensemble du matériel et méthodes est détaillé en annexe.

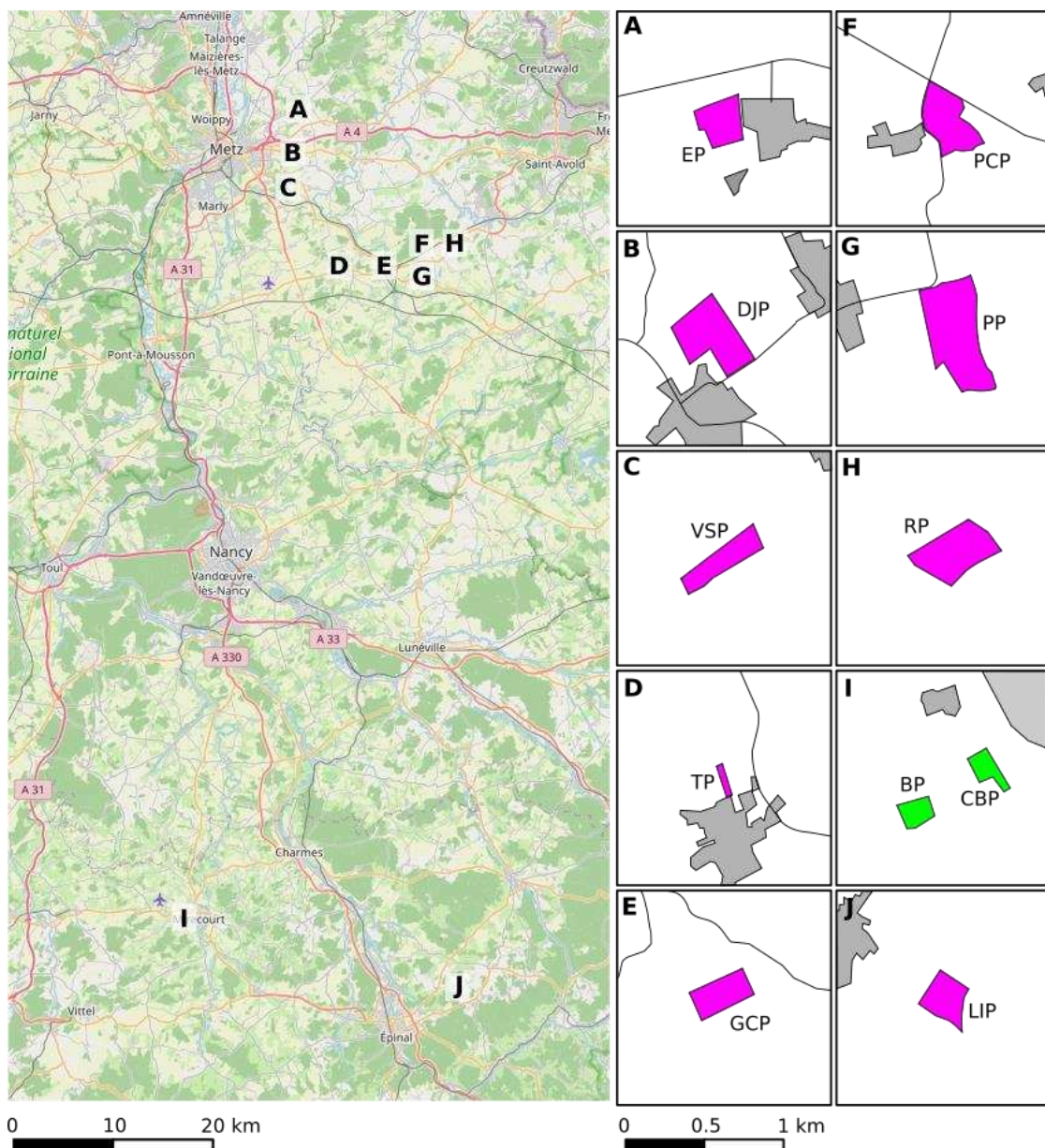


Figure 19 : Cartographie des 11 parcelles prélevées en Lorraine (Zone Atelier Moselle). Les parcelles en agriculture biologique sont représentées en vert, celles en rose ont reçu un traitement au tébuconazole. Les agglomérations apparaissent en gris. Le fond de carte est issu d'OpenStreetMap.

Tableau 9 : Détails des données des 11 parcelles prélevées en Lorraine (ZAM).

	Parcelle	Culture (2019)	Surface (ha)	Matière active (2019)
Lorraine	BP	Blé	3,0	Agriculture biologique
	CBP	Blé	3,1	Agriculture biologique
	PP	Blé	20,5	Tébuconazole
	PCP	Blé	9,3	Tébuconazole
	RP	Blé	12,6	Tébuconazole
	DJP	Blé	22,6	Tébuconazole
	VSP	Blé	7,9	Tébuconazole
	EP	Blé	7,2	Tébuconazole
	TP	Blé	1,5	Tébuconazole
	GCP	Blé	12,1	Tébuconazole
	LIP	Blé	7,3	Tébuconazole

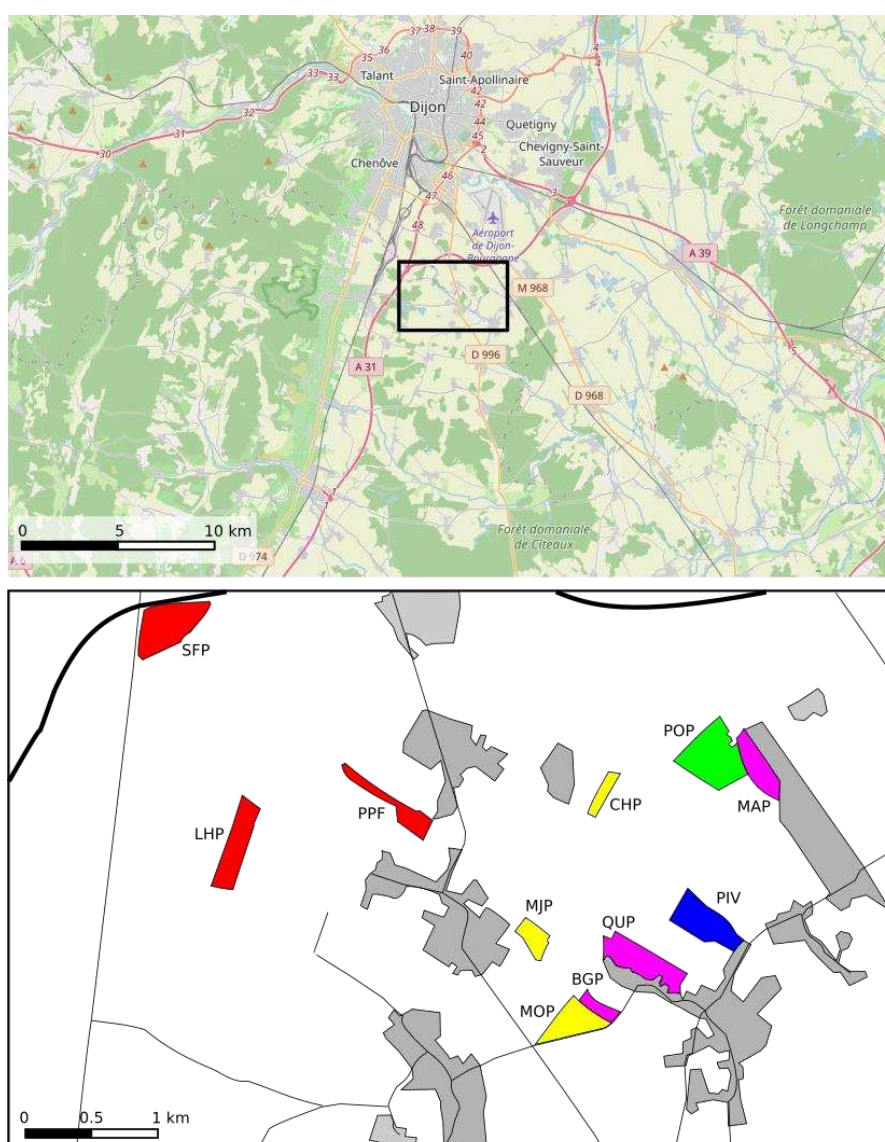


Figure 20 : Cartographie des 11 parcelles prélevées en Bourgogne (zone d'étude gérée par l'UMR 1347 Agroécologie de Dijon). Les parcelles en rouge ont reçu du metconazole, celle en bleu du prothioconazole, celles en rose un mélange de prothioconazole et de tébuconazole. Les agglomérations apparaissent en gris. Le fond de carte est issu d'OpenStreetMap.

Tableau 10 : Détails des données des 11 parcelles prélevées en Bourgogne (zone d'étude gérée par l'UMR 1347 Agroécologie de Dijon).

	Parcelle	Culture (2021)	Surface (ha)	Matière active (2021)
Bourgogne	POP	Jachère	16,3	Agriculture biologique
	MJP	Maïs	4,4	Non traitée
	CHP	Maïs	3,0	Non traitée
	MOP	Maïs	8,9	Non traitée
	PIV	Blé	16,0	Prothioconazole
	QUP	Blé	12,0	Prothioconazole/Tébuconazole
	MAP	Blé	6,4	Prothioconazole/Tébuconazole
	BGP	Blé	2,9	Prothioconazole/Tébuconazole
	PPF	Colza	7,4	Metconazole
	SFP	Colza	16,3	Metconazole
	LHP	Colza	11,5	Metconazole

Une analyse des paramètres du sol a été réalisée dans le but de déterminer d'éventuelles différences entre les deux zones de prélèvements. Les répartitions des différents paramètres des sols, pourcentage de matière organique, pH et CEC sont présentées Figure 21.

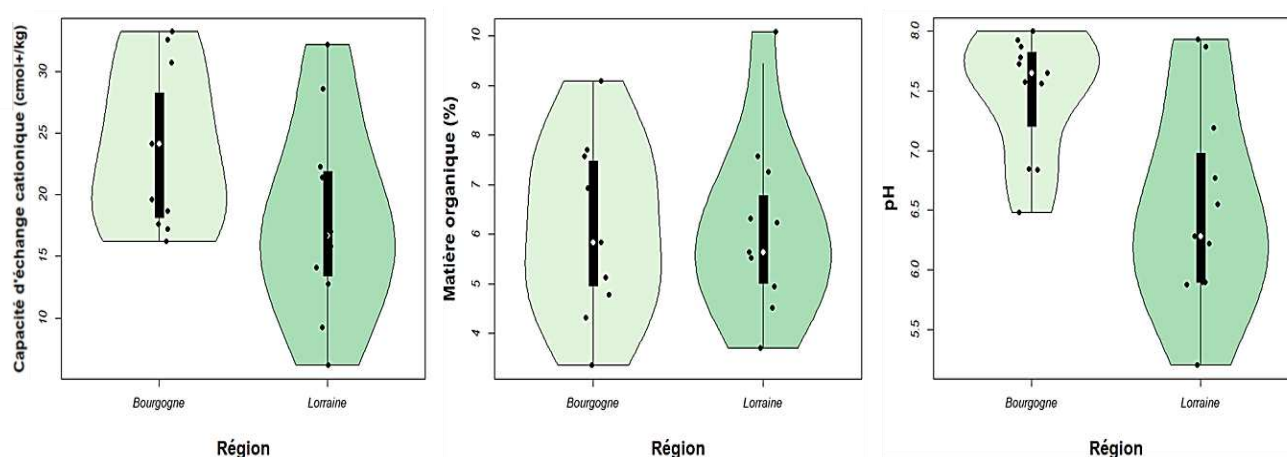


Figure 21 : Diagramme en violon de la répartition des sols pour chaque région en fonction des valeurs de capacité d'échange cationique ($\text{cmol}(+)\cdot\text{kg}^{-1}$), de matière organique (en %) et de pH.

Les distributions des parcelles prélevées dans les 2 régions étaient comparables en termes de matière organique. Les valeurs étaient également relativement comparables entre les 2 régions pour la capacité d'échange cationique. Pour les pH les valeurs moyennes étaient de 6,5 et de 7,5 pour la Lorraine et la Bourgogne respectivement, cette variable étant la plus contrastée.

D'autres mesures telles que la quantité d'azote total et de carbone total n'ont pas montré non plus de contraste marqué entre les deux régions. Une analyse granulométrique a été réalisée sur chaque prélèvement (Figure 22). Une grande majorité des sols analysés étaient des sols à dominante argile limoneuse.

Deux sols de Lorraine, LIP et PCP ainsi qu'un sol de Bourgogne, LHP, présentaient des résultats plus contrastés avec des sols à dominante sable limoneux, limon sableux et argile sableux respectivement (SL, LS et AS sur la Figure 22).

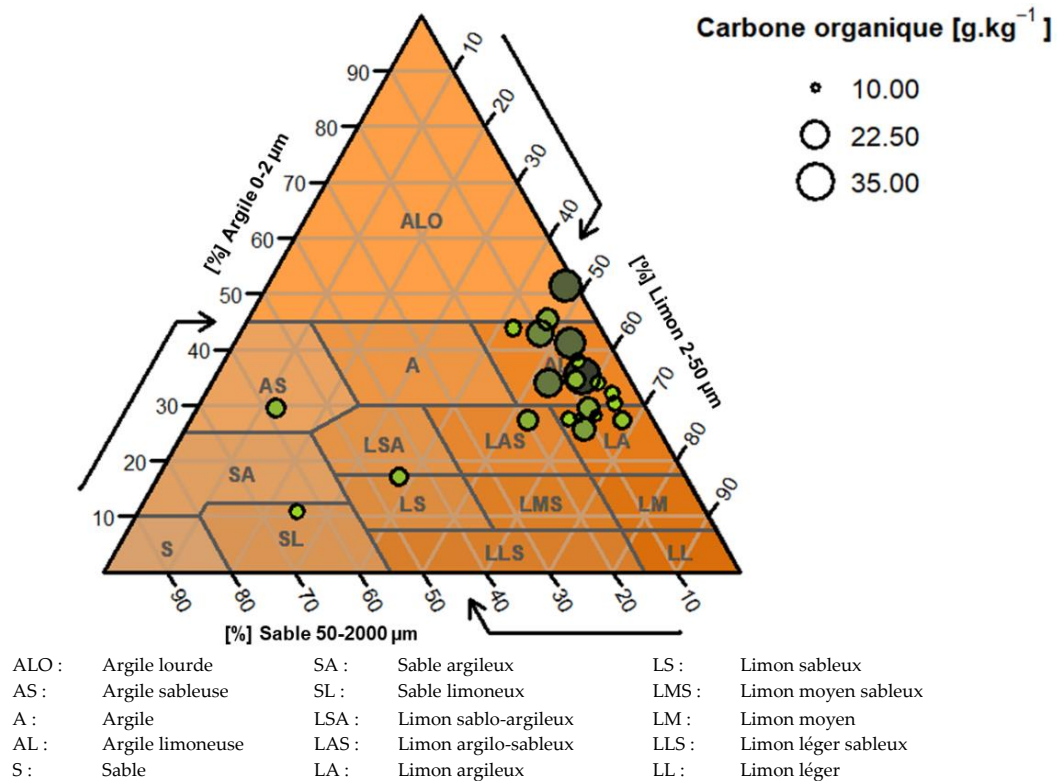


Figure 22 : Triangle de texture des sols de Bourgogne et de Lorraine et diagramme à bulles du carbone organique total retrouvé dans les 22 sols analysés.

Les résultats en termes de quantité de carbone organique étaient cohérents avec les valeurs de matière organique mesurées. Les données observées concernant les caractéristiques des sols, particulièrement les pourcentages de sable, limon, argiles et de CEC, semblent relativement comparables à celles issues de la littérature pour des sols agricoles français (Meite *et al.*, 2022).

Dosage des résidus de fongicides par GC-MS/MS et des éléments traces par ICP-AES.

Le dosage des résidus de fongicides a été réalisé par chromatographie gazeuse triple quadripôle (GC-MS/MS) sur un mélange homogène des 10 prélèvements réalisés pour chacune des parcelles afin de disposer d'un échantillon représentatif de la totalité de la parcelle. Malgré les traitements au metconazole et au prothioconazole appliqués sur certaines parcelles, aucun résidu de ces molécules n'a été détecté dans ces sols. Des résidus de tébuconazole ont par contre été retrouvés dans chacun des sols de Bourgogne et de Lorraine, que ce soit les sols traités, les parcelles non traitées l'année du prélèvement ou les parcelles en agriculture biologique (Figure 23).

Un maximum de 19 ng.g⁻¹ a été observé dans la parcelle DJP (Lorraine). Cinq des 11 parcelles prélevées en Lorraine présentaient également des résidus détectables d'époxiconazole dans des concentrations inférieures à 5 ng.g⁻¹.

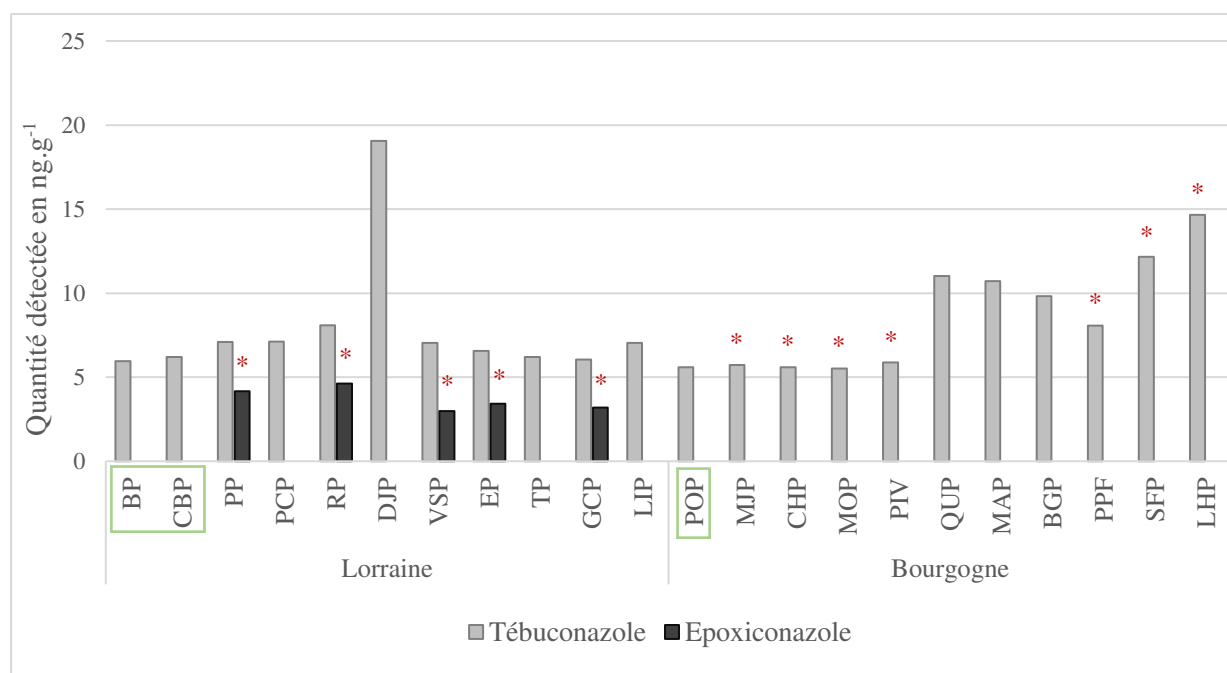


Figure 23 : Dosage en chromatographie phase gazeuse triple quadripôle (GC-MS/MS) des résidus de fongicides triazolés (en ng.g⁻¹ MS de sol) présents dans chaque parcelle. Les parcelles en agriculture biologique sont encadrées en vert ; * : parcelles n'ayant pas été traitées avec la molécule retrouvée l'année concernée.

Ces résultats sont cohérents avec les observations de Hvězdová *et al.* (2018), qui ont montré que sur 75 sols de culture céréalière de République Tchèque, 36% présentaient des résidus de tébuconazole, parmi lesquels 11% présentaient des concentrations supérieures à 10 ng.g⁻¹.

Avec une médiane de 7,05 ng.g⁻¹ de tébuconazole dans les sols, les niveaux de contamination observés dans notre étude sont tout de même relativement faibles par rapport à certaines valeurs de la littérature. Par exemple, Silva *et al.* (2019) ont quantifié 20 ng.g⁻¹ de niveau de contamination médian pour chaque molécule de tébuconazole et d'époxiconazole sur 317 sols agricoles européens. L'époxiconazole a également été détecté dans une étude menée sur 180 sols de culture céréalière en France avec une médiane de 34,6 ng.g⁻¹ (Pelosi *et al.*, 2021). Dans ces différentes études les contaminations maximales observées pour les sols de grandes cultures en Europe étaient de 130 ng.g⁻¹ pour le tébuconazole et 283 ng.g⁻¹ pour l'époxiconazole, des valeurs plus de 10 fois plus importantes que celles observées dans les sols étudiés dans cette étude. Les niveaux maximums de contamination présentés dans la littérature pour ce type d'agriculture restent plus faibles que les maximums observés dans les cultures maraichères (*cf.* partie 4.2.).

Une analyse statistique a été réalisée afin de pouvoir corrélérer la présence de résidus de tébuconazole dans les sols avec le traitement appliqué sur les cultures.

Bien qu'aucune différence statistiquement significative n'ait été mise en évidence, des tendances montrent que les sols non traités l'année des prélèvements et ceux en agriculture biologique sont globalement peu contaminés en tébuconazole (Figure 24). Le même constat peut être fait pour les parcelles traitées au prothioconazole. Le sol le plus contaminé au tébuconazole avait été traité avec cette molécule l'année concernée. Cependant, les autres sols traités au tébuconazole semblent présenter des quantités de résidus de cette molécule plus faible que les sols ayant été traités au metconazole. Une diffusion depuis des parcelles voisines, ou bien une contamination suite à l'utilisation de matériel de traitement commun aux deux molécules pourraient expliquer ces observations.

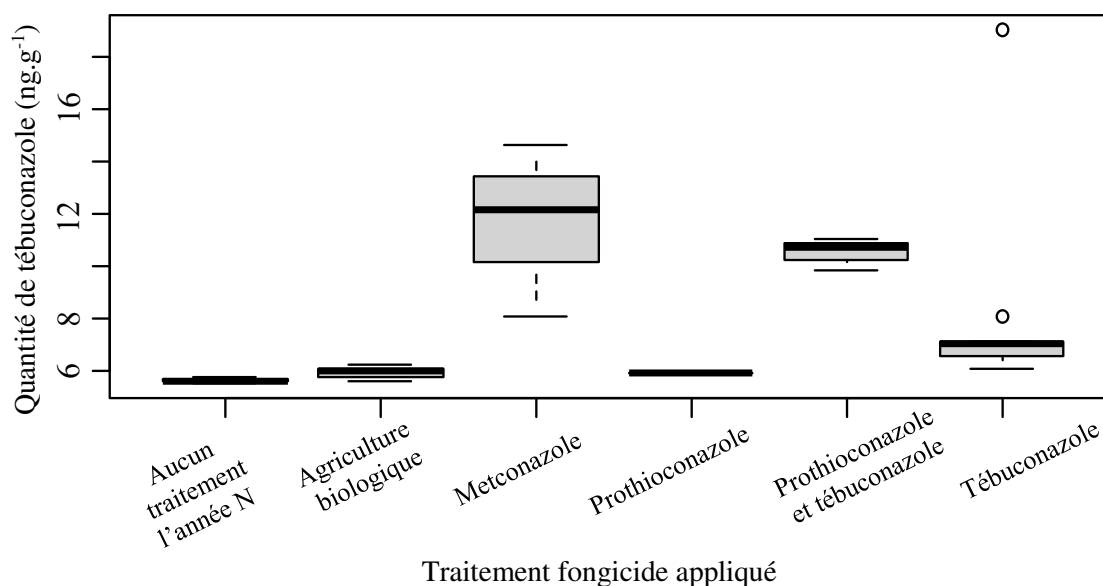


Figure 24 : Quantités de résidus de tébuconazole dans les 22 parcelles étudiées (de Lorraine et Bourgogne) en fonction du traitement appliqué sur les cultures.

Un dosage par ICP-AES des éléments dits majeurs et des éléments traces a été réalisé pour 25 éléments dans chaque parcelle dans l'objectif d'évaluer si la présence de ces éléments dans les sols était susceptible d'influencer l'adsorption des triazolés.

Les valeurs obtenues semblent comparables avec des valeurs classiquement retrouvées dans les sols (Baize, 2000). Certaines valeurs pour l'arsenic, le cuivre et le mercure semblent plus élevées que ce qui est ordinairement observé pour ces types de sol (le mercure pour BP et GCP ; l'arsenic pour DJP, LIP et SFP et le cuivre pour PP, TP, GCP, BP et LIP). Leur utilisation dans d'anciennes formulations fongicides pourrait expliquer ces observations, malgré l'interdiction de leur utilisation en grandes cultures depuis 2016.

Un extrait des valeurs obtenues pour les deux régions pour certains de ces éléments est présenté Tableau 11, la totalité de ces valeurs est présentée en annexe.

Tableau 11: Extrait des valeurs des éléments traces retrouvés dans les sols (en mg.kg-1MS) pour la Lorraine et la Bourgogne. <LDM = inférieur à la limite de détection).

		Eléments traces (mg.kg ⁻¹)						
	Parcelle	As	Cr	Cu	Hg	Ni	Pb	Zn
Lorraine	VSP	18,9	38,9	14,3	< LDM	35,4	28,1	51,6
	EP	15,9	36,8	15,1	< LDM	30,5	28,2	54,3
	TP	18,9	60,2	21,0	< LDM	46,5	35,3	85,2
	DJP	25,1	42,8	17,2	< LDM	33,3	31,3	57,3
	PCP	6,8	23,9	11,1	< LDM	20,5	17,3	63,6
	PP	11,4	42,4	28,7	< LDM	34,8	31,1	74,9
	GCP	< LQM	33,6	23,3	1,4	28,0	24,2	47,8
	RP	23,0	39,9	16,8	< LDM	35,1	38,4	55,3
	LIP	26,8	18,1	20,0	< LDM	8,2	15,3	26,2
	BP	3,9	41,6	40,2	0,7	34,2	31,0	45,4
	CBP	8,5	30,0	10,4	< LDM	20,5	23,7	31,8
Bourgogne	POP	15,6	49,5	18,2	< LDM	33,8	34,3	79,7
	BGP	16,1	34,5	10,3	< LDM	21,5	25,2	49,6
	CHP	8,5	38,1	9,7	< LDM	21,9	28,2	46,1
	PPF	15,6	52,2	15,8	< LDM	33,5	34,7	67,6
	SFP	23,7	54,2	15,0	< LDM	40,1	35,7	74,0
	MJP	9,5	31,5	17,2	< LDM	19,4	22,1	53,2
	LHP	15,2	46,2	17,3	< LDM	31,8	33,4	63,7
	MOP	8,2	29,1	13,4	< LDM	19,2	28,2	49,5
	MAP	13,9	47,6	13,7	< LDM	31,5	32,4	62,9
	QUP	11,8	56,7	16,6	< LDM	32,8	34,7	71,2
	PIV	9,5	34,7	9,8	< LDM	21,4	28,2	47,7

Analyse fongique des prélèvements de sols.

Les 10 prélèvements de sol de chaque parcelle ont étéensemencés sur milieu de culture Dichloran-Glycérol (DG18) pour la pousse fongique et sur des milieux de culture supplémentés en antifongiques Malt-itraconazole à une concentration de 2 mg.L⁻¹ (Mitra) et Malt-voriconazole à une concentration de 1 mg.L⁻¹ (Mvori) (Figure 25). Cette analyse a été faite en triplicat. Différentes conditions de températures ont été appliquées : une culture à 48°C permettant de sélectionner les isolats d'*A. fumigatus* et une culture à 37°C ciblant les espèces susceptibles d'être pathogènes pour l'Homme. Une identification macroscopique et microscopique a été réalisée.



Figure 25 : Exemples de boîtes de culture obtenues après ensemencement de sol et incubation à l'étuve à 48°C (DG18 = Dichloran-Glycérol ; Mitra = milieu malt supplémenté en itraconazole ; Mvori = milieu malt supplémenté en voriconazole), les isolats de la section *Fumigati* sont entourés en rouge.

Au total sur les 2 campagnes de prélèvements, 166 isolats ont été déterminés comme appartenant à la section *Fumigati*. Leur répartition, entre 0 et 36 isolats par parcelle, est présentée Figure 26.

La sensibilité de chaque souche a été évaluée selon la méthode de référence préconisée par l'European Committee on Antimicrobial Susceptibility Testing (EUCAST). Aucun de ces isolats ne s'est avéré résistant. Un séquençage du gène β -tubuline a été réalisé pour les 20 premiers isolats retrouvés sur milieu sélectif (Mitra), permettant de confirmer leur appartenance à l'espèce *A. fumigatus*.

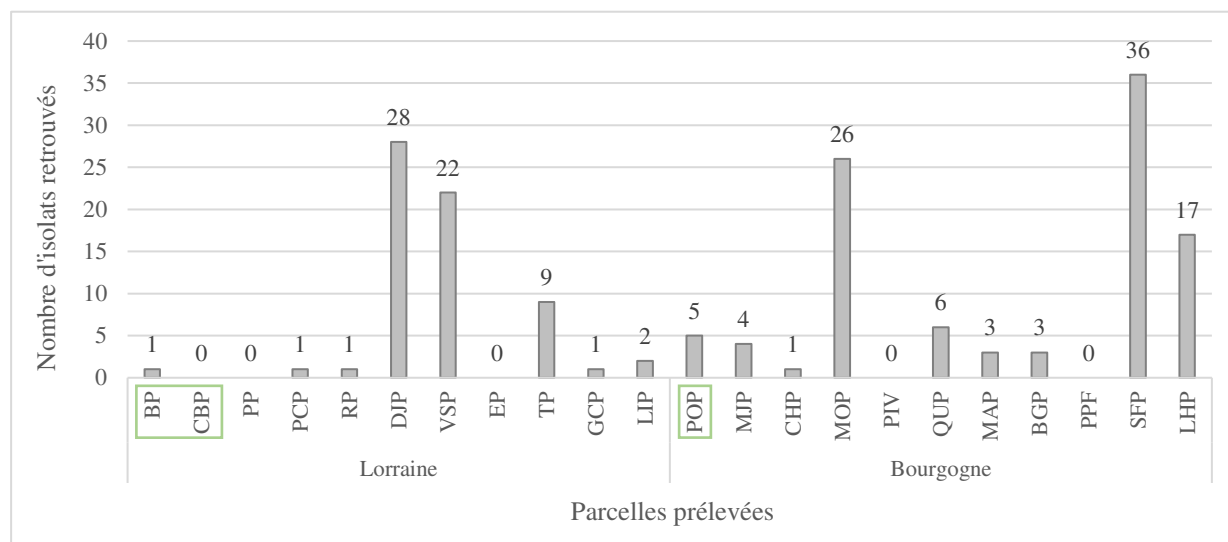


Figure 26 : Nombre d'isolats de la section *Fumigati* retrouvés dans les sols des 2 régions prélevées, les parcelles encadrées en vert sont les parcelles en agriculture biologique.

Dans la littérature, la recherche d'aR-Af dans les cultures céréalières, se fait parfois en lien avec des cas d'Aspergillose décrits chez des patients qui auraient potentiellement pu être colonisés dans leur environnement. Ainsi deux études françaises décrivent des souches résistantes dans les cultures céréalières : 5 souches pan-résistantes aux triazolés, porteuses de la mutation

TR₃₄/L98H, ont pu être mises en évidence dans des champs d'orge (Lavergne *et al.*, 2017) à proximité des logements des patients concernés. Une étude menée dans l'environnement d'un patient agriculteur a montré la présence d'une souche d'*A. fumigatus* résistante sur 128 isolées (0,8%) portant également la mutation TR₃₄/L98H (Rocchi *et al.*, 2014). Pour la première étude citée, aucune information sur les fongicides utilisés n'est communiquée. Pour la deuxième, le champ en question était un champ traité aux triazolés auparavant mais qui n'était pas cultivé l'année des prélèvements.

Des études récentes menées sur la recherche d'aR-Af dans les cultures céréalières à plus large échelle ont montré que les pourcentages de souches résistantes étaient relativement faibles en Europe. Des prélèvements ont été effectués sur des cultures de blé et d'orge en Allemagne sur 3 années consécutives : les pourcentages d'aR-Af présents dans ces sols allaient de 0 à 3% entre 2016 et 2018, la mutation TR₃₄/L98H étant majoritairement la cause de la résistance (Barber *et al.*, 2020). Fraaije *et al.* (2020) ont analysé des sols issus des champs de blé en France, en Allemagne et au Royaume-Uni. Ils ont montré que 2/418 (0,5%) des souches présentaient une pan-résistance aux triazolés due à cette même mutation.

Bien que ces aR-Af puissent être détectés dans les sols des cultures céréalières, les pourcentages de résistance observés en Europe sont inférieurs à certaines valeurs rapportées dans d'autres pays, avec des pourcentages d'aR-Af allant jusqu'à 8,6% dans les rizières en Chine (Cao *et al.*, 2021) et 12,3% dans les rizières en Inde (Chowdhary *et al.*, 2012). Ces niveaux de résistance sont également plus faibles que ceux observés en Europe dans d'autres domaines que la culture céréalière. Par exemple, des études sur des cultures de cucurbitacées et de compost en Italie ont montré des pourcentages de résistance de 21% et 14% respectivement (Mortensen *et al.*, 2010 ; Prigitano *et al.*, 2014 ; Riat *et al.*, 2018).

Toutes ces études sur la détection de la résistance ont été mises en relation avec l'utilisation environnementale des fongicides triazolés. Mais la détection de la résistance semble dépendre directement à la fois des pratiques agricoles utilisées (différentes en fonction des pays) et des domaines étudiés.

Dans notre étude, nous avons voulu voir si la détection des isolats d'*A. fumigatus* présents dans les sols dépendait de la nature de la culture en place sur ces derniers ou du traitement appliqué (Figure 27).

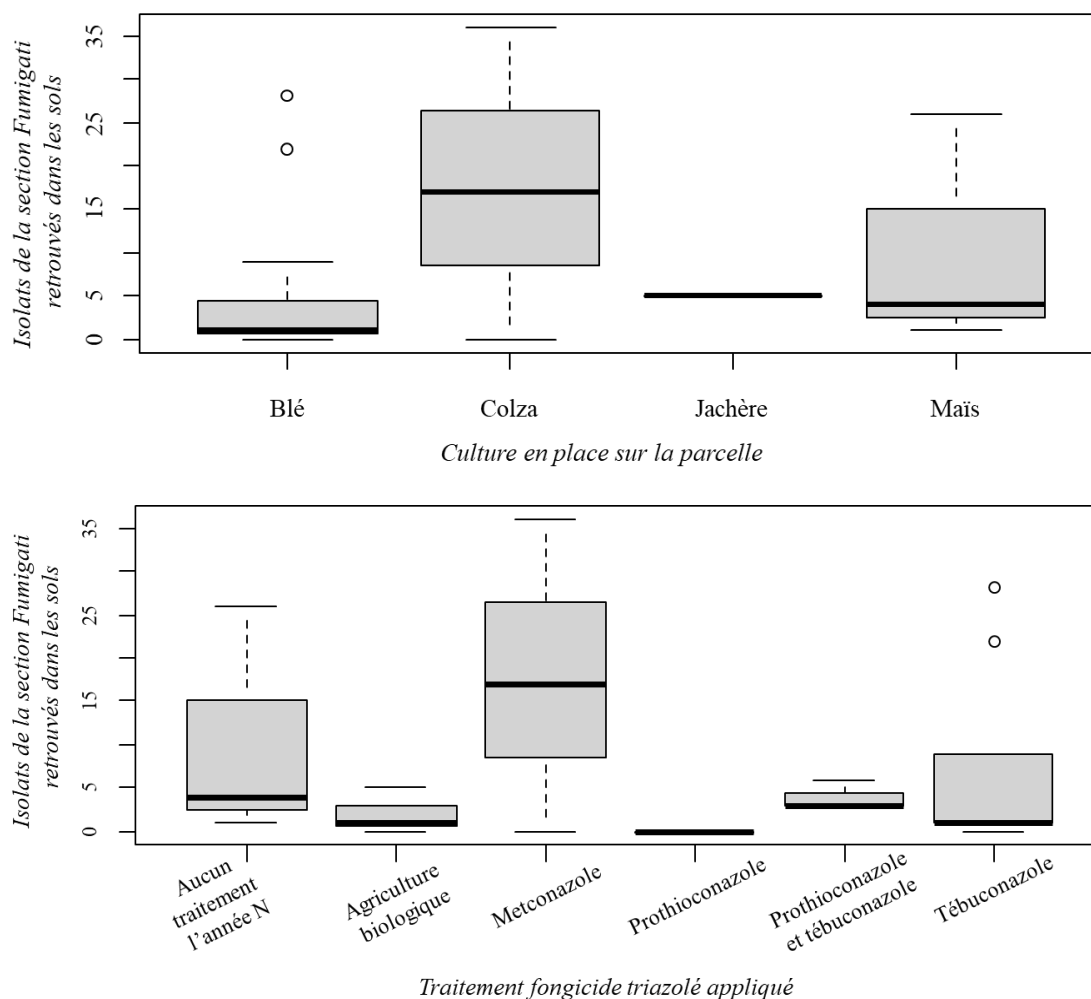


Figure 27 : Nombre d'isolats de la section *Fumigati* retrouvés dans les 22 parcelles étudiées en fonction de la culture en place sur ces parcelles ou du traitement fongicide appliqué.

Le type de céréales cultivé sur les parcelles étudiées n'a pas de lien statistiquement significatif avec la détection des isolats de la section *Fumigati* issus de ces sols. En ce qui concerne la corrélation avec les traitements appliqués, des tendances montrent que les parcelles en agriculture biologique présentaient très peu d'isolats de la section *Fumigati* en comparaison des parcelles traitées, notamment au metconazole. La détection de ces isolats, qui s'est faite en partie sur milieux de culture sélectifs (Mitra et Mvori), a probablement favorisé le développement des isolats qui avaient déjà été exposés aux triazolés lors des traitements des parcelles, par rapport à ceux des parcelles en agriculture biologique.

Evaluation de la proportion d'ADN d'*Aspergillus fumigatus* dans les sols des grandes cultures par qPCR.

Le faible nombre d'isolats identifiés comme appartenant potentiellement à l'espèce *Aspergillus fumigatus* retrouvés après ensemencement des sols des grandes cultures a conduit à l'hypothèse

que ces sols ne réunissaient pas les conditions les plus adaptées au développement de cette espèce. Pour confirmer cette hypothèse, une extraction d'ADN total a été réalisée sur des échantillons de sol représentatifs de chaque parcelle prélevée en Lorraine et en Bourgogne (pool des 10 points de prélèvements de chaque parcelle). Des qPCR ont ensuite été réalisées sur l'ADN extrait de chaque sol : une qPCR panfongique et une ciblant spécifiquement *A. fumigatus*. Les résultats de ces qPCR sont présentés dans la Figure 28.

Une variation importante des quantités d'ADN fongique total a été observée entre les parcelles. Les deux extrêmes sont la parcelle CBP de Lorraine qui présente une quantité d'ADN fongique total 26 fois plus importante que CHP, la parcelle la moins contaminée de Bourgogne. Les sols de Lorraine semblent globalement plus riches en ADN fongique que les sols de Bourgogne.

Les sols en agriculture biologique prélevés en Lorraine présentent des niveaux d'ADN fongique total légèrement plus élevés que les sols traités aux triazolés (Figure 28). Zhang *et al.* (2014) ont montré qu'une exposition des sols au tétraconazole entraînait une diminution de la biomasse microbienne et impactait notamment les populations fongiques, avec des effets persistants dans les sols plusieurs mois après le traitement.

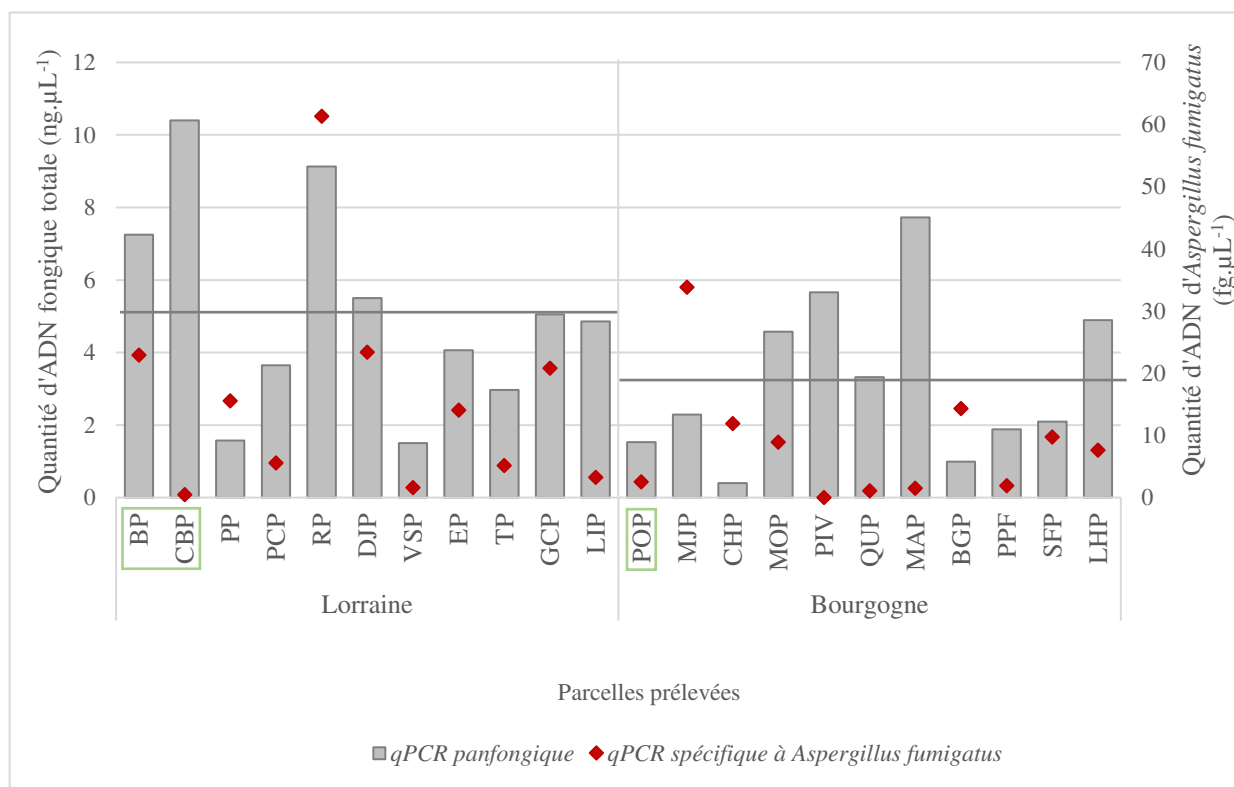


Figure 28 : ADN fongique total retrouvé dans chaque sol des grandes cultures, la moyenne des quantités pour chaque région est représentée par une ligne horizontale (exprimé en ng.µL⁻¹) et quantité d'ADN d'*Aspergillus fumigatus* correspondante (exprimée en fg.µL⁻¹).

La différence observée, bien que non significative (Kruskal-Wallis $p > 0,05$, Figure 29), entre les sols ayant reçu un traitement et les sols non traité l'année des prélèvements ou en agriculture biologique depuis plusieurs années semble aller dans le sens d'un appauvrissement des communautés fongiques dû aux différents traitements appliqués de façon répétée.

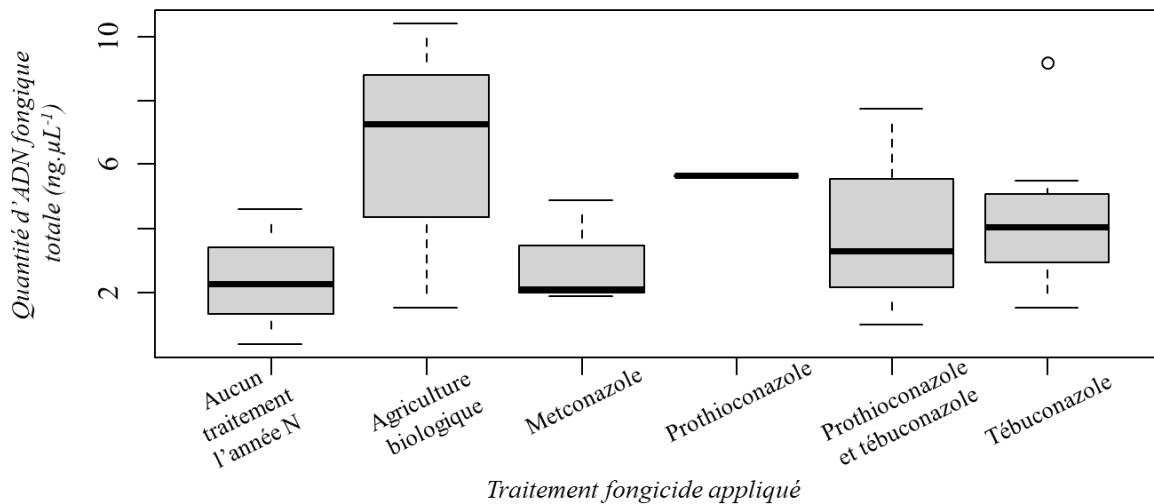


Figure 29 : Quantité d'ADN fongique totale dans les sols en fonction du traitement fongicide appliqué.

Les concentrations d'ADN d'*A. fumigatus* présentes sont de l'ordre de quelques femtogrammes (Figure 28), que ce soit en Bourgogne ou en Lorraine, ce qui représente une très faible proportion de l'ADN fongique total pour chaque parcelle. A notre connaissance, aucune étude n'a été réalisée sur l'évaluation des communautés fongiques des sols par qPCR, particulièrement ciblée sur *A. fumigatus*. Mais les quantités d'ADN déterminées dans les sols des grandes cultures pour cette espèce sont beaucoup plus faibles que celles observées dans les sols des pots de fleurs (cf. partie 4.3.). Ces résultats semblent confirmer que les conditions réunies dans ces parcelles ne sont pas optimales au développement de cette espèce. De fait, ces parcelles ne peuvent pas être considérées comme des « hotspots » potentiels de développement de la résistance. Bien que la détection de souches d'*A. fumigatus* résistantes aux triazolés ait déjà été décrite dans les grandes cultures en Europe, ce phénomène reste relativement rare en culture céréalière en comparaison d'autres domaines où des résidus de ces molécules peuvent être retrouvés, tels que le compost, les déchets issus de bulbes de fleurs ou bien encore le bois traités par exemple (Schoustra *et al.*, 2019 ; Burks *et al.*, 2021).

Caractéristiques physico-chimiques des sols et populations fongiques

Même si les sols des grandes cultures ne semblent pas constituer une niche écologique optimale pour *A. fumigatus*, une analyse en composante principale a été réalisée afin de comparer

l'ensemble des données obtenues pour les sols des grandes cultures et tenter de décrire les tendances concernant les différences de communautés fongiques (Figure 30).

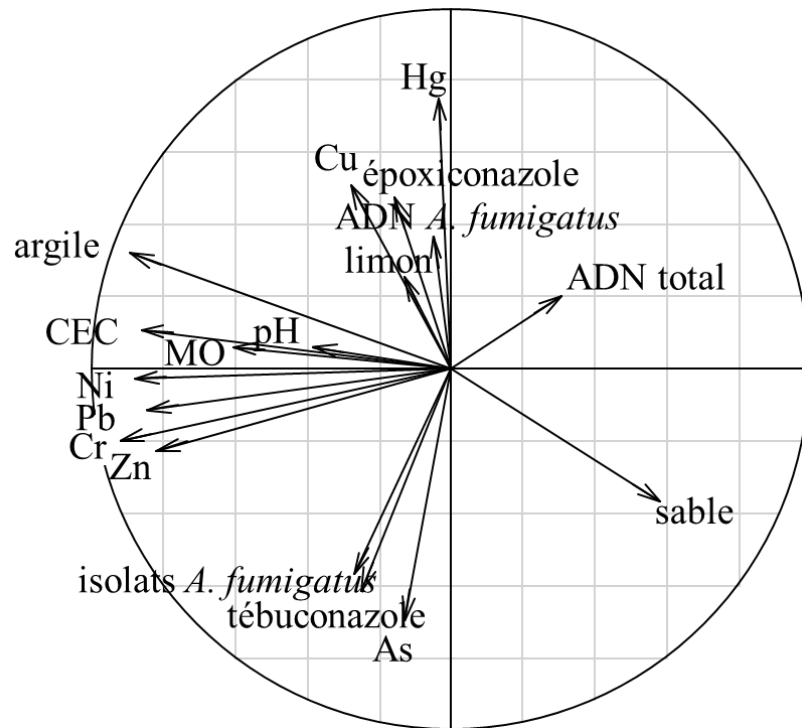


Figure 30 : Cercle des corrélations issu de l'analyse en composante principale des données obtenues sur les sols des cultures céréalières.

Le peu de contraste observé entre les niveaux de contamination en triazolés des sols et leurs caractéristiques physico-chimiques (pourcentage de matière organique ; CEC) n'a pas permis de conclure quant à de potentiels liens entre ces paramètres.

Aucune corrélation significative n'a pu être mise en évidence mais des tendances montrent que les niveaux de contamination des sols à l'arsenic et le nombre d'isolats de la section *Fumigati* retrouvés en cultures pourraient être liés. Des espèces fongiques, dont *A. fumigatus* notamment, sont utilisées pour la remédiation des sols pollués en éléments traces, en particulier l'arsenic (Nierman *et al.*, 2005 ; Maheswari & Murugesan, 2009 ; Pandey *et al.*, 2022) ce qui pourrait expliquer cette tendance. Certains gènes présents chez *A. fumigatus* codent en effet pour des exportateurs d'arsénite, une arsenic méthyltransférase ou bien encore une protéine de résistance à l'arsenic (Nierman *et al.*, 2005). Cependant, aucune preuve de l'utilisation de l'arsenic dans le métabolisme d'*A. fumigatus* ne peut expliquer que sa présence dans les sols favorise le développement de cette moisissure.

Conclusions

La présence des résidus triazolés dans 100% des sols analysés, qu'ils soient issus de l'agriculture biologique, traités l'année des prélèvements ou non traités, démontre l'omniprésence et la persistance de ces molécules. La présence de résidus de tébuconazole dans les sols exploités en agriculture biologique, c'est-à-dire inscrits dans des démarches plus durables depuis de nombreuses années, pourrait indiquer une potentielle diffusion « horizontale » des molécules d'une exploitation traitée à une exploitation voisine. Une contamination issue de l'utilisation d'un même engin agricole sur des parcelles appartenant au même agriculteur peut également être envisagée. Malgré les phénomènes de dégradation, lixiviation et diffusion documentés dans la littérature, une quantité résiduelle minimale ou « quantité stock » pourrait subsister dans les sols. De plus, seules les molécules triazolées mères ont été suivies. Il est fort probable que certains métabolites soient également présents dans ces sols, ce qui augmente le risque d'exposition à ces composés dans l'environnement (Liang *et al.*, 2013 ; Pacholak *et al.*, 2022).

Toutefois ces observations restent difficiles à interpréter, en raison de la complexité à obtenir des informations auprès des exploitants concernant les différentes pratiques agricoles employées (rotation des cultures ; méthodes de traitement ...) ainsi que les données détaillées concernant les traitements appliqués (formulation employée ; fréquence de traitement ...).

La recherche d'aR-Af en culture n'a démontré la présence d'aucune souche résistante. Un faible nombre d'isolats a été retrouvé en culture malgré 3 ensemencements pour chaque sol à 3 conditions de températures différentes. Les analyses de qPCR sur les extraits d'ADN totaux des sols ont confirmé la faible présence d'ADN fongique par rapport aux pots de fleurs (voir partie 4.3.) ainsi qu'une proportion très faible d'ADN d'*A. fumigatus*. Les traitements répétés sur ces cultures peuvent être responsables d'une diminution progressive des quantités d'ADN fongique. Une éventuelle compétition contre des espèces fongiques mieux adaptées (*e.g.* des espèces phytopathogènes) peut expliquer les faibles quantités d'*A. fumigatus* observées.

Pour conclure, au vu des résultats obtenus dans les conditions de cette étude sur les cultures céréalières et bien qu'une contamination des sols aux triazolés (*e.g.* tébuconazole et époxiconazole) ait été constatée dans 100% des parcelles, ce domaine d'application des triazolés ne peut pas être considéré comme un « hotspot » de la résistance pour *A. fumigatus*. Un contact entre les souches fongiques de l'environnement et les résidus et dérivés des triazolés semble inévitable dans ces sols mais les concentrations des résidus observés pour ces molécule

ne semblent pas suffisantes pour induire une pression de sélection. De plus, ces sols ne semblent pas réunir les conditions optimales au développement de la population d'*A. fumigatus*.

4.2. Impact de la présence de résidus de triazolés sur les populations d'*Aspergillus fumigatus* dans les cultures maraîchères

Titre de l'article :

Determination of azole fungal residues in soils and detection of *Aspergillus fumigatus* resistant strains in market gardens of Eastern France.

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Hypothèses de travail :

- L'utilisation des traitements triazolés dans les cultures maraîchères peut être mise en lien avec la présence d'aR-Af dans les sols.
- Les triazolés utilisés en traitement phytosanitaire sont présents dans les sols.

Résumé :

Les triazolés sont utilisés dans de nombreux domaines d'application, notamment dans la culture des fruits et légumes. Afin d'évaluer la présence de ces molécules dans les sols des cultures maraîchères et de déterminer leur éventuel impact sur les populations d'*Aspergillus fumigatus*, des prélèvements de sols ont été réalisés dans 6 exploitations maraîchères de l'Est de la France. Parmi ces exploitations : une était en agriculture biologique, une utilisait des traitements autres que les DMI et les 4 autres utilisaient des traitements à base de triazolés, notamment le difénoconazole. Aucun résidu de triazolés ni aucun aR-Af n'ont été mis en évidence dans les exploitations en agriculture biologique ou utilisant des traitements à base d'autres familles de molécules que les triazolés.

Deux exploitations (notées D et E) parmi celles utilisant des triazolés présentaient des traces de difénoconazole dans 88% des échantillons analysés avec des concentrations allant de 4,6 à 414 ng.g⁻¹. Les dosages de ces résidus ont montré la présence du difénoconazole dans ces parcelles jusqu'à 2 ans après la pulvérisation. Sur ces mêmes sols, 44 aR-Af ont été détectés. Ces isolats présentaient la mutation TR₃₄/L98H ou TR₄₆/Y121F/T289A sur le gène *Cyp51A*.

Il est nécessaire d'identifier les activités professionnelles qui pourraient conduire au développement des aR-Af et de développer des mesures préventives pour endiguer l'émergence de ces résistances et empêcher l'exposition des populations à risque vivant à proximité des activités professionnelles utilisant les triazolés.

Eléments essentiels :

- Les fongicides triazolés sont largement utilisés dans l'environnement pour combattre les moisissures phytopathogènes : on les retrouve notamment dans les cultures maraîchères ;
- 30 prélèvements de sol par parcelle ont été réalisés dans 6 exploitations maraîchères et ont été analysés pour quantifier les résidus de triazolés et pour évaluer la présence d'isolats d'*A. fumigatus* résistants.
- 44 souches résistantes ont été retrouvées sur 2 des 6 exploitations considérées (notées D et E), 87,5% portaient une mutation TR₃₄/L98H et 22,5% une mutation TR₄₆/Y121F/T289A sans qu'aucun génotype commun ne puisse être attribué aux souches résistantes ;
- Le difénoconazole a été retrouvé dans 88% des prélèvements de sols de ces 2 exploitations recourant à l'usage d'un traitement, même celle ayant été traitée l'année N-2 pour la dernière fois, signe de la grande persistance de cette molécule ;
- Les risques d'émergence de la résistance et d'exposition professionnelle sont grands, soulignant l'importance de déterminer quels sont les hotspots impliqués dans ce phénomène.



Determination of azole fungal residues in soils and detection of *Aspergillus fumigatus*-resistant strains in market gardens of Eastern France

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Abstract

Medical azole antifungals are major compounds used to prevent and to treat invasive aspergillosis (IA). Azole fungicides, called DMI (14-alpha demethylase inhibitors), are also widely used for crop protection and have been reported to be linked to azole-resistant *A. fumigatus* (aR-Af) development in the environment. The aim of this study was to determine whether or not market gardens that spray DMIs in Eastern France are also affected by the presence of aR-Af. Forty aR-Afs were detected in soils in only two of the four market gardens using DMIs, with 23% (7/30) and 10% (3/30) of soils containing aR-Af. A total of 87.5% of these isolates had the TR34/L98H mutation and 22.5% the TR46/Y121F/T289A mutation on the *cyp51A* gene. Analyses of residual azole concentrations in soils showed the presence of difenoconazole for up to 2 years after spraying, but only in soils of market gardens where aR-Af was detected. It is very important to identify professional activities that could lead to aR-Af development and to develop preventive measures for at-risk patients living near professional activities using DMIs. We have to better understand why, in some cases, the use of DMI is not linked to aR-Af. Measures should be taken to avoid the use of DMI conferring cross-resistance to preserve the efficiency of human therapeutics.

Keywords DMI fungicides · *Aspergillus fumigatus* resistance · Market gardens · TR34/L98H mutation · TR46/Y121F/T289A mutation · Selection pressure

Introduction

Aspergillus fumigatus is the etiological agent of many diseases, including invasive aspergillosis (IA), which occurs mainly in immunocompromised patients with hematologic malignancies and can reach a mortality rate as high as 50%

(Denning and Bowyer 2013). Medical azole antifungal drugs (itraconazole, voriconazole, posaconazole, and isavuconazole) are major compounds used to prevent and treat filamentous fungal pathogens, with voriconazole recommended as the first-line therapy for treating IA (Groll et al. 2014).

The first azole-resistant *A. fumigatus* (aR-Af) was isolated in the late 1980s (Denning et al. 1997). Since 2008, the number of clinical cases with resistant strains and publications on this subject have both increased considerably (Sweileh et al. 2017). Two routes of resistance selection are described. The first is linked to the long-term treatment of patients with chronic aspergillosis (Arendrup et al. 2010; Camps et al. 2012). However, the emergence of azole-resistant *A. fumigatus* strains is probably due to the other route caused by fungicides in the environment. Azole fungicides, called DMIs (14-alpha demethylase inhibitors), are widely used for crop protection and have been reported to probably induce resistance to *A. fumigatus* directly in the environment (Snelders et al. 2012). Until now, no publication has shown

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that azoles are mutagenic. However, when they are sprayed on crops, selection pressure is exerted on *A. fumigatus* which is widespread. Thus, resistant strains which spontaneously had mutated are preferentially selected. Patients inhale aR-Afs and develop resistant diseases (Snelders et al. 2009). The two main mutations described in the environmental route of resistance are TR34/L98H and TR46/Y121F/T289A (Snelders et al. 2015; Snelders et al. 2008; Van der Linden et al. 2011). They are located on the *cyp51A* gene and confer a multiple triazole resistance. In fact, five azole fungicides (propiconazole, tebuconazole, difenoconazole, epoxiconazole, and bromuconazole) and medical azoles exhibit similar molecular characteristics, leading to a great level of cross-resistance. Thus, colonization and infection of patients with these resistant strains are associated with treatment failure. It is very important to determine how these mutations were selected, and also to identify practices that could lead to resistance development.

DMIs are used for crop protection and as biocides to preserve material (wood, wall-paper paste, clothing, and paint) (Meis et al. 2016). There are more and more studies on the impact of using DMIs in agriculture (cereal and flower crops and compost), whereas its impact on practices in other professional fields remains largely unknown. Therefore, as suggested by Meis et al., it is important to “focus on the questions of ‘where’, ‘when’, ‘how’ and ‘how often’” azole resistance by *A. fumigatus* occurs (Meis et al. 2016). We recently conducted a study in sawmills where propiconazole and tebuconazole were used on a large scale, and TR34/L98H aR-Afs isolation depended on the azole fungicide formulation and residual concentration in soil (Jeanvoine et al. 2017). Another professional field that uses azole fungicides in our region (Bourgogne Franche-Comte region, in Eastern France) is market gardening. Thus, the three aims of this study were to determine whether or not market gardens spraying azole fungicides are also affected by the presence of aR-Afs, to characterize resistant strains (mutations, genotypes), and to assess links between resistance and azole fungicide use.

Material and methods

Environmental sampling

From February to March 2017, six market gardens were included in the study according to their fungicide use. Market gardens consisted of small-scale productions (400 to 2500 m²) of vegetables and fruits, sold directly to consumers and restaurants. A questionnaire dealing with the general organization of the farm operation and vegetable treatment practices (fungicide formulation, frequency of treatment, and sprayed quantities) was given to each market gardener. Four

market gardens used DMIs, one used another fungicide, and the other was an organic farm.

Characteristics of market garden soils using DMIs (collectively referred to as MG-DMI, called C, D, E, and F) and their fungicide treatments are summarized in Table 1. The four MG-DMIs treated their crops three times a year with a fungicide product from three different suppliers (coded 1, 2, and 3 in Table 1), all containing difenoconazole (C and F used the same commercial product, coded 1). Fungicide formulations of products 1 and 3 are similar, but are sold by different suppliers: the difenoconazole concentration (250 g L⁻¹) and the molecules of the mixture are identical with a recommended sprayed fungicide dose of 0.5 L per 10,000 m². The product used by market garden D (coded 2 in Table 1) is composed of a lower difenoconazole concentration than the others (125 vs 250 g L⁻¹) but with a recommended sprayed fungicide dose of 1 L per 10,000 m². It also contains another fungicide of the strobilurin family: azoxystrobin (200 g L⁻¹).

Thirty surface soil samples were taken at all the farms; sampling sites were selected whether fungicide treatments were used or not. For market gardens without azole treatment, the 30 soil samples were distributed throughout the entire farm. For those with azole treatment limited to only certain areas, 20 surface samples were taken in a treated field and 10 in an untreated field. When all the fields were treated with azoles, samples were taken in two different fields not treated the same year, if possible. For each field, the samples were distributed according to a transect over the entire length of the crop.

Soils were seeded as previously described (Jeanvoine et al. 2017) and incubated at 48 °C. Briefly, 2 g of each dissolved soil was seeded on three media: dichloran-glycerol agar (DG18, Oxoid®, Baingstoke, Hampshire, UK), two malt agars supplemented, respectively, with 1 mg L⁻¹ of voriconazole (Maltvori), and 2 mg L⁻¹ of itraconazole (Maltitra).

Azole susceptibility testing and *cyp51A* sequencing

The susceptibility to itraconazole (ITC) and voriconazole (VRC) of *A. fumigatus* isolates isolated on Maltitra and Maltvori was checked using the EUCAST (European Committee on Antimicrobial Susceptibility) microdilution method. Resistance was defined according to the EUCAST breakpoints (> 2 mg L⁻¹) (Arendrup et al. 2012; Hope et al. 2013). Secondly, the susceptibility of one isolate out of 10 isolated on DG18 per soil in market gardens where aR-Afs were detected was also tested using the EUCAST method.

The DNA of azole-resistant isolates was extracted using the DNeasy Plant Mini Kit™ (Qiagen®, Milan, Italy). Then, a part of the beta-tubulin gene (Alanio et al. 2011) was amplified by PCR, purified and sequenced (Applied Biosystems 3130 Genetic Analyzer™ (ThermoFisher®, Waltham, MA))

Table 1 Characteristics of market garden soils using DMIs and fungicide treatment

Market gardens	Type of soil	Organic matter content	Type of treatment and uses (supplier recommendations)			
			Product fungicides (concentrations)	Treatment code	Frequency of treatment (time year ⁻¹)	Sprayed fungicide quantity (L ha ⁻¹)
C	Grass	NA	DFC (250 mg L ⁻¹)	1	3	0.5
D	Clay	1.6–4.5%	DFC (125 mg L ⁻¹)	2	3	1
E	Sandy	1.5–2.5%	Azoxystrobin (200 mg L ⁻¹) DFC (250 mg L ⁻¹)	3	3	0.5
F	Sandy	0.2%	DFC (250 mg L ⁻¹)	1	3	0.5

to validate *A. fumigatus* identification. In order to detect the possible presence of mutations explaining resistance to azole antifungals, the *cyp51A* gene and its promoter were amplified by PCR and sequenced with five sets of primers (Alanio et al. 2011; Mellado et al. 2001).

Finally, susceptibilities to posaconazole (PSC), tebuconazole (TBC), propiconazole (PPC), and difenoconazole (DFC) were also defined by the EUCAST method.

Microsatellite typing

Short tandem repeat (STR) typing was performed for all resistant isolates and a selection of susceptible isolates using nine highly polymorphic microsatellite markers (STRAfs 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, 4C) amplified with three triplex PCRs (de Valk et al. 2005). The reference strain AF293 (CBS 101355) was used as a control. PCR products were analyzed on the Applied Biosystems 3130 Genetic Analyzer with GeneScan™ 400 HD ROX™ size standard (ThermoFisher®, Waltham, MA, USA). Amplicon sizes were determined with GeneMapper software and then transformed in repeat number.

Chemical analysis of soils: DMI dosage

At each farm, three deep samples were taken with auger, the same device as for pedological analyses. Each sample was collected as a drilled 40-cm soil core and then divided into four parts to obtain four depths of azole measures. Five DMIs (propiconazole (PPC), tebuconazole (TBC), difenoconazole (DFC), epoxiconazole (EPC), bromuconazole (BMC)) were sought in all the auger samples at four depths (0–10, 10–20, 20–30, and 30–40 cm). A total of 10 surface samples per site with AR-Afs were also analyzed.

DMIs were extracted by using the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe), and the dosage was performed by gas chromatography (GC) with a GC system 7890A™ (Agilent Technologies®, Massy,

France) coupled with detection by triple quadrupole mass spectrometry (7000 GC-MS/MS model; Agilent Technologies®) as previously described (Jeanvoine et al. 2017). Validation of the method was studied through linearity of the calibration curve established from spiked samples (coefficient of determination > 0.9972), limit of detection calculated at a signal-to-noise ratio of 3 (in the range of 0.7 (EPC) to 4.2 ng g⁻¹ (DFC), precision (19% on average for the 5 studied triazoles), and trueness (between 91 and 107%). For collected samples, analyses were made in triplicate and concentrations were expressed in µg g⁻¹ of soil in dry weight.

Statistical analysis

Statistical analyses were performed using RStudio software (version 3.2.2, Boston, MA). Wilcoxon tests were used to compare resistance and minimal inhibition concentration (MIC) values. Microsatellite genotype distances were calculated using Bruvo's distance (Bruvo et al. 2004). A minimum spanning network was calculated via the *bruvo.msn* function on the *poppr* library (Kamvar et al. 2015).

Results

Market gardens and crop treatment

The four MG-DMIs sprayed difenoconazole on celery and carrots (D, E, F), cabbage (D), or apples (C): C, E, and F in all fields in 2016 and D in one field in 2015 and in another in 2016. Sampling in MG-DMI C was done in an orchard, in three fields of apple trees. Soil was covered with grass. The soils of MG-DMI D, E, and F had recently been plowed. The clay soil of D contained 4.5% of organic matter, and the sandy soils of E and F contained less 2% and 0.2% of organic matter, respectively (Table 1).

Resistance analysis

In total, 566 *A. fumigatus* were detected among the 180 soil samples seeded on DG18 media. For market gardens without DMIs, 5 CFU were detected for garden A and 338 CFU for garden B on DG18 media and only 2 isolates on Maltitra for market garden B. In the four MG-DMIs, 223 *A. fumigatus* were detected on DG18 media, 152 isolated on Maltitra and Maltvori media. Forty isolates of *A. fumigatus* (verified by beta-tubulin sequencing) cultured on Malt supplemented by azoles were really ITC, VRC, and/or PSC-resistant according to breakpoints defined by the EUCAST committee. Other strains isolated on Malt supplemented by azoles had MIC ≤ 1 mg L⁻¹ for ITC and VRC, except for one strain with MIC = 2 mg L⁻¹ for VRC.

The 40 aR-Afs were found in market gardens D and E. For these two market gardens, susceptibilities of 25 *A. fumigatus* isolated on DG18 media were also tested and 4 were resistant for one or more medical azoles (ITC, VRC, and PSC). Thus, the percentages of soils with aR-Afs were 23% (7/30) in D and 10% (3/30) in E, for a total of 44 aR-Afs.

Among the 44 aR-Afs, 34 isolates had the TR34/L98H mutation (77.5%), nine the TR46/Y121F/T289A mutation (22.5%), and one had no mutation in the *cyp51A* gene. Twenty-seven aR-Afs were highly resistant with MIC for ITC and/or VRC ≥ 16 mg L⁻¹. Fifteen (13 TR34/L98H and 2 TR46/Y121F/T289A) were pan-resistant to 3 medical azoles (Table 2). Among aR-Afs, all isolates had high MIC for DFC (≥ 8 mg L⁻¹) except 3 (with MIC = 4 mg L⁻¹). MIC values for VRC and DFC were significantly higher for strains carrying the TR46/Y121F/T289A mutations (Wilcoxon tests: *p* values < 0.01). For ITC, strains carrying the TR34/L98H mutation had higher MIC (*p* value = 0.050).

Susceptible isolates had low MIC for fungicides (≤ 4 mg L⁻¹) except two isolates (numbered 103 and 104 in Table 2 with MIC = 8 mg L⁻¹ for TBC and/or PPC).

Strain genotyping

In total, 43 genotypes were defined from the 65 analyzed isolates (Table 1 in supplemental data). STRAf 3A was the most variable locus with a minimum of 10 repeats and a maximum of 101 repeats. The 21 susceptible strains had 21 different genotypes, with two groups of two and three genotypes with similarities (Bruvo's distance, cutoff < 0.2 represented by a thicker link and dotted lines in Fig. 1).

The 44 aR-Afs were characterized by 22 genotypes, with some isolates genotypically indistinguishable (for example, numbers 36 and 40 from a market garden D field sprayed in 2015, and 66 from a market garden D field sprayed in 2016, Fig. 1 and Table 2). Genotypes showed diversity between aR-Afs with different mutations conferring resistance, but also in the TR34/L98H group. However, for aR-Afs, four groups of genotypes were defined (Bruvo's distance, cutoff < 0.2) and

presented similarities (Fig. 1). Three isolates from market garden D, two TR46/Y121F/T289A and one TR34/L98H, were grouped together and differed only by 38 repeats in STRAf 3A locus. No link was found between resistance/susceptibility profile and genotype.

Dosage of DMI in surface samples

Concerning the dosage of DMIs in drilled soil cores, no azoles were detected in the two market gardens (A and B) that had declared no use of azoles on the initial questionnaire. DFC was only detected in the soil of market gardens D and E, with 1/12 and 3/12 positive samples, respectively. No DFC was detected in market gardens C and F while both used this molecule to treat crops.

The dosage of DMI in the surface samples was carried out for all the samples containing resistant strains: seven in market garden D and three in E; and in the surface samples without resistant strains, three in D and seven in E (Table 3). Only DFC was detected in all of the 20 surface samples, with quantities ranging from 4.6 to 414.5 ng g⁻¹ of soil. In market garden E samples, there was no significant difference in DFC concentrations between substrates with and without resistant strains. Conversely, in market garden D, a slight significant difference was established (Wilcoxon test, *p* value = 0.0497), with the two higher DFC concentrations (360.9 and 414.5 ng g⁻¹ of soil) in substrates without resistant strains.

Discussion

This study, carried out in market gardens, showed the presence of aR-Afs harboring TR34/L98H and TR46/Y121F/T289A mutations, with a diversity of genotypes between susceptible and resistant strains and also between aR-Afs. These strains were found only in crops using azole-based fungicides and for which DFC was found in soils.

The primary mutation was TR34/L98H (77.3% of cases), which is actually the most common environmental mutation (Berger et al. 2017). Other aR-Afs had the TR46/Y121F/T289A mutation, more recently described and less common, and one did not have a mutation on *cyp51A*. Observed differences between MIC for medical azoles (higher for VRC in strains with TR46/Y121F/T289A and higher for ITC for strains with TR34/L98H) have already been described in publications (Garcia-Rubio et al. 2017; Howard et al. 2009; Lavergne et al. 2015). In our study, TR46/Y121F/T289A strains also showed high MIC for DFC. In our previous study in sawmills, where TBC and PPC were detected in soils, no TR46/Y121F/T289A strains were isolated (Jeanvoine et al. 2017). Thus, perhaps depending on the type of fungicide used, the adaptation of exposed strains could be different, with more serious consequences if the selected mutation is correlated

Table 2 Susceptible and resistant analyzed strains isolated in soils and their MIC for three medical antifungal treatments (itraconazole (ITC), voriconazole (VRC), posaconazole (PSC)) and three IDMs (tebuconazole (TBC), difenoconazole (DFC), propiconazole (PPC))

Market garden	Soil sample (year of treatment)	Isolated on	Susceptible or resistant and cyp51A mutations	MIC (mg L ⁻¹)						Genotyping number (Fig. 1)		
				ITC	VRC	PSC	TBC	DFC	PPC			
D	6 (2016)	DG18	susceptible	1	0.5	0.25	4	2	8	92		
	7 (2016)	DG18	susceptible	0.5	0.25	0.25	2	1	4	93		
	10 (2016)	DG18	susceptible	1	0.5	0.25	2	2	8	94		
	15 (2016)	DG18	susceptible	0.5	0.5	0.25	2	2	4	95		
				0.5	0.25	0.25	1	1	4	96		
				1	0.5	0.25	2	2	8	97		
				MaltVori	resistant TR34/L98H	0.0312	2	2	16	16	>16	32
				4	1	2	16	16	>16	33		
				>16	2	2	16	>16	>16	34		
				>16	>16	2	16	>16	>16	35		
				2	4	2	16	8	>16	36		
				2	2	2	16	16	>16	37		
				2	2	2	16	16	>16	38		
	2	2	2	8	8	>16	39					
	2	2	2	8	8	>16	40					
	16 (2016)	DG18	susceptible	0.25	0.5	0.25	2	1	4	98		
		MaltVori	resistant TR46/Y121F/T289A	2	>16	2	16	8	>16	41		
		MaltItra		4	>16	2	16	>16	>16	42		
				2	>16	2	16	>16	>16	43		
				2	>16	2	16	>16	>16	44		
	17 (2016)	DG18	susceptible	0.25	0.25	0.25	2	1	4	99		
				resistant TR34/L98H	2	2	1	16	16	>16	88	
				MaltItra	4	16	2	16	8	>16	45	
					4	>16	2	>16	8	>16	46	
				MaltVori	2	>16	1	16	16	>16	47	
					>16	2	2	8	8	>16	48	
					>16	>16	2	16	8	>16	49	
					>16	4	2	8	8	>16	50	
					>16	4	2	16	16	>16	51	
					>16	4	2	8	16	>16	52	
		>16	4	2	8	8	>16	53				
		>16	>16	2	16	16	>16	54				
			resistant TR46/Y121F/T289A	8	>16	2	>16	>16	>16	55		
				2	>16	2	16	16	>16	56		
	18 (2016)	DG18	susceptible	0.5	0.5	0.25	2	2	4	100		
	19 (2016)	DG18	susceptible	0.25	0.5	0.25	1	2	4	101		
		MaltItra	resistant TR34/L98H	2	2	2	8	8	>16	57		
		MaltVori	resistant TR46/Y121F/T289A	2	>16	16	2	16	>16	58		
	20 (2016)	DG18	susceptible	0.25	0.5	0.25	1	2	4	102		
	21 (2015)	DG18	resistant TR34/L98H	>16	4	1	16	16	>16	89		
MaltItra				4	4	2	8	8	>16	59		
MaltVori				2	2	2	16	8	>16	61		
				resistant TR46/Y121F/T289A	1	>16	2	8	8	16	60	
					2	>16	2	8	>16	>16	62	
25 (2015)	MaltItra	resistant TR34/L98H	4	4	2	8	16	>16	63			
28 (2015)	DG18	susceptible	0.25	2	0.25	8	4	8	103			

Table 2 (continued)

Market garden	Soil sample (year of treatment)	Isolated on	Susceptible or resistant and cyp51A mutations	MIC (mg L ⁻¹)						Genotyping number (Fig. 1)	
				ITC	VRC	PSC	TBC	DFC	PPC		
E	12	DG18	susceptible	0.25	1	0.125	4	2	8	104	
				0.5	1	0.125	4	4	4	105	
				1	1	0.25	2	4	4	106	
				>16	>16	1	>16	16	>16	90	
				MaltItra	2	2	2	16	8	>16	64
				MaltVori	8	2	2	8	8	>16	65
				8	1	2	8	4	>16	66	
				2	4	2	8	4	>16	67	
				>16	2	2	16	16	>16	68	
				0.25	0.25	0.5	1	1	4	91	
				>16	2	2	>16	>16	>16	87	
				MaltVori	2	>16	2	16	16	>16	69
				DG18	0.25	0.5	0.5	2	2	4	107
				MaltItra	2	8	4	8	16	>16	70
				DG18	0.25	0.5	0.125	1	4	4	108
				DG18	0.25	0.5	0.5	2	1	4	109
DG18	0.25	0.5	0.5	2	2	4	110				
DG18	0.25	0.25	0.5	2	2	4	111				
MaltItra	2	16	2	8	4	>16	71				

with strong cross-resistance to VRC, first line therapy treatment (Tissot et al. 2017). Globally, aR-Afs had high MIC for DFC (≥ 8 mg L⁻¹) and cross-resistance with medical azoles (ITC, VRC, PSC) with high MIC for ITC and/or VRC \geq

16 mg L⁻¹. This cross-resistance has recently been described after exposure to DFC and TBC (and not for other DMIs) in experimental conditions (7 weeks under fungicide pressure) (Zhang et al. 2017).

Fig. 1 Minimum spanning network of nine microsatellite loci STRAf using Bruvo's distance. Numbers are genotyping numbers (information about strains are in both Table 1 and Table 1 in supplemental data). Each circle represents a specific genotype, with a size proportional to genotype frequency. Different colors represent the different isolates (susceptible or resistant and their mutations on cyp51A). Link thickness is proportional to genotype similarity. Dotted lines represent groups of genotypes with Bruvo's distance cutoff < 0.2 . Numbers are the genotype numbers of isolates (Table 2)

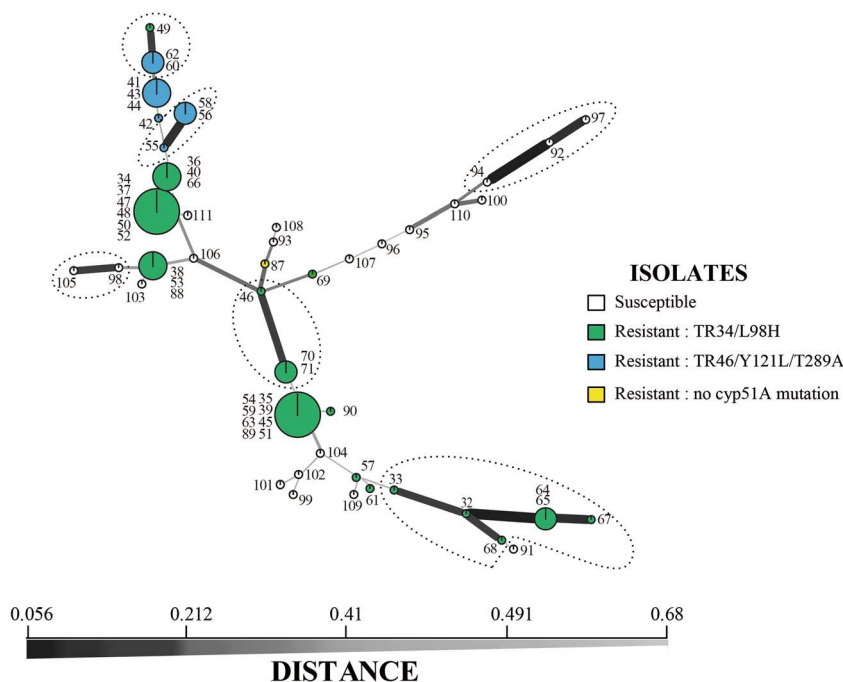


Table 3 Amounts of difenoconazole (DFC) in surface samples in market gardens D and E and number of resistant strains and their mutations on *cyp51A*

Sample number	DFC concentration in surface samples (ng g ⁻¹)	Number of resistant strains and mutations
Market garden D		
25*	4 (<LD)	1 TR34/L98H
28*	4.6	6 TR34/L98H
24*	6.3	/
21*	9.4	3 TR34/L98H-2 TR46/Y121F/T289A
17	22.3	11 TR34/L98H-2 TR46/Y121F/T289A
16	58.7	4 TR46/Y121F/T289A
15	61.1	9 TR34/L98H
19	90.6	1 TR34/L98H-1 TR46/Y121F/T289A
1	360.9	/
5	414.5	/
Market garden E		
2	22.2	/
8	33.9	/
26	34.3	/
16	37.5	1 TR34/L98H
14	44.2	1 TR34/L98H-1 Ø mutation
24	53.3	/
23	66.9	/
28	73.8	1 TR34/L98H
5	86.3	/
27	143.7	/

A few publications have advanced the hypothesis of a common ancestor of TR34/L98H aR-Afs spreading across countries (Badali et al. 2013; Chowdhary et al. 2015), and Alvarez-Moreno et al., for example, found genotypically undistinguishable isolates in different sites in Colombia, thus promoting a clonal spread of aR-Afs (Alvarez-Moreno et al. 2017).

In our study, the genotypic diversity observed between aR-Afs in market gardens is actually quite different. Even if greater diversity was found between susceptible genotypes, there does not seem to be any genotype related to resistance level. We did not find any genotypically undistinguishable isolates in different market gardens but only one common genotype in two different fields (1.5 km apart) of the same market garden (D). Some isolates detected from the same field (not in the same sample) had the same genotype and the same mutations but not exactly the same resistance profile. STRAf loci 3A showed the greatest diversity, as has already been shown for agricultural strains (Ashu et al. 2018). This microsatellite is located on chromosome 4, like the *cyp51A* gene. However, we will probably never be able to find a link between STRAf loci 3A and resistance profile because of an important number of possible recombinations during sexual reproduction.

Few studies have been carried out on vegetable and/or fruit crops (Chowdhary et al. 2014; Prigitano et al. 2014; Sharma

et al. 2015). In a Chinese study conducted in a market garden with fields sprayed with fungicides (Ren et al. 2017), three resistant isolates (two TR46/Y121F/T298A and one TR34/L98H/S297T/F495I) were found in greenhouses with *Luffa* and strawberry plants. In this study, only 2% (3/144) of fungicide-sprayed soils had resistance, with *A. fumigatus* isolated in 40.3% of soil samples. In our study, results were different with 17.9% of soils containing *A. fumigatus* and 10% of soils with aR-Afs. This percentage of soils with resistance varies according to the studies, depending on the number of samples and probably depending on the type of soils, crops, and fungicides. Moreover, the detection of aR-Afs on DG18 (and not by means of a pre-selection with azole-supplemented media) showed that we globally underestimate the presence of aR-Afs.

The four MG-DMI sprayed fungicides contained DFC. Market gardens C, E, and F used fungicides with the same formulation, but aR-Afs were only detected in market garden E. AR-Afs were primarily detected in market garden D which used another fungicide with DFC mixed with azoxystrobin, an inhibitor of mitochondrial respiration. By analogy with SOS response in bacteria (Baharoglu and Mazel 2014), which allows adaptation and evolution when environmental conditions require them, we hypothesize that the observed differences in resistance between the D sites

and other MG-DMIs could be explained by the use of this product. The combination of two fungicides (difenoconazole and azoxystrobin) probably increases stress in strains causing a larger number of mutations (as is the case in bacteria following activation of the SOS response). Moreover, in market garden D, DFC was detected in soils treated in 2016 and 2015, 1 or 2 years before the study, proving that azoles can remain in agricultural soils (Berger et al. 2017). The half-life value of DFC in soil ranges from 103 to 1600 days according to the Pest Management Regulatory Agency (Canada, 2011 Report ERC2011-06) and depends on the frequency of applications, climate, and soil composition (Alvarez-Moreno et al. 2017). Types of soils in the four MG-DMIs were different (containing grass, clay, or sand), and this difference could account for the different results observed between markets C, E, and F. Soils in market garden C were covered with grass which could serve as a retention barrier for fungicide explaining why no aR-Afs were isolated. Soils E and F were very sandy, and only three aR-Afs were found in market garden E. These types of soils are very permeable to water providing better drainage. Therefore, azoles are more diluted due to their hydrophobicity (Komarek et al. 2010). Water and azole molecules do not remain on the surface for very long, which may not allow enough time for aR-Af development. Other aR-Afs ($n = 37$) were isolated in D soils, which contained a considerable amount of clay. This probably facilitated contact between DFC and *A. fumigatus*.

The presence of aR-Afs in market gardens raises the question of workers' exposure. We published a case of immunocompromised farmer who live near his fields and develop an IA with aR-Afs (Rocchi et al. 2014). But, IA does not concern only patients with hematological malignancies and can occur in immunocompetent host after work-related accident in a professional activity using DMIs, as was the case for a wood-worker in our region (Jeanvoine et al. 2016). These two case reports showed that professional activities using DMIs, such as farming or sawmills, may represent a risk of developing infection with aR-Afs.

Market gardens can, indeed, be even more worrying because fields of crops are sometimes closely surrounded by houses. We now isolate resistant strains even in the corridors of the University hospital of Besançon (11 aR-Afs from 1248 air samples (0.9%) in the last 3 years) (unpublished personal data). Several factors could play an important role for infection with aR-Afs including distance to occupational sites where DMI are used, rural, peri urban or urban habitat, occupational activities, level of susceptibility to infection (pulmonary diseases, immunosuppression). Infection with resistant strains could be possible near occupational sites in rural area, with high abundance of aR-Afs but low density of at risk population, or also in hospital, with higher number of at risk subjects but lower abundance of aR-Afs.

Conclusion

Although further studies are clearly needed to identify all possible sources of resistance selection, from our point of view, it is also important to better understand why in some cases, using DMIs does not promote a resistant population. Studies like this one, including detection and genotyping of aR-Afs and quantification of residual DMI in soils, are necessary along with a pedological analysis to define the different types of soils. As therapeutic options for treatment of severe invasive fungal infection in human are very few, better knowledge of parameters involved in resistance, better management of azole fungicides environmental use, and even prohibition of those able to promote cross-resistance in pathogenic fungi are necessary to preserve efficiency of azole medical antifungals.

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DFC difenoconazole, NA not available

LD limit of detection (4.2 ng g⁻¹)

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4.3. Origine de la présence de souches d'*Aspergillus fumigatus* résistantes dans les massifs de tulipes

Titre de l'article:

Azole-resistant *Aspergillus fumigatus* in the hospital: Surveillance from flower beds to corridors

Revue : American Journal of Infection Control, volume 48

Indices de publication : IF=1,92 – Q1

Etat : Publié, 2019

Hypothèses de travail :

- La provenance des isolats d'*Aspergillus fumigatus* résistants présents dans les couloirs du CHU est attribuée à une source extérieure à l'établissement.
- La source de ces isolats peut potentiellement être liée à la présence de massif de plantes ornementales aux abords du CHU.
- Un risque plus important de détecter des résistances est lié à la présence de bulbes de fleurs traitées aux triazolés.

Résumé en français :

La recherche d'*Aspergillus fumigatus* résistants aux triazolés (aR-Af) est réalisée dans l'air intérieur de l'hôpital de Besançon depuis 2015 et a été faite dans des échantillons de sol et de poussière en 2019. Au total, 83 aR-Af présentent une mutation TR₃₄/L98H : 1 dans l'air de l'unité de réanimation médicale, 16 dans les couloirs principaux, 59 issus de prélèvements de terre réalisés dans des bacs de fleurs contenant des tulipes importées des Pays-Bas, et 5 provenant de prélèvements de terre réalisés dans des bacs contenant des arbustes cultivés en pots.

Eléments essentiels :

- Un nombre croissant d'*A. fumigatus* résistants a été détecté dans les couloirs du CHU de Besançon au fil des années ;
- Des prélèvements environnementaux (air et sol de parterres de fleurs) ont été réalisés aux abords du CHU afin de les analyser par culture pour déterminer les sources potentielles des isolats résistants ;

- Aucun isolat résistant n'a été retrouvé dans les prélèvements d'air tandis que 71% des isolats provenant des prélèvements de sols était résistants, porteur de la mutation TR₃₄/L98H dans leur gène *Cyp51A* ;
- Les isolats d'*A. fumigatus* résistants ont principalement été retrouvés dans des pots contenant des bulbes de tulipes traités aux triazolés.



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Brief Report

Azole-resistant *Aspergillus fumigatus* in the hospital: Surveillance from flower beds to corridorsChloé Godeau^a, Gabriel Reboux PhD^{a,b}, Emeline Scherer PhD^{a,b}, Audrey Laboissiere^a, Clothilde Lechenault-Bergerot MSc^a, Laurence Millon^{a,b}, Steffi Rocchi PhD^{a,b,*}^a Chrono-Environnement UMR CNRS 6249, Bourgogne Franche-Comté University, Besançon, France^b Parasitology-Myiology Department, University Hospital, Besançon, France

Key Words:

Triazole resistance
Tulips
TR34/L98H

Screening has been performed for azole-resistant *Aspergillus fumigatus* in the indoor air of the hospital since 2015 and in soil and dust samples since January 2019. In total, 83 azole-resistant *A. fumigatus* isolates with a TR₃₄/L98H mutation have been obtained: 1 from the air of the intensive care unit, 16 from the main corridors, 59 from pots of tulips imported from the Netherlands, and 5 from the soil of trees grown in pots.

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Azole-resistant *Aspergillus fumigatus* (ARAF) have emerged worldwide over the last 20 years and pose an increasing public health problem. ARAF emerge principally in situations in which fungicides with chemical structures similar to medical compounds are used for the treatment of crops and materials in the environment.

The number of clinical cases involving these strains is increasing at the University Hospital of Besançon (UHB), particularly in cystic fibrosis patients (16% of patients with *A. fumigatus*-positive cultures in 2017). Since 2015, the number of ARAF isolates obtained in air of corridors has also increased.

There are 2 possible reasons for this increase in the number of ARAF: (1) the location of the hospital in a geographic area, with high levels of agricultural activity and hotspots of resistance,^{1,2} and (2) the presence of flower beds in the outdoor spaces of the hospital (flowers have been banned in hospital corridors and rooms since 1982), because bulbs have been reported to be a source of ARAF.^{3,4}

Therefore, the primary objective of this study was to develop new measurements of air contamination to determine whether ARAF are brought to the hospital from the rural environment (with resistance hotspots) by prevailing winds. We also investigated the possible role of plants, trees, and flower beds within and around the hospital as sources for ARAF and the spread of these fungi to the hospital.

METHODS

Over the last 17 years, weekly air samples have been collected from the hematology departments and corridors of the hospital (n = 25 air samples per week).⁵ Since December 2018, air samples have also been collected monthly from the intensive care unit.

Since January 2019, external samples have been collected with a homemade dust vane sensor equipped with 2 wipes, placed on a second floor terrace (the hematology units being located on this floor), to capture dust carried by the prevailing westerly wind (Fig 1). This easy-to-use inexpensive device was developed for this study to be able to collect outdoor dust for 1 month, even in windy conditions, and allowed both culture and qPCR analyses to detect *A. fumigatus*, according to a previously described protocol.⁶

Dust samples were also collected by aspiration on a filter placed at the end of a vacuum cleaner, close to the emergency door of the intensive care hematology department, in the corridor along zone A, and in an area in which hematology consultations take place.

We identified 4 soil sampling areas within the hospital and in the surrounding environment. Zone A corresponds to an outdoor relaxation area within the hospital with flowerbeds, zone B consists of the flowerbeds in front of the hospital entrance, zone C is an indoor area below the hospital reception area, in which there are trees grown in pots, and zone D corresponds to an outdoor area, at level -1, below zone A, with flower beds (Fig 1).

ARAF were screened (in the air, dust, and soil samples) by culture on 2 media containing itraconazole (2 mg/L) and voriconazole (1 mg/L). The EUCAST method was used to test for resistance. *Aspergillus* identification and *cyp51A* gene mutation were assessed by sequencing the

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Conflicts of interest: None to report.

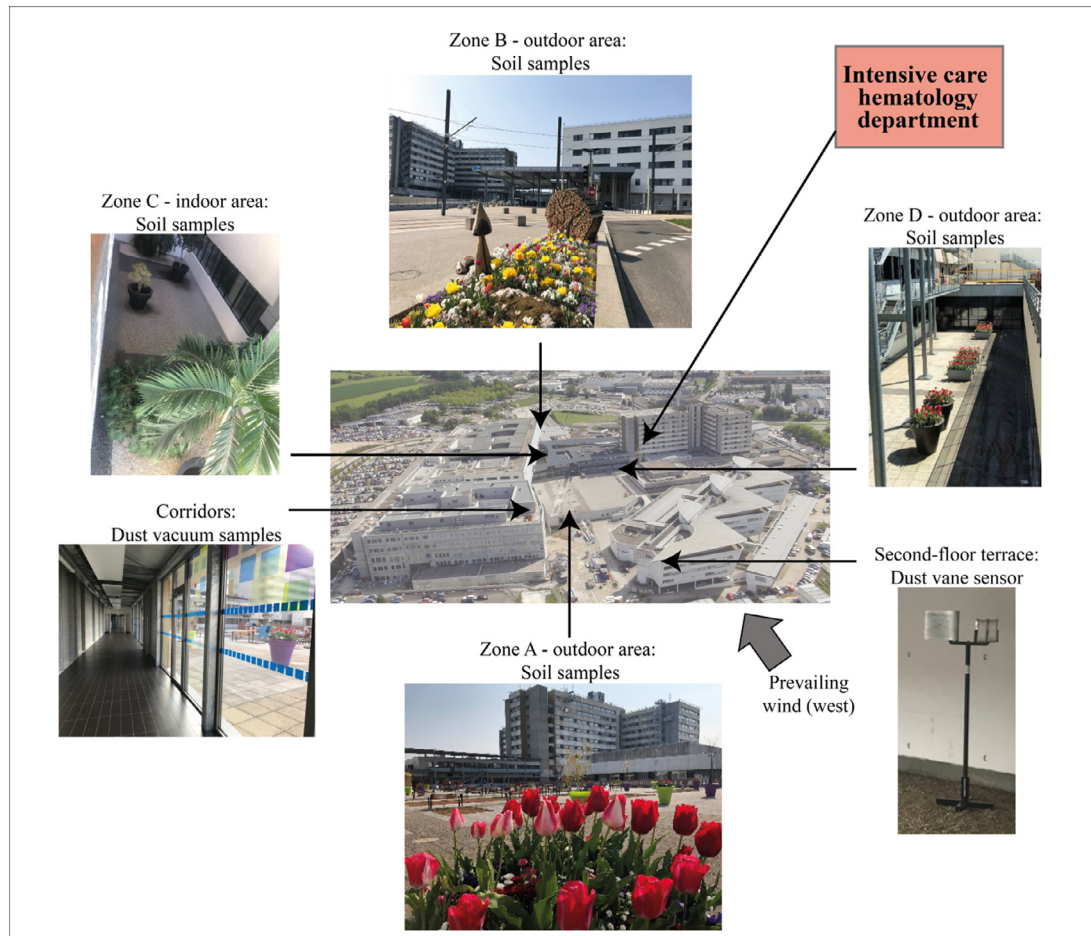


Fig 1. Aerial view of the hospital and photographs of the various areas sampled. The prevailing wind is westerly and represented by an arrow. (Source of the aerial view: Besançon Town Hall).

β -tubulin gene and *cyp51A* and its promoter and/or with the Fungiplex Aspergillus and Fungiplex Azole-R Bruker kits (Bruker Daltonik GmbH, Bremen, Germany).

RESULTS

Several cases of IA are diagnosed annually at UHB (eg, 6 in 2016, 16 in 2017, 12 in 2018, and 6 in 2019), and 3 cases of IA owing to ARAf (TR₃₄/L98H mutation) have been diagnosed since 2012: 2 in immunocompromised patients from hematology department (1 farmer using triazoles and 1 living in a farm in the middle of cultivated fields) and 1 immunocompetent woodworker who suffered facial destruction as a result of a work accident with a piece of wood.^{2,7,8} For now, we did not change antifungal regimens for prophylaxis or treatment, however, since 2015 we have extended the antifungal susceptibility testing (E-test) to every *A fumigatus* strains isolated in at-risk patients.

Seventeen ARAf isolates have been obtained from the indoor air of UHB: 1 in 2015, 1 in 2016, 7 in 2017, 3 in 2018, and 5 in 2019 (first half of the year). All of the isolates had the TR₃₄/L98H mutation (*cyp51A* sequencing). One was detected in the intensive care unit, and the other 16 were found in the main corridors. Two ARAf isolates with the TR₃₄/L98H mutation were also detected in dust collected by aspiration at the front of the intensive care hematology department and in the corridor along zone A.

The 85 soil samples yielded 137 isolates from zones A, B, and D that grew on azole media: 90 *A fumigatus* and 47 *Neosartorya fisheri* (zone B). Overall, 71% (64/90) of the *A fumigatus* isolates were resistant to at least 1 azole, and all of the isolates had the TR₃₄/L98H

mutation: 59 ARAf were obtained from tulip pots imported from the Netherlands, and 5 were found in the soil of pots containing trees.

The external sensor detected no ARAf, suggesting that the ARAf detected in the hospital are not carried in by the prevailing winds from surrounding hotspots of resistance.

DISCUSSION

The configuration of the area experiencing ARAf contamination (zones A and D) and the absence of ARAf detection by the outdoor sensor suggest that the prevailing wind is blocked by the walls of the buildings around the esplanade, which create an obstacle, and that this results in the resuspension of ARAf spores from the flower beds into the air. To confirm the hypothesis that the flower beds or imported tulips were the actual 'source' of ARAf isolates obtained from hospital corridors or patient clinical specimens, typing analysis (genotyping or whole-genome sequencing) are needed. The results showed the interest of increasing vigilance on the surveillance of resistant strains in our hospital. The hospital committee for the control of nosocomial infections has already decided to stop planting bulbs in the outdoor areas surrounding the hospital. In 2020, other alternatives will be proposed, such as the planting of other types of flowers of controlled origin or of bulbs produced through organic farming methods.

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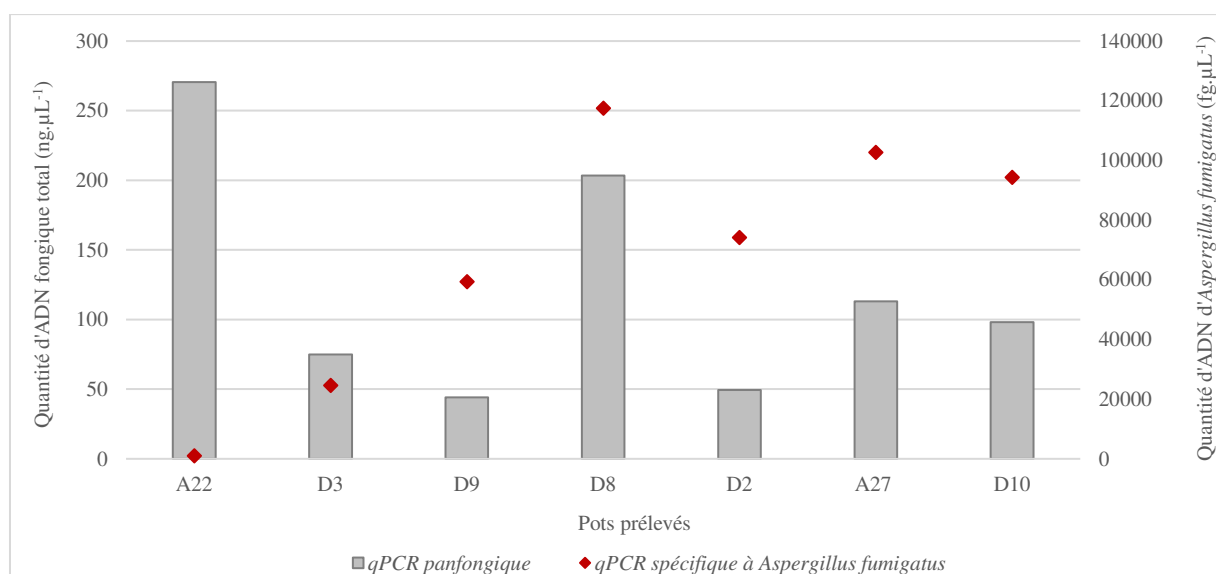
The Intersection of Hand Hygiene, Infusion Pump Contamination and High Alarm Volume in the Healthcare Environment

Résultats complémentaires de l'article :

Objectif : Afin de pouvoir comparer les résultats de qPCR obtenus pour les sols des grandes cultures (ne présentant aucune résistance), des qPCR ont été réalisées sur certains sols des pots de tulipes (qui présentent globalement le plus de résistance).

Sur l'ensemble des sols des pots prélevés dans cette étude, 7 ont été choisis pour évaluer les quantités d'ADN fongique total et d'*A. fumigatus* que l'on pouvait retrouver dans ces sols par qPCR. Parmi ces sols, 6 présentaient des isolats d'*A. fumigatus* en culture et chacun d'eux contenait au moins un aR-Af (notés D3 ; D9 ; D10 ; A27 ; D8 et D2 dans la Figure 31). Le dernier sol choisi (A22) ne présentait aucun isolat d'*A. fumigatus* en culture.

Le niveau d'ADN fongique retrouvé dans ces sols était de l'ordre de plusieurs centaines de $\text{ng}\cdot\mu\text{L}^{-1}$ ce qui est en moyenne 30 fois plus important que pour les sols des grandes cultures, probablement le signe d'une population fongique riche (Figure 31). Les quantités d'ADN d'*A. fumigatus* allaient jusqu'à plusieurs centaines de milliers de $\text{fg}\cdot\mu\text{L}^{-1}$. En moyenne ces sols présentaient des quantités d'ADN d'*A. fumigatus* 5000 fois plus importantes que les sols des grandes cultures.



A22 : Pas de souche résistante

D3 : 1 souche résistante

D9 : 3 souches résistantes

D8 : 4 souches résistantes

D2 : 4 souches résistantes

A27 : 5 souches résistantes

D10 : 8 souches résistantes

Figure 31 : ADN fongique total (exprimé en $\text{ng}\cdot\mu\text{L}^{-1}$) retrouvé dans 7 sols issus des pots de fleurs des zones A et D et quantité d'ADN d'*Aspergillus fumigatus* correspondante (exprimée en $\text{fg}\cdot\mu\text{L}^{-1}$). Les sols ont été classés par ordre croissant de souche(s) d'*Aspergillus fumigatus* résistantes retrouvées en culture.

L'ADN d'*A. fumigatus* a été retrouvé dans tous les sols analysés, avec une quantité beaucoup plus faible pour le sol noté « A22 » pour lequel aucun isolat n'avait été retrouvé en culture. La nature même du sol utilisé pour la plantation de ces fleurs en pot (terreau) semble particulièrement favoriser le développement des populations fongiques.

4.4. Quels moyens pour limiter l'émergence de la résistance environnementale ?

4.4.1. *Les bulbes comme source de résistance, rétablissement d'une population sensible aux fongicides en changeant les pratiques*

Titre de l'article :

One year later: The effect of changing azole-treated bulbs for organic tulips bulbs in hospital environment on the azole-resistant *Aspergillus fumigatus* rate

Revue : Medical Mycology, volume 59

Indices de publication : IF=3,353 - Q1

Etat : Publié, 2021

Hypothèses de travail :

- La présence de bulbes traités aux triazolés dans les massifs de fleurs situé aux abords du CHU est la source de diffusion d'isolats d'*Aspergillus fumigatus* résistants.
- En remplaçant les bulbes de fleurs traités aux fongicides azolés par des bulbes issus de l'agriculture biologique, la source d'isolats résistants sera stoppée.
- Une population d'isolats sensibles remplacera la population résistante en l'absence de cette pression.

Résumé en français :

Les bulbes de plantes traités aux azolés ont déjà été évoqués comme une explication potentielle de la diffusion mondiale d'*Aspergillus fumigatus* résistants aux azolés (aR-Af). Nous avons précédemment souligné la présence d'un taux élevé d'aR-Af (71% des *A.fumigatus* isolés de milieux supplémentés en azolés) dans des parterres de fleurs contenant des bulbes traités aux azolés aux abords de l'hôpital. Ici nous montrons que la plantation de bulbes d'origine biologique peut être une solution pour réduire le taux d'aR-Af (passant de 71% à moins de 3%). Ces résultats suggèrent que le remplacement des bulbes traités par des bulbes biologiques peut être suffisant pour retrouver une population majoritairement sensible en seulement 1 an.

Eléments essentiels :

- Une analyse des sols provenant de pots de fleurs bordant le CHU de Besançon a été effectuée un an auparavant. Sur les isolats d'*A. fumigatus* retrouvés, 71% étaient résistants aux triazolés (*cf.* partie 4.1) ;
- Les isolats ont été principalement retrouvés dans des pots de terre contenant des bulbes de tulipes traités aux triazolés. La décision a été prise de les remplacer par des bulbes issus de l'agriculture biologique sans pour autant changer le sol ;
- Un an plus tard, le même protocole a été réalisé sur ces mêmes pots de fleurs : seulement 3% des isolats d'*Aspergillus fumigatus* retrouvés dans les sols étaient alors résistants ;
- Le remplacement des bulbes traités par des bulbes issus d'agriculture biologique semble favoriser le rétablissement d'une population sensible (*wild type*) en un an.



Brief Report

One year later: The effect of changing azole-treated bulbs for organic tulips bulbs in hospital environment on the azole-resistant *Aspergillus fumigatus* rate

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Abstract

Azole-treated plant bulbs have already been evoked as a potential explanation of the worldwide spread of azole-resistant *Aspergillus fumigatus* (ARAF). We previously pointed out the presence of a high rate of ARAF (71% of *A. fumigatus* detected on azole-supplemented media) in flower beds containing azole-treated bulbs at the hospital's surroundings. We show here that planting organic bulbs can be a solution to reduce ARAF burden (from 71% rate to below 3%). The results suggest that replacing treated bulbs with organic bulbs may be sufficient to regain a population that is predominantly susceptible in just 1 year.

Lay Summary

Antifungal resistance is increasingly observed in fungal pathogens. This study argues that planting organic bulbs in hospitals' outdoor surroundings could be a good alternative to continue to beautify green spaces, without the risk of dissipating antifungal-resistant fungal pathogens.

Key words: *Aspergillus fumigatus*, azole resistance, organic tulips bulbs, hospital environment, TR₃₄/L98H qPCR detection.

Fungal aerocontamination monitoring is carried out for 18 years in our hospital, with weekly air samples collected from the haematology departments and general corridors.¹ Given the global emergence of triazole resistance in *Aspergillus fumigatus*, in both clinical as well as environmental isolates and extension of the phenomenon to all countries in Europe and to all continents, this environmental air monitoring has been extended in 2015 to include research on azole-resistant *A. fumigatus* (ARAF). Since 2017, several ARAF with TR₃₄/L98H mutation were detected in the air of general corridors each year: 7 in 2017, 3 in 2018 and 5 in 2019.

In 2019, a study was conducted to determine whether plant, tree and flower beds, ornamenting a new terrace at a central location in hospital, could be sources of ARAF, especially since the presence of tulip bulbs was described as a potential route

of ARAF dissemination.^{2,3} We performed 85 soil samples and 71% (64/90) of *A. fumigatus* detected on azole-supplemented media were resistant, had TR₃₄/L98H mutation and most of them (92%) were detected in pots containing tulips.⁴ After information taken from the gardener and ornamental plant seller, we knew that the bulbs had been treated with azole fungicides.

Following these results, it was decided to order bulbs from organic farming through the same plant manufacturer. Since soil in the pots had not been changed, we expected a similar resistance rate, or at best, a small decrease in the number of resistant strains. The objective at this stage was to annually monitor the pots in order to assess (or not) the presence of ARAF and evaluate the fungal contamination induced by organic plants.

In February 2020, controls were made in 10 pots, already sampled in 2019, containing or not ARAF (Table 1). The isolation

Table 1. Comparative distribution of *A. fumigatus* isolates found, including resistant ones (ARAF), between the 2019 sampling campaign (treated bulbs) and same sampling plots controlled in 2020 (organic bulbs)

	2019 sampling		2020 sampling	
	Number of <i>A. fumigatus</i> isolated on antifungal supplemented media	Number of ARAf per plate*	Number of <i>A. fumigatus</i> isolated on antifungal supplemented media	Number of ARAf per plate*
Pot 10-D	15	14	30	0
Pot 11-D	9	8	13	1
Pot 8-D	13	7	20	0
Pot 9-D	13	6	9	1
Pot 27-A	8	5	11	0
Pot 6-A	1	1	1	0
Pot 25-A	2	2	0	0
Pot 4-D	2	2	7	0
Pot 1-A	0	0	0	0
Pot 15-A	0	0	0	0
Total	63	45	91	2

* 2 g of each substrate, dissolved with 10 ml of 0.1% Tween-80 solution (Merck), vortexed for 1 min and seeded on media. Resistant isolates are highlighted with shaded entry.

of *A. fumigatus* was carried out as previously described on malt agar supplemented by itraconazole (2 mg l⁻¹) and voriconazole (1 mg l⁻¹) at 48°C.⁴ The EUCAST method (broth microdilution) was used to assess minimal inhibition concentration (MIC).⁵ The DNA of azole-resistant isolates characterized by EUCAST was extracted using the DNeasy Plant Mini KitTM (Qiagen®, Milan, Italy) and identification of *A. fumigatus* isolates was validated by PCR amplification and sequencing a part of beta-tubulin gene.⁶ The search for the main ‘environmental’ mutations on *cyp51A* gene (TR₃₄/L98H and TR₄₆/Y121A/T289A) was done with the Fungiplex Azole-R Bruker kit (Bruker Daltonik GmbH, Bremen, Germany).

This kit has been previously evaluated on 97 DNA extracts of our ARAf collection for which *cyp51A* gene sequencing^{6,7} was previously performed (84 TR₃₄/L98H, 9 TR₄₆/Y121A/T289A and four resistant isolates without *cyp51A* mutation, from clinical and environmental samples). After various DNA dilution tests, qPCR was validated for an optimal DNA concentration between 0.2 and 10 ng/μl, with 100% of strains carrying TR₃₄/L98H or TR₄₆/Y121A/T289A detected and 100% of resistant strains without TR mutation not detected.

In the 2020 sampling campaign, only two pots (Table 1) present one ARAf identified with MIC of 4 mg l⁻¹ for voriconazole and >16 mg l⁻¹ for itraconazole. Both strains had TR₃₄/L98H mutation.

Hypothesis was that ARAf was brought by tulip bulbs and could spread in pots, resistant isolates may be able to persist over months in the environment,⁸ with duration of persistence not known. In our study, the number of ARAf found in flowers beds had greatly decreased, from 71% (rate of resistant isolates out of the number of isolates detected on antifungal media) in 2019 to <3% in 2020. It seems that the simple act of removing the azole-treated bulbs is enough for a susceptible population to re-

establish itself in 1 year, gaining the upper hand over the resistant ones that were largely dominant.

For TR₃₄/L98H isolates, fitness cost has never been described yet. For others ARAf (mutations from patients acquired resistance route or other mechanisms without *cyp51A* mutations) observations are more mixed: *In vitro*, the growth of the mutants (G54W and M220K) was similar to those of the parental strain.⁹ In opposition, azole-resistant isolates (from patient-acquired resistance route) showed reduced growth rates, abnormal colony morphology or lack of sporulation when grown in the absence of triazoles.¹⁰ In these cases, ARAf are not able to compete with wild-type isolates in non-azole environment.¹¹ It is possible that the same thing happened in the flower pots, where, when the source of azole (bulb) is removed, a sensitive population becomes dominant.

For now, only four studies were interested in the ARAf linked to the bulbs.^{2-4,12} and they emphasized that ARAf can be disseminated by international trade. Although the observation of resistance related to bulbs is not observed everywhere,¹³ it may be time to find ways to limit this diffusion. This study was limited to one hospital and even if the decrease in ARAf rate may also be favored by environmental factors that occurred in 2020 (such as weather) the results justify a precautionary principle, to stop putting fungicide-treated bulbs in hospital surroundings.

Conversely, in the most recent study on bulbs conducted in Japan, the authors have also investigated a method of disinfecting plant bulbs contaminated with ARAfs, using benomyl and prochloraz.¹² But, in this context where we show that fungicides in agrosystems have an unintentional impact on public health, the overuse of fungicides is certainly not a good way to go in the long term. However, the planting of ornamental plants from organic agriculture could be a good alternative to reduce risk

to dissipate ARAf in the hospital environment where numerous patients at risk are hospitalized in hematology ICU.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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Le changement des pratiques d'une agriculture intensive (utilisant les triazolés) vers une agriculture biologique a permis, pour le cas des plantes ornementales, de réduire les quantités d'*A. fumigatus* résistantes. Endiguer le phénomène de cette diffusion de la résistance est particulièrement important quand il est associé à l'exposition directe d'une population à risque de développer des pathologies fongiques graves, par exemple aux alentours de l'hôpital. Dans d'autres types d'agriculture, les alternatives d'agriculture biologique ne sont pas si aisées à mettre en place de façon systématique. Il apparaît cependant indispensable de prendre conscience qu'un usage plus raisonné de ces molécules est essentiel, par exemple en culture céréalière. De plus, trouver des moyens applicables à plus petite échelle (*e.g.* industrie du bois, cultures maraîchères) pour limiter la diffusion de ces molécules triazolées et éviter les impacts dont elles sont responsables est également nécessaire.

4.4.2. *Cyclodextrines, présentation et intérêt pour un usage environnemental*

Limiter la pollution environnementale qui touche tous les compartiments et peut trouver sa source dans de nombreux domaines, l'industrialisation, l'agriculture intensive, la déforestation ou encore l'utilisation des combustibles fossiles (Hayzoun *et al.*, 2014 ; Perera, 2018 ; Kopittke *et al.*, 2019 ; Sánchez-Bayo & Wyckhuys, 2019 ; Austin *et al.*, 2019) est devenu une nécessité.

La contamination des sols par les fongicides triazolés s'inscrit pleinement dans cette problématique. Des « technologies vertes » sont ainsi à développer dans le but de répondre à ce défi. Le cahier des charges du développement de ces technologies réside dans le fait de diminuer l'exposition des écosystèmes aux polluants en utilisant des ressources renouvelables et des biomatériaux (Fenyvesi & Sohajda, 2022). Dans cette optique, nous nous sommes intéressés aux cyclodextrines, des molécules cages issues de la dégradation de l'amidon, afin d'évaluer leur potentielle capacité à limiter la pollution environnementale aux triazolés.

Les cyclodextrines.

Les cyclodextrines (CD) sont des molécules cages composées de résidus glucopyranoses. Ces molécules sont produites grâce à la digestion enzymatique de l'amidon (*e.g.* de maïs ou de pomme de terre) (Slomińska & Sobkowiak, 1997). Elles présentent une structure de cylindre conique avec une cavité indéformable et hydrophobe qui leur confère la capacité d'encapsuler d'autres molécules au moyen de relation de type « hôte-invité ».

Il existe 3 molécules natives (Figure 32), qui diffèrent par le nombre de résidus qui les constituent, l' α -CD, le β -CD et le γ -CD (D'Souza & Lipkowitz, 1998).

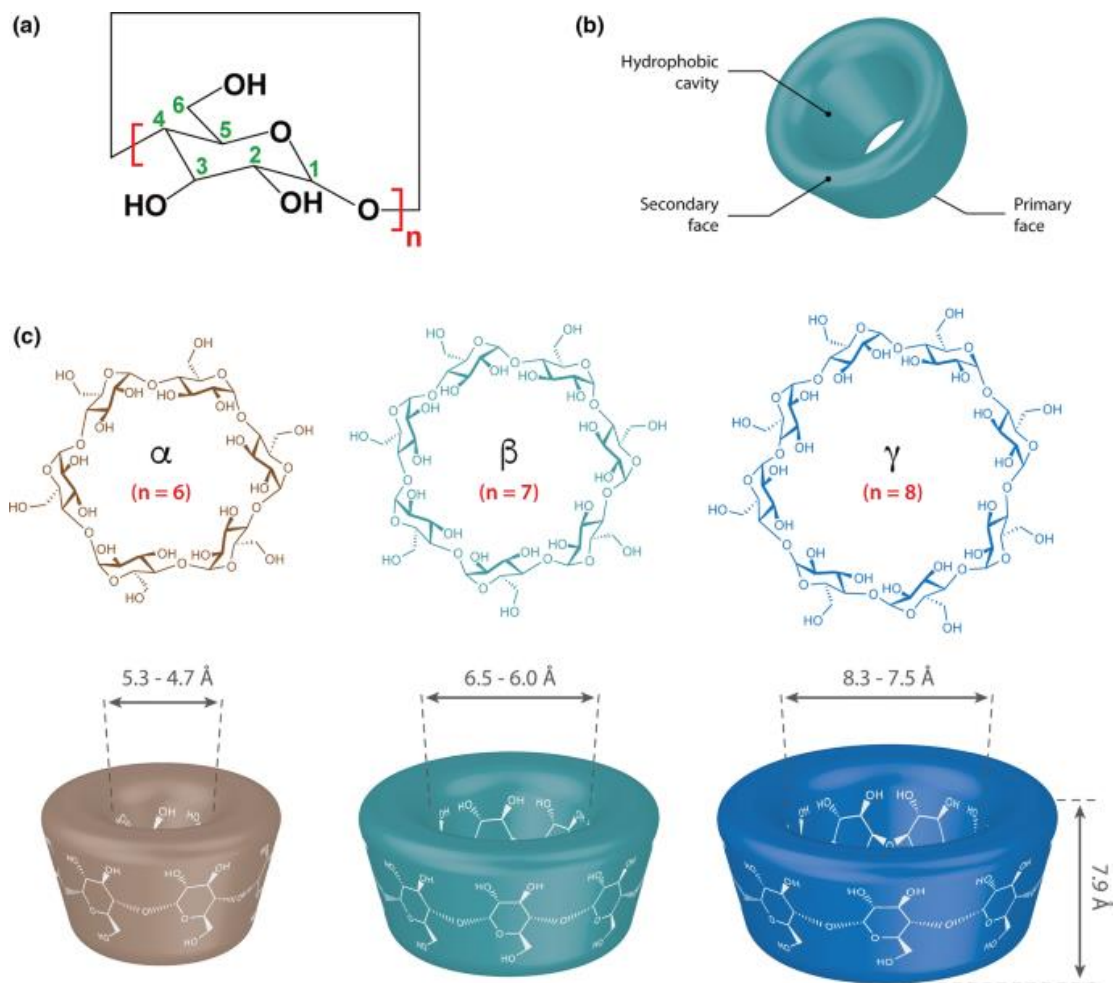


Figure 32: Représentation schématique de (a) la structure chimique et (b) de la structure tridimensionnelle des cyclodextrines ; (c) structures chimiques et dimensions des α - ; β - et γ -cyclodextrines ($n=6, 7$ et 8 respectivement). (Crini *et al.*, 2018)

Les différentes tailles de cavités que l'on observe pour cette famille de molécules permettent d'envisager des interactions avec une multitude d'autres composés de structures chimiques et de dimensions différentes. Ces CDs natives peuvent être modifiées afin d'obtenir de nouvelles molécules. On trouve de nombreux dérivés dont des CDs acétylées, alkylées comme par exemple les CDs di- ou triméthylées (DIMEB ou TRIMEB), ou hydroxyalkylées avec notamment la 2-hydroxypropyl- β -cyclodextrine (HP- β -CD), ou bien même des polymères de CDs (Yoshida *et al.*, 1988 ; Eastburn & Tao, 1994 ; Frank *et al.*, 2002 ; Liu *et al.*, 2021). Leurs caractéristiques en font des molécules de choix qui ont trouvé leur place, depuis les années 1970, dans de nombreux domaines d'applications (Szejtli, 1988, 1998).

Les domaines d'application des cyclodextrines.

Les CDs présentent de nombreux avantages pour une utilisation dans les domaines pharmaceutiques. Leurs capacités d'inclusion en font des véhicules particulièrement efficaces pour le transport des molécules actives des médicaments (Bibby *et al.*, 2000 ; Bilensoy, 2011). Les CDs permettent également d'atténuer les goûts désagréables et les mauvaises odeurs, mais aussi d'augmenter la biodisponibilité des principes actifs qu'elles contiennent en les rendant plus solubles (Duchene & Wouessidjewe, 1992 ; Uekama *et al.*, 1998). Leur flexibilité dans les voies d'administration et leur non toxicité font qu'elles sont actuellement utilisées dans une quarantaine de formulations (Morin-Crini *et al.*, 2015). Certaines de leurs propriétés sont également utilisées dans le domaine de la cosmétique (Buschmann & Schollmeyer, 2002 ; Dodziuk, 2006).

Le secteur alimentaire est également un domaine où les cyclodextrines sont largement mises à profit depuis plusieurs décennies. Elles trouvent un intérêt dans la préservation des arômes, en tant qu'exhausteurs de goût ou comme outils de préservation des saveurs (Goubet *et al.*, 1998 ; Astray *et al.*, 2009). Comme dans les autres domaines, les CDs peuvent également permettre de limiter les odeurs de certains aliments et d'éviter l'amertume. Les 3 cyclodextrines natives sont répertoriées comme additifs alimentaires sous les numéros E457, E459 et E458 pour l' α -CD, la β -CD et la γ -CD respectivement (Mortensen *et al.*, 2016 ; Matencio *et al.*, 2020).

Les CDs peuvent également être utilisées dans les secteurs du textile, du plastique, du papier ou bien encore de la photographie (Szejtli, 2004 ; Dodziuk, 2006 ; Bhaskara-Amrit *et al.*, 2011 ; Voncina & Vivod, 2013).

L'usage des CDs concerne de nombreux champs d'application, mais leur emploi pour la préservation de l'environnement n'a commencé que dans la fin des années 1990 (Morin-Crini *et al.*, 2015). Pourtant ces molécules sont biodégradables, non toxiques et leur coût a été largement diminué au fil du temps, parallèlement à l'augmentation de leur production.

Les effets bénéfiques qu'elles peuvent avoir dans la dépollution des eaux sont largement décrits dans la littérature (Boving & McCray, 2000 ; Landy *et al.*, 2012 ; Morin-Crini & Crini, 2013 ; Sikder *et al.*, 2019 ; Tian *et al.*, 2021 ; Cova *et al.*, 2021). Leur capacité à dépolluer l'eau en agissant comme des filtres contre les résidus de pesticides, les métaux lourds ou bien encore les perturbateurs endocriniens a été démontrée (Salipira *et al.*, 2007 ; Mahlambi *et al.*, 2010 ; Sikder *et al.*, 2019 ; Rubin Pedrazzo *et al.*, 2019 ; Song *et al.*, 2021).

Malgré ces constats sur leur efficacité, les CDs sont sous-utilisées dans le domaine de la protection et de la dépollution de l'environnement. Peu d'études ont été menées sur leur capacité à interagir avec les fongicides triazolés afin d'éviter leur diffusion environnementale (Stepniak *et al.*, 2015). Pourtant, disposer de ce genre « d'outils » ouvrirait de nouvelles perspectives indispensables concernant la protection des écosystèmes.

4.4.3. *Evaluation de l'interaction potentielle cyclodextrines/triazolés grâce au modèle biologique A. fumigatus et à la modélisation moléculaire*

Titre de l'article :

Reducing the unintentional impact of triazole fungicides on *Aspergillus fumigatus*: are cyclodextrins a solution?

Revue : Journal of Environmental Science and Public Health, volume 5

Indices de publication : IF=3,6

Etat : Publié, 2021

Hypothèses de travail :

- Les cyclodextrines sont des molécules capables d'interagir avec les triazolés par formation d'un complexe d'inclusion.
- Ce complexe d'inclusion empêche l'action fongistatique des molécules triazolées sur des isolats d'*Aspergillus fumigatus*.
- L'usage environnemental des cyclodextrines pourrait permettre de limiter la diffusion des triazolés dans les écosystèmes.

Résumé en français :

Les triazolés comptent parmi les fongicides les plus utilisés en agriculture, pour la protection des matériaux et des cultures. Leur large spectre d'action en fait des substances de choix pour la prévention et le traitement de maladies fongiques telles que la septoriose ou la fusariose. Cependant, leur diffusion dans l'environnement peut conduire à la sélection de résistance aux triazolés non seulement chez les pathogènes des cultures, mais aussi chez des organismes non ciblés, comme un champignon non phytopathogène, *Aspergillus fumigatus*. Ce champignon est un pathogène humain opportuniste associé à une mortalité élevée en cas de résistance aux traitements pharmacologiques à base de triazolés. Il est donc nécessaire de trouver des moyens pour limiter l'impact involontaire des fongicides sur ce pathogène. Nous décrivons ici l'impact des dextrines linéaires (maltodextrine) ou cycliques (cyclodextrines, CDs) et leur interaction avec deux fongicides (difénoconazole et tébuconazole), à travers des mesures de croissance de 20 souches d'*A. fumigatus*. Trois dextrines natives (α -CD, β -CD et γ -CD) et deux modifiées

(hydroxypropyl- β -CD (HP- β - CD) et heptakis-(2,3,6-tri-O-méthyl)- β -CD (TRIMEB)) ont été utilisées. Dans chaque expérience, la concentration minimale inhibitrice (CMI) a été déterminée selon la méthode préconisée par l'European Committee on Antimicrobial Susceptibility Testing (EUCAST). Des observations microscopiques ont également été réalisées pour mettre en évidence l'effet de la présence des dextrans par rapport à des milieux classiques sans dextrans. L'HP- β -CD est la dextrine cyclique la plus efficace. Elle a réduit l'activité antifongique des triazolés, alors que la maltodextrine et l' α -CD n'ont eu aucun effet. Ces observations ont été approfondies grâce à la modélisation moléculaire afin de clarifier les interactions cyclodextrine/fongicide. L'utilisation de cyclodextrines est une approche prometteuse pour limiter l'émergence de la résistance aux fongicides de type triazolés.

Eléments essentiels :

- Les cyclodextrines sont étudiées afin d'évaluer leur capacité à interagir avec 2 triazolés, le difénoconazole et le tébuconazole en utilisant *A. fumigatus* comme modèle biologique ;
- Une diminution de l'activité des triazolés (et donc une augmentation de la concentration minimale inhibitrice (CMI)) sur la moisissure en présence de cyclodextrine est le signe d'une interaction cyclodextrine/triazolé potentielle ;
- Une dextrine linéaire (maltodextrine) et 5 cyclodextrines (α -CD ; β -CD ; γ -CD ; HP- β -CD et TRIMEB) ont été étudiées ;
- L'HP- β -CD induit l'augmentation de CMI la plus importante alors que la dextrine linéaire n'a pas d'effet, signe d'une inclusion potentielle du triazolé dans la cavité de l'HP- β -CD, inclusion confirmée par modélisation moléculaire ;
- Le piégeage des triazolés au sein de la cavité de l'HP- β -CD par formation d'un complexe d'inclusion semble être une bonne piste d'étude pour limiter la diffusion environnementale de ces composés ;



Research Article

Reducing the Unintentional Impact of Triazole Fungicides on *Aspergillus fumigatus*: Are Cyclodextrins a Solution?

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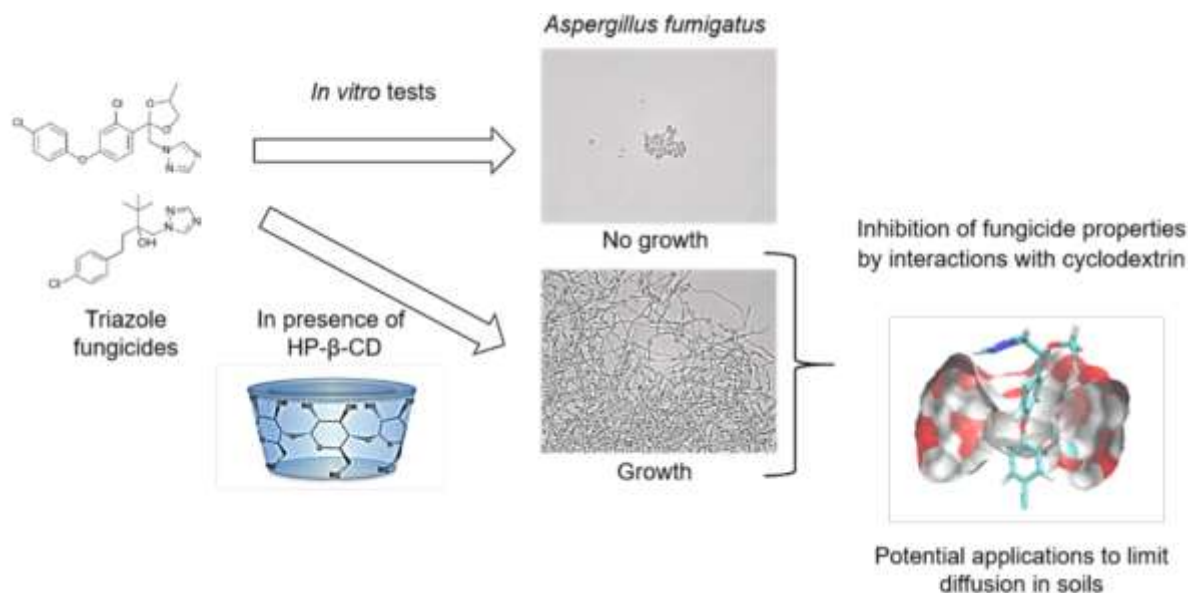
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Graphical Abstract



Abstract

Triazoles are among the most widely used fungicides in agriculture, for the protection of materials and crops. Their broad spectrum of action makes them the substances of choice for preventing and curing fungal diseases such as *Septoria* or *Fusarium* wilt. However, their spread in the environment can lead to the selection of triazole resistance not only in crop pathogens, but also in non-target organisms, such as a non-phytopathogenic fungus *Aspergillus fumigatus*. This fungus is an opportunistic human pathogen associated with high mortality in cases of resistance to pharmacological treatments based on triazoles. There is therefore a need to find ways of limiting the unintended impact of fungicides on this pathogen. We describe here the impact of linear (maltodextrin) or cyclic (cyclodextrins, CDs) dextrins and their interaction with two fungicides (difenoconazole and tebuconazole), through measurements of growth in 20 *A. fumigatus* strains. Three native (α -CD, β -CD and γ -

CD) and two modified (hydroxypropyl- β -CD (HP- β -CD) and heptakis-(2,3,6-tri-O-methyl)- β -CD (TRIMEB)) cyclodextrins were used. In each experiment, the minimum inhibitory concentration (MIC) was determined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method. Microscopical observations were also performed to highlight the effect of the presence of dextrins. HP- β -CD was the most effective cyclic dextrin. It reduced the antifungal activity of triazoles, whereas maltodextrin and α -CD had no effect. Those observations were more investigated thanks to molecular modeling in order to clarify cyclodextrin/fungicide interactions. The use of cyclodextrins is a promising approach for limiting the emergence of resistance to triazole antifungal drugs.

Keywords: Triazoles; Cyclodextrins; Interaction; *Aspergillus fumigatus*; Molecular Dynamics Simulation

1. Introduction

Pesticides use is increasing worldwide, to intensify agricultural production to meet the needs of growing populations requiring more and more food [1]. Fungicides are widely used in agricultural development, to prevent or cure fungal infections of plants that can decrease production yields [2]. Triazole fungicides are often the treatment of choice, because of their broad spectrum of action and their specific targeting of fungi [3, 4]. Almost 30 different molecules have been authorized by national, European and international agencies (e.g. the US Food and Drug Administration) and are used to protect crops or materials against fungal damage [5, 6]. New formulations are continually being released onto the market [7].

Following their application on crops, triazoles act on the aerial parts of the plant and persist in soils for a few days to several months, depending on the molecule used, the characteristics of the soil and meteorological conditions [8, 9]. During this period, these fungicides may have unintended effects on *Aspergillus fumigatus*, a non-phytopathogenic fungus naturally present in soils, through the exertion of selection pressure [10]. This can lead to an increase in the size of the population of *A. fumigatus* strains with pan-resistance to triazoles [11].

This resistance phenomenon is a matter of concern, because this fungus can cause disease in humans [12]. Indeed, *A. fumigatus* is an opportunistic pathogen, and is the fungus most frequently implicated in invasive pulmonary aspergillosis, a fungal infection with a high mortality rate [13, 14]. The phenomenon of resistance to medical treatments based on triazoles has been known for more than 20 years and is frequently

described [15]. This resistance has been reported worldwide and is linked to the use of triazole fungicides in the environment, given the close chemical similarities of medical and agricultural triazoles [16-19]. The number of people at risk of developing opportunistic diseases is steadily increasing around the world. Over the last decade, medical advances in immunosuppression techniques, chemotherapy, organ transplantation and the use of biotherapies in chronic inflammatory diseases, in particular, have resulted in a major expansion of the population of immunocompromised individuals [20]. This observation highlights the importance of limiting the emergence of resistance in opportunistic pathogens. New solutions to limit the spread of triazole resistance are therefore required, to limit public health implications [21].

One possible approach to preventing triazole selection pressure would be to limit the overdispersion of triazole fungicides in the soil, which isn't needed, and to prevent contact between active fungicides and *A. fumigatus*. Cyclodextrins (CDs), synthetic cyclic molecules derived from starch by enzymatic degradation [22, 23], can be used to catch fungicides before their uncontrolled dissipation in the environment. These oligosaccharides have a remarkable ability to form host-guest interactions in solution and in the solid state (possible formation of inclusion complex). Their specific properties and their lack of toxicity and biocompatibility with humans have led to their use in numerous applications in the food industry, pharmaceuticals, medicine, biotechnology, cosmetics, hygiene and toiletries, the textile industry, catalysis, chromatography, and remediation, for example [24-34]. Crini et al. [35] have shown that cyclodextrin polymers can interact

with five triazole molecules. In a preliminary study, we showed that some dextrans interacted with difenoconazole, limiting its antifungal activity [36]. Complex formation and stability of inclusion compounds in CDs can be studied by molecular dynamics simulation [37-41], which are especially useful when guest solubility limits experimental investigations.

Here, we pursue our work on this topic, broadening the experimental conditions used and performing *A. fumigatus* growth tests to assess the impact of linear and cyclic dextrans and their interaction with two triazole fungicides, difenoconazole (DIFENO) and tebuconazole (TEBU), which are particularly widely used in the environment. Molecular dynamics simulations are also conducted to better investigate interactions between cyclodextrins and these fungicides.

2. Materials and Methods

2.1 Fungal strains

Twenty *A. fumigatus* strains were used, isolated in 2017 from a sampling campaign carried out on market gardens in the east of France. These strains were characterized by sequencing the β -tubulin and *cyp51A* genes, to check species identification and the absence of genetic mutations conferring triazole resistance [42-43].

2.2 Fungicides

Difenoconazole (DIFENO, 1-[[2-[2-chloro-4-(4-chlorophenoxy) phenyl]-4-methyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole) and tebuconazole (TEBU, 1-(4-chlorophenyl)-4,4-dimethyl-3-((1,2,4-triazol-1-yl)methyl)pentan-3-ol) were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Their structure and main characteristics are presented in Table 1.

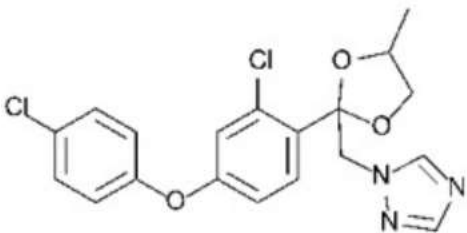
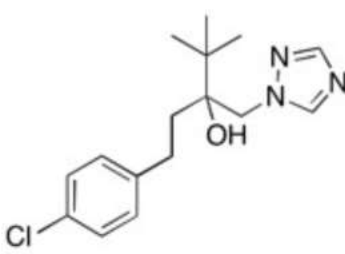
	Difenoconazole	Tebuconazole
Chemical structure		
Molecular formula	C ₁₉ H ₁₇ Cl ₂ N ₃ O ₃	C ₁₆ H ₂₂ ClN ₃ O
Molecular weight (g/mol)	406.2	307.82
Solubility in water (mg/L at 20°C)	15	36
Log K _{ow} (at 25°C)	4.36	3.7

Table 1: Chemical characteristics of difenoconazole and tebuconazole.

2.3 Commercial dextrans

A linear dextrin (maltodextrin, MALTO, Glucidex® D19 from Roquette, Lestrem, France), three native (α -CD, β -CD and γ -CD, purity $\geq 98\%$) and two modified (HP- β -CD and TRIMEB, purity $\geq 95\%$) cyclodextrins (from CycloLab, Hungary) were used in this study. MALTO (purity $\geq 99\%$) has a dextrose equivalent

value of 19 ($n \approx 6$ in Figure 1). α -CD, β -CD and γ -CD contain six, seven and eight glucose units, respectively. HP- β -CD (hydroxypropyl- β -cyclodextrin) and TRIMEB (heptakis-(2,3,6-tri-O-methyl)- β -cyclodextrin) are commercial products derived from β -CD. Their structures are shown in Figure 1 and they are described in Table 2.

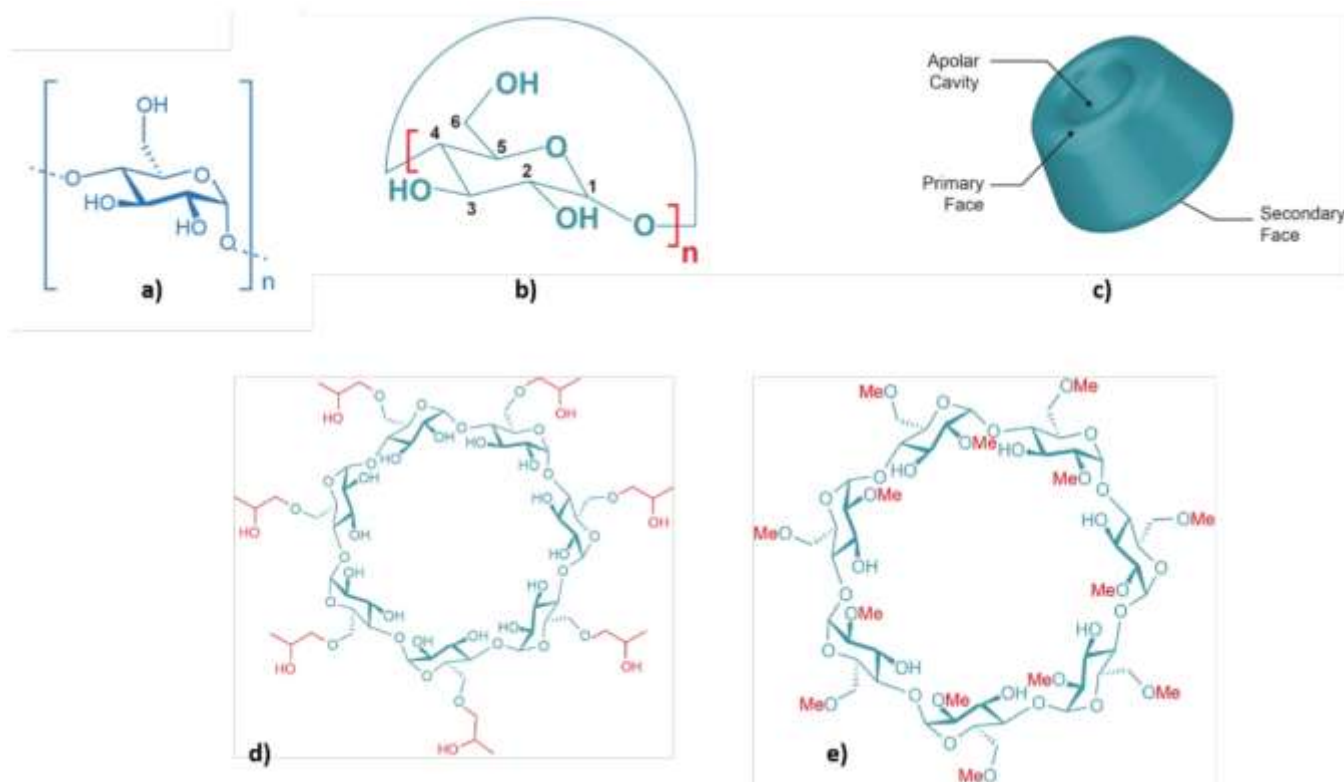


Figure 1: Chemical structures of linear and cyclic dextrans; a) maltodextrin (MALTO; $n \approx 6$); b) α -cyclodextrin (α -CD, $n = 6$), β -cyclodextrin (β -CD, $n = 7$) and γ -cyclodextrin (γ -CD, $n = 8$); c) general structure of native α -, β -, and γ -cyclodextrins; and of two modified β -cyclodextrins: d) hydroxypropyl- β -cyclodextrin (HP- β -CD) and e) heptakis-(2,3,6-tri-O-methyl)- β -cyclodextrin (TRIMEB).

Cyclodextrin	Degree of substitution ¹	Molecular weight ²	Solubility ³
α -cyclodextrin (α -CD)		972	145
β -cyclodextrin (β -CD)		1135	18.5
γ - cyclodextrin (γ -CD)		1297	232
Hydroxypropyl- β -cyclodextrin (HP- β -CD)	0.65	1400	>600
Randomly methylated β -cyclodextrin (TRIMEB)	3.0	1430	>500

¹Mean number of substituents per glucopyranose repeat unit

²Molecular weight in Daltons

³Solubility in pure water at 25°C, in g/L

Table 2: General description of cyclodextrins.

2.4 Determination of minimum inhibitory concentrations

All 20 strains were tested with the EUCAST method to determine minimum inhibitory concentrations (MICs) for medical triazoles [44]. For itraconazole and voriconazole, clinical breakpoints were used to define resistance (a strain was defined as resistant if the MIC for these two molecules exceeded 2 mg/L [45]).

In each EUCAST plate, two reference strains were used (a sensitive strain: CBS 101355, and an environmental strain with the TR34/L98H mutation, pan-resistant to antifungal triazoles). Briefly, the different strains were plated in 96-well microplates and exposed to a range of antifungal concentrations, from 0.0312 to 16 mg/L generated by a 10-well two-

fold dilution series in RPMI 1640 (Roswell Park Memorial Institute; Sigma Aldrich, Saint Louis, USA) medium. MICs were determined, by eye, after 48 hours of incubation at 37°C, as the lowest triazole concentration inhibiting fungal growth.

We then performed the EUCAST method with DIFENO and TEBU in same conditions, to measure the MICs of triazole fungicides for the same strains. In addition to the reference RPMI medium used for the EUCAST method, we used RPMI medium supplemented with maltodextrin and each of the five CDs to estimate the dextrin/triazole interaction potential and its limits. These media were prepared such that there were 10 molecules of dextrin per molecule of fungicide, in accordance with our preliminary results [36].

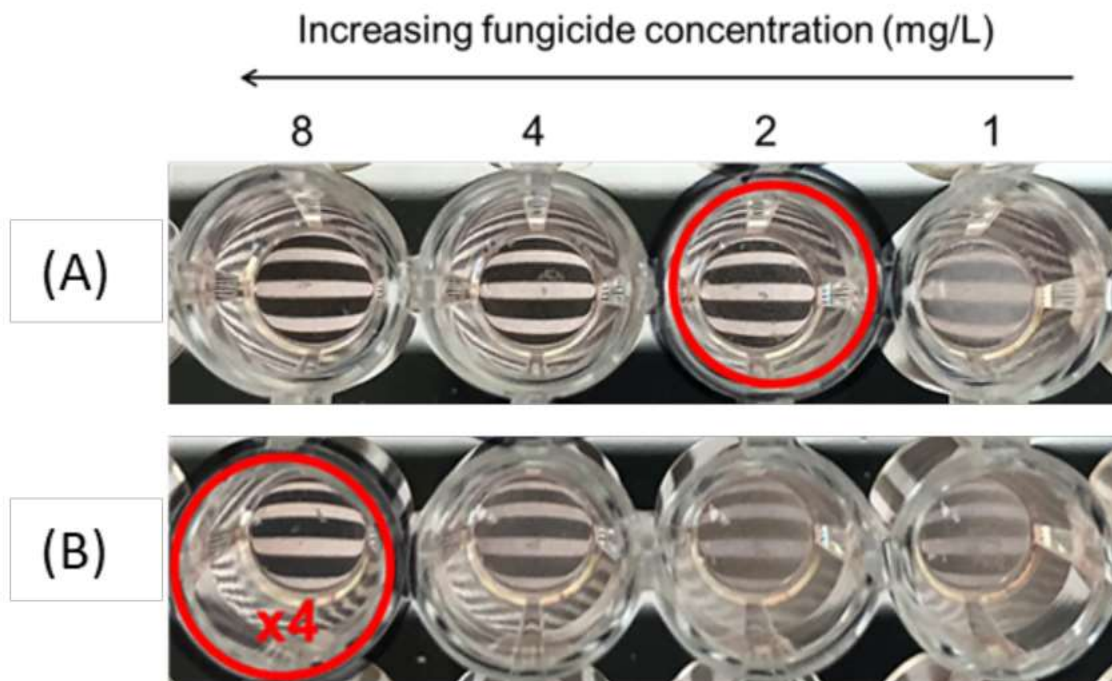


Figure 2: Picture of part of the EUCAST plate for the 4E007 strain with difenoconazole in (A) RPMI medium, (B) medium supplemented by HP- β -CD at a molar ratio of 10 dextrin molecules per molecule of fungicide. Minimum inhibitory concentrations are indicated by a red circle for each set of conditions: “x4” (increase by a factor of 4), corresponding to the increase in MIC relative to the triazole in RPMI alone.

The MICs obtained with culture medium consisting solely of RPMI with DIFENO or TEBU were compared with those obtained with each of the dextrin-supplemented media. For each fungicide, we assessed the impact of MALTO or CDs in culture media by observing the increase in MIC (Figure 2).

2.5 Microscopy analysis of fungal growth

The effect of each culture medium on fungal strain growth was also investigated in more detail, by performing a microscopy analysis. After the 48 hours of incubation required for the susceptibility test, 40 μ L of culture medium present in the well were transferred to a microscope slide and the slide was sealed. The slides were observed under a light

microscope (Leica microsystems, France), and image analysis was performed with Leica LAS V4.9 software. Microscopy observations were performed in every media at the concentration of fungicides determined as MIC for the strain concerned with the medium containing only RPMI: 1 mg/L for DIFENO and 2 mg/L for TEBU.

2.6 Statistical analysis

Log₂-transformed MIC values were used for statistical analysis. The Akaike information criterion (AIC) was used to select the most parsimonious models (including random effects and/or interactions between variables). A linear mixed-effects model (LMM) was applied incorporating a random effect of

strain, to assess differences in fungal growth according to the dextrin used (relative to RPMI medium without dextrins) and fungicides. Statistical analyses were performed, and table and graphical display were generated with R-3.4.4 statistical software for Microsoft® Windows and lme4 [46], nlme [47], MuMIn [48], gtsummary and ggeffects [49] libraries.

2.7 Simulation methodology

Simulations were conducted between α -CD, β -CD, γ -CD or HP β -CD on the one hand and TEBU or DIFENO on the other hand. The high-resolution X-ray structures of α -CD, β -CD and γ -CD were used as initial 3D models for molecular dynamics simulations. The following protein data bank (PDB) codes were taken from research collaboratory for structural bioinformatics (RCSB) as 2XFY for α -CD [50], 5MK9 for β -CD and 5MKA for γ -CD [51]. The structures of TEBU and DIFENO molecules were also downloaded from RCSB [52]. HP- β -CD structure used had one side substitution of seven hydroxypropyl, it was converted from PubChem to PDB format using Online SMILES Translator of NIH site (<https://cactus.nci.nih.gov/>). All structures were then subjected to quantum chemistry calculations using the Gaussian09 suite of programs [53]. Optimization of all molecules was done at DFT/B3LYP /6-31g* level of theory. Partial charges were calculated within the same level of theory using the restrained electrostatic potential scheme [54]. The software package Antechamber [55] was used for the automatic parameterization of force field parameters with general Amber force field atom type [56,57]. Several systems were built for molecular dynamics (MD) which are presented in supplementary data (Table S-2) together with their equilibrium protocol.

Finally, production runs for the complexes of each systems were performed for 200 nanoseconds (ns) under the condition of constant pressure and temperature. An integration step of 2 femtoseconds was used in all simulations with the bonds to hydrogen atoms converted to rigid constraints. The trajectories for structural coordinates were saved every 1 picosecond for data analysis. MD trajectories were analysed using visual molecular dynamics [58] and Chimera [59]. The molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) method [60, 61] were applied to estimate the binding free energy of the complexes.

3. Results

3.1 Characteristics of the fungal strains

The susceptible reference strain, CBS 101355, had a MIC of 4 mg/L for both fungicides, whereas the resistant reference strain had a MIC of 16 mg/L for both fungicides. The 20 strains tested had MICs no higher than that of the susceptible reference strain, for both DIFENO and TEBU. MICs were between 1 (12/20 strains) and 2 (8/20 strains) mg/L for DIFENO, and between 2 (12/20 strains) and 4 (8/20 strains) mg/L for TEBU, depending on the strain tested. Thus, for statistical analysis, strains were considered to be the random factor accounting for MIC variations and were added to the random part of the model.

3.2 Effect of the presence of cyclodextrins in the culture medium

The MICs of DIFENO and TEBU in dextrin-free RPMI culture medium were not significantly different from those obtained in RPMI medium supplemented with MALTO ($p = 0.8$) or α -CD ($p = 0.3$).

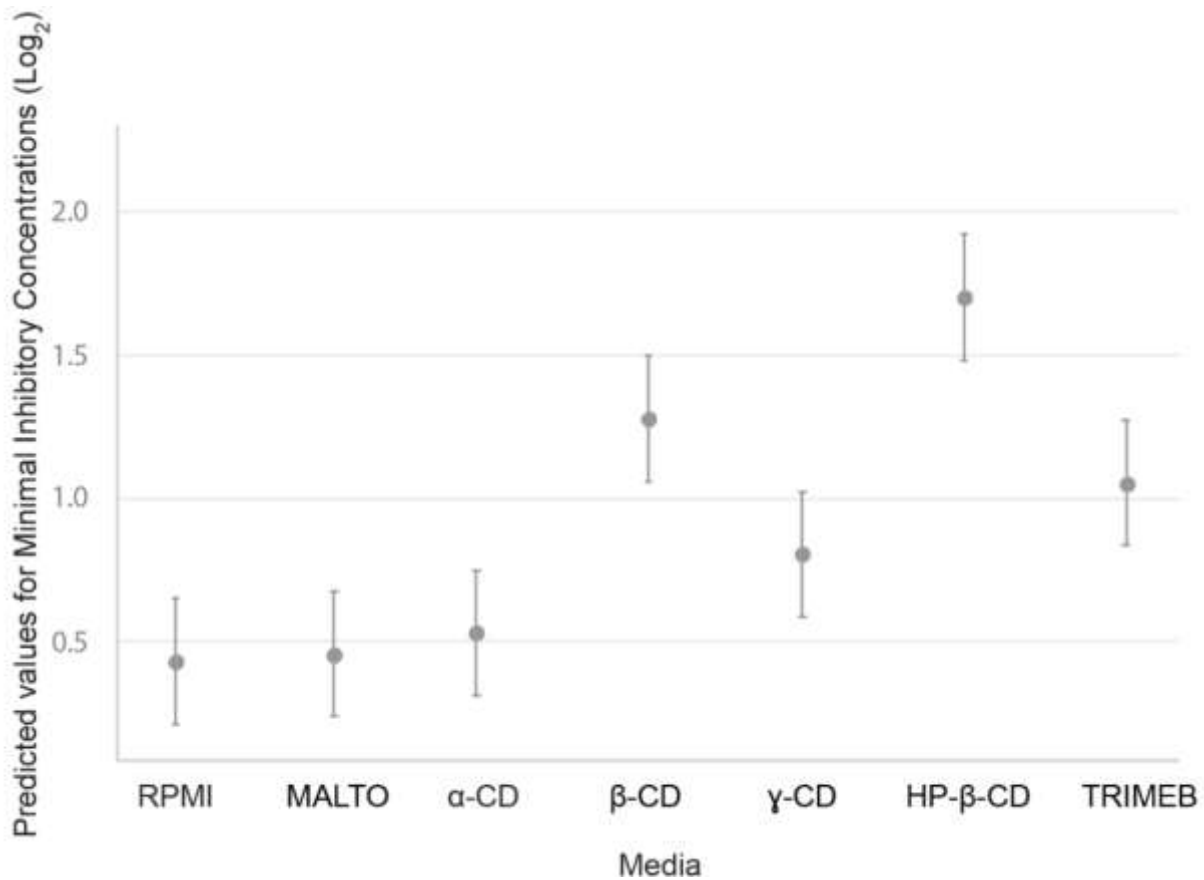


Figure 3: Predicted values of minimum inhibitory concentrations as a function of medium (RPMI reference medium or RPMI supplemented with dextrans), with strain integrated into the mixed-effects model as a random effect. The dextrans used were MALTO: maltodextrin; α-CD: α-cyclodextrin; γ-CD: γ-cyclodextrin; TRIMEB: heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin; β-CD: β-cyclodextrin; and HP-β-CD: hydroxypropyl-β-cyclodextrin.

By contrast, the MICs obtained in media supplemented with the other dextrans tested (β-CD, γ-CD, HP-β-CD and TRIMEB) were significantly higher than those obtained in dextrin-free RPMI medium ($p < 0.001$). The outputs of the LMM (estimates, confidence intervals and p-values) are presented in the supplemental data (Table S-1). Strains (random effects) accounted for 0.40 of the variance, and the fixed model accounted for 0.396. The predicted MICs for the fixed part of the model (medium) are shown in Figure 3.

In several strains, the MICs of both DIFENO and TEBU were higher in media supplemented with TRIMEB, β-CD, or HP-β-CD than in dextrin-free RPMI medium (Table 3). For DIFENO, an increase in MIC was observed for 14/20, 17/20 and 20/20 for TRIMEB, β-CD and HP-β-CD, respectively. The effect seemed particularly strong with HP-β-CD, increasing MICs relative to TRIMEB and β-CD.

For TEBU, the effects on growth of TRIMEB, β-CD and HP-β-CD were less pronounced than for

DIFENO: an increase was observed for 9/20, 16/20 and 19/20 strains, respectively. Only one strain displayed an increase in MIC by a factor of 4, on medium supplemented with HP-β-CD against 9 for DIFENO. MICs for DIFENO were higher in γ-CD-supplemented medium than in the reference medium (higher MICs for 12/20 strains), whereas no effect on

the MIC of TEBU was observed. Overall, increase in MICs were larger for DIFENO than for TEBU. Regardless of the fungicide considered, an order of effectiveness, in terms of the increase in MIC, was observed for the dextrins added to the culture medium: MALTO ≈ α-CD < γ-CD < TRIMEB < β-CD << HP-β-CD (Figure 3).

Strains	Media supplemented with cyclodextrins*							
	γ-CD		TRIMEB		β-CD		HP-β-CD	
	TEBU	DIFENO	TEBU	DIFENO	TEBU	DIFENO	TEBU	DIFENO
4E007	-	x2	-	x2	x2	x2	x2	x4
4E008	-	-	-	-	x2	x2	x2	x2
4E009	-	x2	x2	x2	x2	x2	x2	x4
4E010	-	-	-	-	x2	x2	x2	x4
4E011	-	x2	x2	-	x2	x2	x2	x4
4E012	-	-	x2	x2	x2	-	x2	x2
4E013	-	-	-	-	x2	x2	x4	x4
4E014	-	x2	-	x2	x2	x2	x2	x2
4E015	-	x2	-	x2	-	x2	x2	x2
4E016	-	x2	x2	x2	-	x2	x2	x4
4E017	-	x2	-	x2	-	x2	x2	x2
4E018	-	-	x2	-	x2	x2	x2	x4
4E019	-	x2	-	x2	x2	x2	x2	x2
4E020	-	-	-	-	x2	-	x2	x2
4E022	-	-	x2	x2	-	x2	x2	x2
4E023	-	x2	-	x2	x2	-	x2	x2
4E024	-	-	x2	x2	x2	x2	x2	x2
4E025	-	x2	-	x2	x2	x2	x2	x2
4E026	-	x2	x2	x2	x2	x2	x2	x4
4E027	-	x2	x2	x2	x2	x2	x2	x4

*γ-CD: γ-cyclodextrin; TRIMEB: heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin; β-CD: β-cyclodextrin; HP-β-CD: hydroxypropyl-β-cyclodextrin
 “x2” or “x4” indicates the factor increase in MIC in each set of conditions

Table 3: Increases in minimum inhibitory concentrations (MICs) observed on EUCAST plates for 20 *A. fumigatus* strains in different conditions, in the presence of the fungicide difenoconazole (DIFENO) or tebuconazole (TEBU). MICs were measured in the presence of four cyclodextrins added to the culture media at a molar ratio of 10:1.

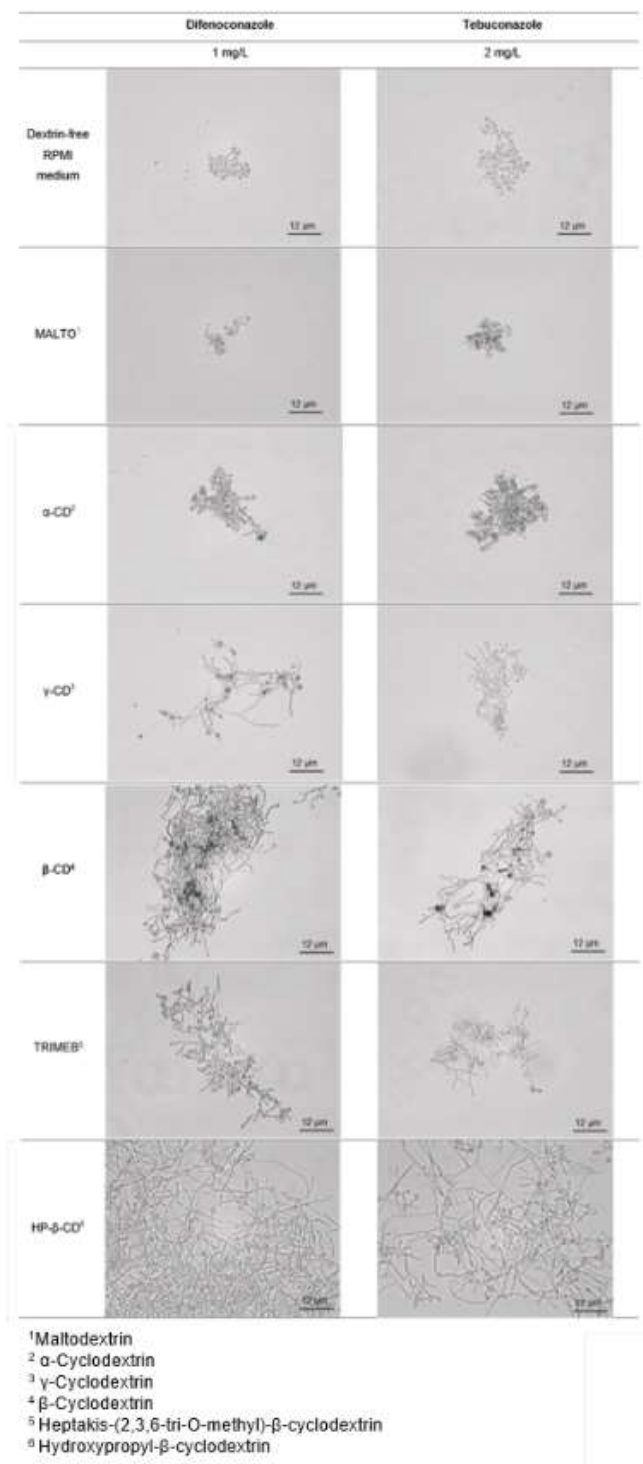


Figure 4: Micrographs of *A. fumigatus* (strain 4E026), comparing its growth between dextrin-free RPMI medium and RPMI medium supplemented with linear or cyclic dextrans at a molar ratio of 10:1. The observations presented were obtained with a fungicide concentration of 2 mg/L for tebuconazole and 1 mg/L for difenoconazole, corresponding to the MICs of these fungicides in dextrin-free RPMI medium. The slides were observed under a light microscope, at a magnification of x 400.

Microscopy analysis of strain 4E026 were performed to assess the germination of fungal spores in each set of conditions, images are presented in Figure 4. Those observations were performed at fungicide concentrations found to inhibit fungal growth in dextrin-free RPMI: 1 mg/L DIFENO and 2 mg/L TEBU. The absence of an effect of α -CD and MALTO on the recovery of fungal growth was confirmed by microscopy (Figure 4), for both fungicides. For γ -CD-supplemented medium, hyphal development was visible in the presence of DIFENO. For β -CD and its derivatives, in the presence of DIFENO or TEBU, fungal growth was greater in medium supplemented with HP- β -CD than in media supplemented with β -CD or TRIMEB, in accordance with Table 3. Similar increases in MIC relative to the reference medium were observed with β -CD, TRIMEB and HP- β -CD with TEBU for the 4E026 strain (Table 3), but microscopy revealed more extensive hyphal development with HP- β -CD.

3.3 Incorporation of antifungal agents in cyclodextrins

In order to test the stability of cyclodextrins in our simulations, the root mean square deviation was calculated (RMSD) for the optimized CDs as a function of MD simulations time. Whatever the system, cyclodextrins were found very stable since RMSD displayed values less than 2Å. γ -CD and HP- β -CD being a bit more mobile due to the larger diameter of γ -CD and the high flexibility of hydroxypropyl groups of HP- β -CD. The interaction of antifungal agents with cyclodextrins and their random incorporation were investigated on the course of the MD simulation runs. Distances between the centers of mass of the CDs and the ligands were analyzed as a function of time (Figure 5). During the 200 ns of MD simulation, DIFENO was found to enter in β -CD, γ -CD and HP- β -CD but not in α -CD. The time scale necessary for the molecules to incorporate into CDs is also a marker of the strength of the interaction between antifungals and CDs. Lesser this time is and stronger the antifungal/CD interaction is. DIFENO incorporated β -CD after about 70 ns, γ -CD after 20 ns and bound in less than 10 ns to HP- β -CD.

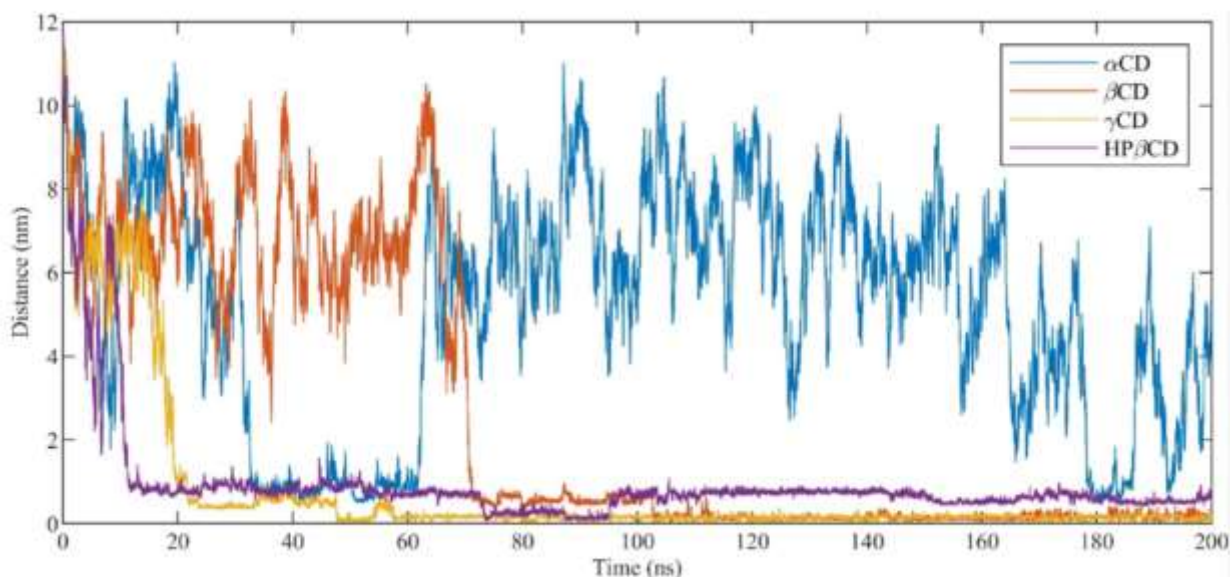


Figure 5: Distance between the center of mass of cyclodextrins (α -CD, β -CD, γ -CD and HP- β -CD) and difenoconazole.

For TEBU, no incorporation in α -CD occurred during the MD simulation run. The interaction of TEBU with CDs is found weaker than the one observed with DIFENO. Indeed, the time scales which characterize the binding and insertion of the ligands are found

greater for TEBU with respect to DIFENO, for example the highest time scale was 140 ns for γ -CD/TEBU interaction instead of 20 ns for γ -CD/DIFENO interaction.

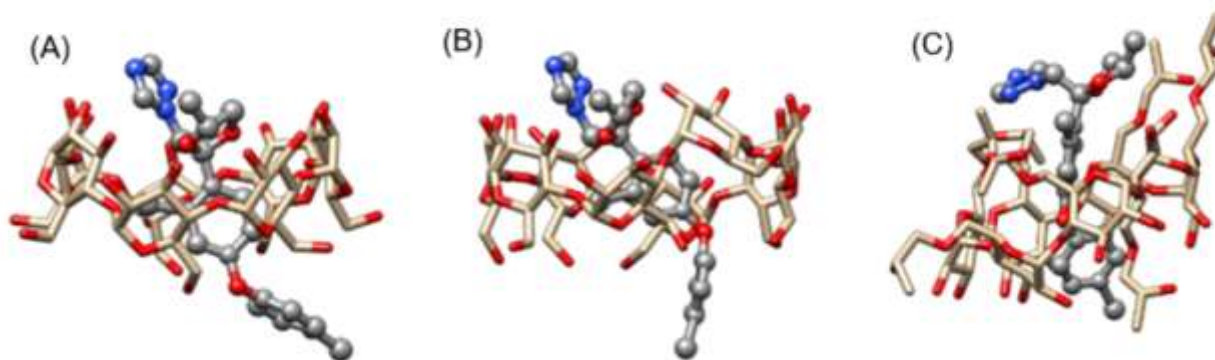


Figure 6: Most favorable configurations of DIFENO obtained in (A) β -CD, (B) γ -CD and (C) HP- β -CD.

A visual inspection of the fungicide/CD complexes have been carried out together with the analysis of all changes of the distances between CDs and antifungal drugs. Figure 6 illustrates the most favourable configurations of DIFENO obtained in β -CD, γ -CD and HP- β -CD: whatever the CD, the chlorobenzene group of DIFENO, which due to its hydrophobicity seems predisposed to interact with the hydrophobic cavity of the CD, appears to have a strong interaction with it. The binding free energy of all systems that formed complexes were also calculated according to the MM-PBSA method.

The binding energies of DIFENO with β -CD and γ -CD (-26.74 ± 0.04 and -27.10 ± 0.04 Kcal/mol, respectively) looked very similar. They were found greater than those obtained for TEBU which were between $-23,76 \pm 0,03$ Kcal/mol for β -CD and -19.69

± 0.04 Kcal/mol for γ -CD. Due to the large flexibility of the hydroxypropyl chains which move constantly, two main conformations of HP- β -CD were observed during the simulations: a “closed” one where one or two hydroxypropyl group(s) block the access to the cavity and an “open” conformation where hydroxypropyl groups point toward the solvent and do not prevent the entry of the ligands into the CD cavity. For DIFENO, in the open situation of the cavity we found the highest binding energy value (-30.46 ± 0.1 Kcal/mol) while the other configuration gave only -22.30 ± 0.05 Kcal/mol.

4. Discussion

An effect of the interaction between dextrans and triazole molecules on the growth of *A. fumigatus* was demonstrated in this study. A decrease in the antifungal activity of DIFENO and TEBU fungicides

in the presence of cyclodextrins was observed *in vitro*. The inhibitory effect was greatest for HP- β -CD, whereas maltodextrin had no effect. A lower biocidal activity in the presence of CDs than in the presence of the biocide alone has already been reported in several studies reviewed by Nardello-Rataj and Leclercq [62]. The authors reported an antagonistic effect of the formation of a complex between HP- β -CD and a series of p-hydroxybenzoic acid esters against *Candida albicans*: antimicrobial activity was lower in the presence of the CD, whereas solubility in water was higher and the concentration of free p-hydroxybenzoic acid esters was lower, with an increase in the binding constant, suggesting that the degree of inactivation depended entirely on the proportion of the biocide encapsulated. The authors also reported that biocides with a higher solubility in water (e.g. thimerosal and bronopol) displayed only slight inactivation, whereas lipophilic substances (e.g. phenolic residues) were strongly inactivated in the presence of HP- β -CD, in assays with various strains.

In details, with native cyclodextrins, fungal growth remained inhibited with α -CD sign that there is probably no interaction with fungicides, surely because its cavity is too small for the formation of complexes with these two triazole molecules. For γ -CD, a resumption of fungal growth was visible only with DIFENO, suggesting that the cavity of this molecule is too large and too flexible for the formation of a stable inclusion compound with TEBU but more appropriate for the inclusion of DIFENO. Indeed, the binding energies obtained in the present work for DIFENO are larger than those obtained for TEBU. The effect on fungal growth was greatest in the presence of β -CD, resulting in an increase in MIC for 17/20 strains for DIFENO and 16/20 strains for

TEBU. The β -CD cavity size was, therefore, considered optimal relative to the other native cyclodextrins. We infer from these results that a cyclodextrin/triazole interaction occurs, potentially through the formation of an inclusion complex in the hydrophobic cavities of the cyclodextrin molecules. This mechanism would also explain the lack of effect of linear maltodextrin, which has no cavity.

Several studies have already reported interactions between CDs and fungicides [63-68]. However, to our knowledge, publications on the interaction between triazole fungicides and CDs are rare. Our results are consistent with those of Stepniak et al. [69], who studied the host-guest interaction of β -CD with TEBU and demonstrated, by calculating the constant for complex formation, a high affinity of β -CD molecules for the non-polar functional groups of TEBU (para-chlorobenzyl and tert-butyl groups) present in the hydrophobic cavity. Balmas et al. [70] also demonstrated the formation of a stable complex between TEBU and β -CD, arguing that this complex could be used to control plant diseases in agriculture through the prolonged release of fungicide. However, our results temper the statements of Stepniak et al. [69]. These authors examined the effect of TEBU within β -CD macromolecules in cultures of only one strain of *A. fumigatus*; they observed a toxic action not only of the free fungicide at a given dose, but also of the fungicide included in β -CD at the same dose. We found that DIFENO and TEBU had clear fungicidal activity against 20 strains of *A. fumigatus*, but that this activity was weaker in the presence of β -CD, as shown by increases in both MICs (Table 3) and hyphal development, as observed under the microscope (Figure 4, comparison between reference medium and medium implemented with β -CD).

However, the presence of β -CD molecules in the culture medium had a statistically significant effect on fungal growth.

These observations led us to test two β -CD derivatives, TRIMEB and HP- β -CD, both of which allowed a recovery of fungal growth. TRIMEB was less efficient than β -CD, but the hydroxypropyl groups grafted onto the glucose units in HP- β -CD seemed to promote interactions with TEBU and DIFENO (Table 3). Indeed, HP- β -CD had an effect on all strains, with both fungicides, resulting in a loss of antifungal efficacy against *A. fumigatus*. This effect was particularly marked with DIFENO, and this interaction was probably the most favorable theoretical “host-guest” interaction in this study. Indeed, DIFENO has a higher electron density than TEBU and is more lipophilic (see log K_{OW} values in Table 2), favoring its interaction with the hydrophobic cavities of CDs. The HP- β -CD-DIFENO system showed the highest binding energy value (-30.46 ± 0.1 Kcal/mol) in molecular simulation in the open state, supporting the hypothesis of the formation of an inclusion complex between these two molecules. It results in the significant loss of antifungal efficiency observed, reflected in the higher MICs obtained (Table 3), together with the higher rates of spore germination and hyphal development (Figure 4). The interaction rendered the fungicides less effective, probably by blocking their active sites.

5. Conclusion

Triazoles are used for the treatment of foliar fungal diseases. Their presence in the soil is not required and has an undesirable side effect on *A. fumigatus*, potentially favoring the emergence of resistance in this microorganism. In this study, we highlight the

potential role of cyclodextrins in limiting this phenomenon. Indeed, cyclodextrins can encapsulate triazoles before they spread in the soil, without affecting the efficacy of foliar treatment. This would eliminate the potential selection pressure exerted on *A. fumigatus* by triazoles in the soil. This study sheds light on the cyclodextrin/triazole interaction and the effect on *A. fumigatus*, providing a strong first argument in favor of their use in the environment. Molecular modeling confirmed the interaction between cyclodextrins and fungicides. They reveal that the binding energy of fungicides to HP- β -CD is significantly larger than to β -CD and γ -CD, α -CD being not favorable at all. DIFENO has a better affinity than TEBU to these CDs. The chlorobenzene group of this fungicide appears to be a key chemical group for their binding to CDs. Other experiments are underway to investigate the interaction between cyclodextrins and other triazoles.

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Conflict of Interest

The authors have no conflict of interest to declare.

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Supplementary Materials

Effects	Characteristics	Estimates	95% CI ¹	p-value
Fixed				
	Fungicides:			
	Difenoconazole (reference)	-	-	-
	Tebuconazole	0.84	0.75, 0.94	<0.001
	Media ² :			
	RMPI (reference)	-	-	-
	MALTO	0.03	-0.15, 0.20	0.8
	α -CD	0.10	-0.07, 0.27	0.3
	β -CD	0.85	0.68, 1.0	<0.001
	γ -CD	0.38	0.20, 0.55	<0.001
	HP- β -CD	1.3	1.1, 1.4	<0.001
	TRIMEB	0.63	0.45, 0.80	<0.001
Random				
	Strains	0.40	-	-
	Residuals	0.396	-	-

¹CI = Confidence interval

²RMPI (reference medium used), supplemented with α -cyclodextrin (α -CD), β -cyclodextrin (β -CD), γ -cyclodextrin (γ -CD), hydroxypropyl- β -cyclodextrin (HP- β -CD) or heptakis-(2,3,6-tri-O-methyl)- β -cyclodextrin (TRIMEB).

Table S-1: Outputs of the linear mixed-effects model using strains, media and fungicides to explain observed minimal inhibitory concentrations. In the model, RMPI medium and difenoconazole were used as references.

System*	Cyclodextrin	Ligand	Side of cubic box (Å)	Water
α CD	α	-	13.31	75776
β CD	β	-	13.51	78159
γ CD	γ	-	13.68	80987
HP β CD	β	-	13.90	84595
α CD-DIFENO	α	DIFENO	13.31	75249
β CD-DIFENO	β	DIFENO	13.51	78141
γ CD-DIFENO	γ	DIFENO	13.68	80970
α CD-TEBU	α	TEBU	13.31	75259
β CD-TEBU	β	TEBU	13.51	78141
γ CD-TEBU	γ	TEBU	13.68	80969
HP β CD-DIFENO	β	DIFENO	13.90	84579

Table S-2: Box size and molecular content of each studied system.

* α -cyclodextrin (α -CD), β -cyclodextrin (β -CD), γ -cyclodextrin (γ -CD), hydroxypropyl- β -cyclodextrin (HP- β -CD), difenoconazole (DIFENO), tebuconazole (TEBU). For all systems, the following equilibration protocol was applied. All simulations were performed using the Gromacs 5.1.2 MD package [1] with the AMBER14sb force field [2]. All systems were immersed into a cubic periodic box of approximately 14 nanometer (nm) side filled of TIP3P water molecules [3]. The cut-off distance for non-bonded interactions was set to 1.2 nm. All systems were first relaxed by steepest descent method at 5000-step with an energy convergence criterion of 10 kJ/mol. . They were then heated from 0 K to 310 K for 100 picoseconds (ps) and equilibrated at 1 atmosphere (atm) with a relaxation time of 2 ps for 150 ps at the NPT ensemble. Pressure of 1 atm was maintained by the isotropic Parrinello-Rhman barostat [4] using Langevin dynamics. Velocity rescale thermostat [5] was used at the temperature of 310 K. All bonds were constrained using the LINCS algorithm [6].

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Résultats complémentaires de l'article :

Objectif : Dans le but d'évaluer l'efficacité des cyclodextrines β -CD et HP- β -CD sur d'autres triazolés, des tests ont été réalisés sur 4 autres molécules : metconazole, propiconazole, bromuconazole et époxiconazole.

L'efficacité de deux cyclodextrines, β -CD et HP- β -CD a été plus largement étudiée avec 4 autres molécules triazolées fréquemment retrouvées dans l'environnement. Ainsi la sensibilité de 6 souches d'*A. fumigatus* parmi celles précédemment étudiées a été testée pour le metconazole, le propiconazole, le bromuconazole ou l'époxiconazole en présence ou en absence de cyclodextrines. Les tests ont été réalisés avec les mêmes ratios molaires que ceux étudiés précédemment (10 :1) pour chaque molécule triazolée (Tableau 12). Ces résultats ont confirmé les capacités des cyclodextrines à interagir avec elles.

Tableau 12 : Facteur d'augmentation des concentrations minimales inhibitrices (CMI) observées pour 6 souches d'*Aspergillus fumigatus* testées selon la méthode EUCAST en présence des fongicides metconazole, propiconazole, bromuconazole et époxiconazole. Les CMI ont été mesurées en présence des cyclodextrines β -CD et HP- β -CD ajoutées au milieu de culture à un rapport molaire de 10 :1.

Souche	Metconazole		Propiconazole		Bromuconazole		Epoxiconazole	
	β -CD	HP- β -CD	β -CD	HP- β -CD	β -CD	HP- β -CD	β -CD	HP- β -CD
4E007	-	x2	x2	x2	-	-	-	x2
4E009	-	x2	x2	x2	-	x2	-	x2
4E011	x2	x2	x2	x2	-	-	-	x2
4E016	x2	x2	x2	x2	-	-	x2	x2
4E026	-	x2	x2	x2	-	-	x2	x2
4E027	x2	x2	x2	x2	-	-	-	x2

La présence de β -CD dans le milieu de culture inhibe l'action fongistatique du propiconazole pour toutes les souches testées. Son action est en revanche partielle concernant le metconazole et l'époxiconazole. Aucun effet n'a été observé concernant le bromuconazole. La comparaison des deux cyclodextrines concernées a permis de confirmer que l'HP- β -CD semble être la plus efficace, un effet de la présence de cette cyclodextrine étant observé pour trois des quatre triazolés testés pour toutes les souches. Seul le bromuconazole ne voit pas son action modifiée sur les souches fongiques. Ces résultats ont été confirmés par microscopie avec une absence d'effet de la présence des cyclodextrines en présence de bromuconazole sur la pousse fongique en comparaison des autres triazolés testés. Contrairement aux autres triazolés étudiés, la structure même de la molécule de bromuconazole semble peu favorable à la création d'une interaction de type hôte-invité avec les cyclodextrines testées, probablement en raison de la

présence de l'atome de brome de rayon ionique élevé (196 pm) générant un encombrement stérique plus important.

4.4.4. Rôle potentiel du chanvre dans la lutte contre la pollution environnementale aux triazolés

Titre de l'article :

Adsorption of a triazole antifungal agent, difenoconazole, on soils from a cereal farm:
Protective effect of hemp felt

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Hypothèses de travail :

- Le chanvre est un matériau susceptible de bloquer la diffusion des molécules triazolées dans les sols après leur application.
- Les capacités d'adsorption de ces supports de chanvre peuvent être améliorées par un « greffage » de cyclodextrines en surface.
- L'utilisation environnementale de ces matériaux de chanvre greffés peut diminuer la diffusion environnementale des triazolés et limiter la pression de sélection de ces molécules sur les espèces fongiques des sols.

Résumé en français :

Le difénoconazole est un fongicide triazolé largement utilisé en agriculture pour la protection de diverses cultures. La présente étude a évalué, par une technique en batch, la capacité de deux sols à adsorber le difénoconazole. Les modèles de Freundlich et de Henry (modèles d'isothermes d'adsorption) répondent bien aux données d'adsorption, ce qui suggère une adsorption multicouche du difénoconazole. Les indices d'ubiquité des eaux souterraines (GUS) sont compris entre 1,8 et 2,8, ce qui indique que le difénoconazole présente un risque modéré de contamination des ressources en eaux souterraines. L'adsorption, dans les expériences de percolation et de lixiviation a également été testée en laboratoire sur une colonne de sol. Nous avons effectué des tests sur des sols seuls, sur des sols recouverts de feutre de chanvre (brut

ou modifié chimiquement par le greffage de molécules de cyclodextrines) comme matériau adsorbant pour limiter la dissipation de cette molécule dans le sol, et également sur les feutres seuls. Le sol seul a adsorbé jusqu'à 99% du difénoconazole, alors que le dépôt du feutre de chanvre modifié sur le sol a permis de limiter le stockage dans le sol à 26%, probablement en raison de la formation d'un complexe d'inclusion avec les cyclodextrines au sein du feutre. Le piégeage des molécules de difénoconazole par le feutre n'est pas irréversible, puisque le lavage de celui-ci avec une solution de CaCl_2 a entraîné la libération de 60% de la quantité initialement fixée sur le chanvre brut. Cependant, seulement 22% de la quantité fixée sur le chanvre modifié a été libérée, en raison d'interactions potentiellement plus fortes avec ce matériau (interactions type hôte/invité entre le difénoconazole et cyclodextrine). Ces mêmes lavages, réalisés à l'eau, entraînent une lixiviation du difénoconazole nettement plus réduite qu'avec la solution de chlorure de calcium (conditions extrêmes). En conclusion, cette étude a montré que l'utilisation de matériaux innovants à base de chanvre pourrait permettre de limiter l'accumulation de fongicides dans les sols.

Eléments essentiels :

- Le difénoconazole est une molécule triazolée très utilisée dans l'environnement comme fongicide, induisant une forte pollution environnementale ;
- L'adsorption de ce composé dans les sols semble suivre le modèle théorique de Freundlich et être particulièrement influencée par la quantité de matière organique du sol ;
- Lors de la percolation d'une solution de difénoconazole à travers le sol, plus de 85% de la quantité introduite se fixe dans le sol, signe de sa forte affinité pour ce dernier et de son potentiel contaminant ;
- Le risque de pollution peut potentiellement également concerner les eaux souterraines, du fait d'une lixiviation, avec un indice GUS calculé jusqu'à 2,6 ;
- L'utilisation, en surface du sol, d'un matériau de chanvre greffé par des cyclodextrines permet de limiter l'adsorption du triazolé par le sol à 26%, le matériau piégeant une grande partie du difénoconazole ;
- Ces matériaux de chanvre présentent de bonnes capacités de rétention des triazolés permettant de limiter la pollution environnementale malgré un phénomène de désorption constaté.



Adsorption of a triazole antifungal agent, difenoconazole, on soils from a cereal farm: Protective effect of hemp felt

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ABSTRACT

Difenoconazole is a triazole fungicide widely used in agriculture for the protection of various crops. The present study evaluated by batch technique, the high capacity of two soils to adsorb difenoconazole. The Freundlich and Henry models fitted the adsorption data well, suggesting a multi-layer adsorption of difenoconazole. Groundwater ubiquity score (GUS) were between 1.8 and 2.8, indicating that difenoconazole had a moderate risk of contaminating groundwater resources. Adsorption in percolation and leaching experiments were also tested in the laboratory with a soil column. We performed tests on soils alone, on soils covered by hemp felt (raw or chemically modified by the grafting of cyclodextrin molecules) as an adsorbent material for limiting the dissipation of this molecule in the soil, and at the same time on felts alone. Soil alone adsorbed up to 99% of the percolating difenoconazole, whereas the use of modified hemp felt made it possible to limit soil storage to 26%, probably due to the formation of an inclusion complex with cyclodextrins. The trapping of difenoconazole molecules by the felt was not irreversible, as leaching with a CaCl₂ solution resulted in the release of 60% of the amount initially fixed on raw hemp. However, only 22% of the amount fixed on modified hemp was released, due to potentially stronger interactions with this material (host/guest type interactions between difenoconazole and cyclodextrin). In conclusion, this study showed that the use of innovative hemp-based materials could permit to limit the accumulation of fungicides in soils.

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1. Introduction

Azoles are very versatile fungicides widely used to control fungal infestations in crops. Triazole fungicides are commonly used in agricultural systems, such as market gardening, cereal crops, ornamental cultures and vineyards, commonly because they have a very broad spectrum of efficiency, against the main diseases of wheat, barley, rye, oat and triticale, for example. Indeed, triazole fungicides are essential to ensure global food security, and their use is forecasted to intensify. In Europe, triazole-based formulations are the most widely used plant protection products (on grains, cereals, fruits and vegetables), due to their protective and curative actions (Zubrod et al., 2019; Jorgensen et al., 2018).

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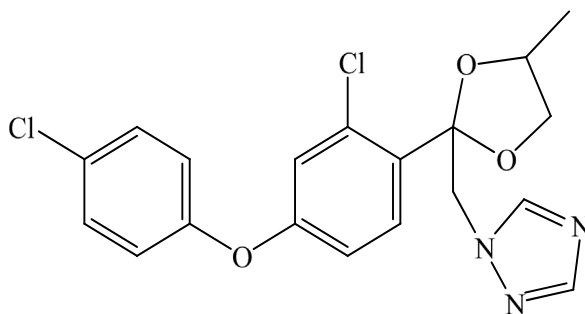
In France, various triazoles are available. Difenoconazole, the second molecule of this family to be released on the market, is widely used as a broad-spectrum environmental fungicide, particularly since the withdrawal of epoxiconazole and propiconazole (Chowdhary and Meis, 2018; European Crop Protection, 2016). The dissipation of this type of fungicide in treated fruits, for example, seems inevitable, as does contamination of the soil, water (including drinking water) and other environmental compartments, as suggested by Agnihotri et al. (1994), who reported that 70% of the pesticide applied inevitably reaches the soil during the spraying process, or by El Azhari et al. (2018), who reported that pesticides enter the soil with precipitation, reaching aquatic ecosystems. Due to their particular physicochemical properties, such as lipophilicity and stability (e.g. against hydrolytic degradation), fungicides have a moderate-to-high potential to interact with organic carbon and may, therefore, also be adsorbed by sediments. Elfikrie et al. (2020) found pesticides in the Tengri river basin in Malaysia, the highest mean concentration (4493.1 ng g^{-1}) being recorded for propiconazole. Fungicides have also been identified in foods. Difenoconazole and propiconazole residue levels in wheat grain have been found to be below 0.01 mg kg^{-1} , in China, suggesting that the use of these two pesticides can be considered safe in conditions of good agricultural practice in Chinese fields (Zhang et al., 2015). By contrast, levels of difenoconazole residues in peaches, which are widely grown around the world, range from 0.054 to 0.11 mg kg^{-1} (Dong et al., 2019). For the highest values in this range, the possibility of a risk to human health of consuming peaches cannot be excluded. Difenoconazole residues are also found in soils, chilli peppers, tomatoes and lettuces for example, posing potential risks to human health (Mukhopadhyay et al., 2011; Elgueta et al., 2020).

With regard to health, Pereira et al. (2019) evaluated the sperm quality of rats exposed to difenoconazole. Sperm number in the testis and the caput/corpus of the epididymis, daily sperm production, progressive motility, acrosomal integrity, and percentage of morphologically normal spermatozoa were all lower in rats exposed to difenoconazole than in unexposed rats.

The intensive use of triazole fungicides, such as difenoconazole, has also created a problem of resistance in fungal pathogens of plants. Brazil is one of the largest producers of papaya worldwide. Stem-end rot, caused by *Lasioidiplodia theobromae*, is one of the most important diseases of papaya. It can be controlled effectively with demethylation inhibitor (DMI) fungicides, but the occurrence of DMI resistance decreases fungicide efficiency. Difenoconazole has been one of the most widely used DMIs in papaya orchards since the start of the 2000s. Li et al. (2020) reported that the most tolerant isolates of this pathogen have a resistance factor for difenoconazole 240 times that of the most sensitive isolates, necessitating the implementation of resistance management strategies. Rotations and the use of mixtures of fungicides with different modes of action has frequently been used to manage fungicide resistance in many crop systems. However, the use of these phytosanitary agents has also resulted in resistance phenomena in fungi other than plant pathogens. Indeed, triazoles are also used as antifungal agents in human medicine, to treat serious fungal infections caused by *Aspergillus fumigatus*, in particular, a microscopic fungus ubiquitous in the environment and capable of causing respiratory disease. The triazole fungicides used in agriculture to protect cereals, vegetables, grapevines and horticultural crops can accumulate in the soil, with ecotoxicological effects on soil microbial communities, leading to the unintentional and undesirable selection of resistant *A. fumigatus* populations (Rocchi et al., 2018).

Various strategies can be adopted to limit the diffusion and accumulation of triazoles in soils. When used as a soil amendment, biochar (a form of charcoal) enhances plant growth and soil fertility and can sequester contaminants. Cheng et al. (2017) studied the effect of biochar on difenoconazole bioavailability and the composition of the microbial community in contaminated soils. They demonstrated that soil applications of biochar significantly decreased the bioavailability of difenoconazole to plant and soil microbial communities, and minimized the amounts of difenoconazole residues in agricultural produce. More recently, Wang et al. (2020) reported the adsorption of difenoconazole onto agricultural polyethylene film used in the dryland cultivation of vegetables and other crops for its ability to store water and enhance moisture levels, thereby increasing crop yield. The authors suggested that strong hydrophobic interactions between the polyethylene film and difenoconazole molecules could account for their findings.

In a previous work, an innovative hemp-based material in the felt form was used as an efficient filter for the removal of triazole fungicides by a batch technique. Adsorption results showed its good ability to retain fungicides (Lechenault-Bergerot et al., 2019). Here we studied two agricultural plots in the Franche-Comté region dedicated to cereal crops, for which treatment with difenoconazole-based formulations was envisaged. It was, therefore, essential to have prior knowledge of how the molecule was likely to behave in these soils. Thus firstly, the physicochemical properties of the soils, which are known to regulate the processes involved in the dissipation of pesticides, were determined. Secondly, the difenoconazole adsorption onto soils and its desorption process were studied using two methods: a batch technique and a percolation method, in the absence or presence of a hemp felt used as a barrier against the penetration of difenoconazole in soil. In the batch method, the fungicide and the soil remained in constant contact for a fixed period of time, whereas, in the percolation method, the fungicide infiltrates the soil. The percolation method more closely resembles the real-life situation in the field (spraying of the treatment solution, gradual penetration into the soil). Hemp is already used in horticulture and agriculture, as a mulch to prevent weed growth and to retain soil moisture. In this study, we investigate the development and application of an innovative product, i.e. hemp felt, for environmental benefits, in order to retain pesticides, and thus to control their accumulation in the soil. This could indirectly reduce the risks of bioavailability and toxicity soil fauna, or underground water contamination. Two hemp-based materials were used, namely a felt made of 100% hemp fiber, and a felt of the same composition coated with cross-linked cyclodextrin-1,2,3,4-butanetracarboxylic polymer, to provide inclusion properties. Indeed, cyclodextrins, biosynthetic molecules derived from starch, have the remarkable ability of forming host-guest interactions with organic substances in solution or in the solid state. To our knowledge, this is the first report of fungicide removal with a modified hemp-based material in felt form.



Scheme 1. Chemical structure of difenoconazole

2. Experimental procedures

2.1. Triazole

Difenoconazole is the common name for 1-[[2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole (IUPAC nomenclature). The commercial formulation (CAS number 119445-68-3, analytical standard Pestanal[®]) of this compound was purchased from Sigma Aldrich (Saint Quentin Fallavier, France) and used without purification. The chemical structure of difenoconazole is shown in [Scheme 1](#). This molecule has the following characteristics: molecular formula: C₁₉H₁₇Cl₂N₃O₃, molecular weight: 406.2 g mol⁻¹, solubility in water: 15 mg L⁻¹ at 20 °C, and Log K_{ow}: 4.36 at 25 °C.

Difenoconazole solution

A stock solution of difenoconazole in methanol (Sigma Aldrich, Saint Quentin Fallavier, France) was prepared at a concentration of 100 mg L⁻¹. Aqueous solutions in 0.01 M CaCl₂ (ProLabo, Paris, France) were prepared by diluting the stock solution to concentrations between 1 and 15 mg L⁻¹.

2.2. Soil characterization

Soil samples were collected from two agricultural plots (*Douce Terre* (DTP) and *Champ Marie* (CMP), GAEC du bas des Champs, Brussey, France) of a cereal farm likely to use triazole fungicides to protect its wheat and rapeseed crops (10 points per plot, approximately every 15 or 20 m, 2 to 3 kg of soil per point), freeze-dried and ground to 2 mm before use. Quantification revealed an absence of triazoles, except for trace amounts of tebuconazole (mean concentrations of 8.65 and 9.91 and ng g⁻¹ for DTP and CPM, respectively).

The mixture of the 10 samples from each plot formed a composite soil sample, which was then used for the determination of total element concentrations by inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Fischer Scientific, iCAP 6500 radial model, Courtaboeuf, France). The protocol was as follows: 500 mg of sample was mineralized with a mixture of 2 mL of 65% nitric acid and 5 mL of 37% hydrochloric acid in closed tubes placed in a block digestion system (DigiPREP system, SCP Sciences, Courtaboeuf, France). The sample was gradually heated to a final temperature of 100 °C. Then, ultrapure water (Millipore Milli-Q Integral 3 system, Molsheim, France) was added to a final volume of 50 mL. Finally, the solution was passed through a filter with 1 μm pores before analysis. The organic matter (OM) content of the composite soil samples was estimated by comparing mass before and after the calcination of 10 g of dried soil at 550 °C for 4 h. The pH was also measured with a WTW[®]-pH meter, after shaking 10 mL of dry soil in 50 mL of reverse osmosis (RO) water (1:5 suspension, v:v) for 20 min and then allowing the suspension to rest overnight. Granulometric analyses were performed with a LS 230 laser diffraction particle size analyzer (Beckman Coulter, Villepinte, France). The physicochemical properties and metal contents of the composite soils are summarized in [Table 1](#). A preliminary detailed ICP analysis showed that concentrations were similar for the two soils. The main elements present were Al, Fe, Ca, K and Mg.

2.3. Hemp material

Hemp was kindly provided by Eurochanvre, a hemp processing company in Franche-Comté (Arc-les-Gray, Haute-Saône, France). The raw hemp (RH) consisted of a felt made of 100% hemp fiber. It had the following characteristics: thickness of 5 mm, fibers composed of 65% cellulose, 20% hemicellulose, 8% lignin, 5% pectin, and 2% impurities. Before use or chemical modification, this material was thoroughly washed in RO water and dried at 60 °C until a constant weight was reached (chemical analysis: 41.4% C, 0.28% N, and 0.06% S) ([Loiacono et al., 2017a](#)).

Modified hemp felt with inclusion properties was synthesized by introducing molecules of hydroxypropyl-β-cyclodextrin (HPβCD, Roquette, Lestrem, France) through a cross-linking reaction with 1,2,3,4-butanetetracarboxylic acid

Table 1
Physicochemical properties and total element analysis of soils (*Douce Terre* (DTP), *Champ Marie* (CMP)).

	DTP	CMP
Clay (%)	7.73	10.66
Silt (%)	69.68	74.67
Sand (%)	22.61	14.71
Texture	silt loam	silt loam
pH _{water}	6.70	6.10
Organic matter (%)	3.86	3.51
Elements ($\mu\text{g g}^{-1}$)		
Al	13399	9300
As	7.69	8.93
B	11.24	6.06
Ca	2364	1400
Cd	0.21	0.26
Co	11.37	8.32
Cr	25.69	18.49
Cu	7.36	9.33
Fe	15400	12845
K	2725	1634
Mg	1750	1327
Mn	695	672
Na	34.26	26.75
Ni	13.71	10.37
P	446	495
Pb	17.32	16.60
S	171	187
Si	78.78	75.02
Sr	9.84	7.36
Ti	127.24	150.80
Zn	36.64	35.63
Hg, Sb, Se, Sn	<LDM ^a	< LDM

^aLimit of detection

(BTCA). The method used to produce a hemp felt coated with hydroxypropyl- β -cyclodextrin-1,2,3,4-butanetetracarboxylic cross-linked polymer (modified hemp, MH) and its chemical and spectroscopic characterization have been described in detail elsewhere (Loiacono et al., 2017b, 2018). Briefly, the RH sample was impregnated and roll-squeezed in a roll padder, in an aqueous solution containing sodium hypophosphite as a catalyst, BTCA as the crosslinking agent, and HP β CD. A cross-linking reaction based on esterification (Fig. 1) occurred during the synthesis, such that the carboxyl groups of BTCA, the hydroxyl groups of HP β CD hydroxyl and the cellulose hydroxyl groups of the hemp fibers (in the RH) were cross-linked. This resulted in a cross-linked polysaccharide network containing cyclodextrin molecules and carboxyl groups. We used a modified hemp felt (MH) with a coating rate of $35 \pm 5\%$, assessed on the basis of the weight gain of the sample (elemental analysis: 51.3% C, 0.27% N, and 0.05% S).

RH and MH disks of 2.5 cm in diameter were used in the adsorption experiments. These disks had a mass of 0.277 ± 0.069 g for RH and 0.408 ± 0.042 g for MH.

2.4. Adsorption test

The experiments were conducted with two adsorption-oriented methods: a batch method and a percolation technique. The first set of experiments was performed with a batch method, in which the kinetic and adsorption isotherms of the soils were determined. In each experiment, 10.000 ± 0.001 g of each soil was added to 50 mL of aqueous difenoconazole solution in 0.01 M CaCl₂ in a tightly closed amber glass flask, and the mixture was stirred with a magnetic stirrer. The experiments were conducted at 25 ± 1 °C, in triplicate (repeatability was checked; relative standard deviation below 20%). The soil was allowed to sediment out and the supernatant was collected, centrifuged for 10 min at 2800 g and subjected to extraction and chromatographic analyses. For kinetic experiments, two concentrations were tested: 1 and 15 mg L⁻¹ (the first being a commonly used per-hectare rate, and the second being an extreme rate), for various times, ranging from 15 to 480 min, to determine equilibrium. For adsorption isotherms, the effect of difenoconazole concentration was tested for various doses (1, 2.5, 5, 7.5, 10, 12.5 and 15 mg L⁻¹) and a contact time of 2 h (this time is sufficient to reach equilibrium).

For the percolation procedure (Scheme 2), we used a glass column with an internal diameter of 2.5 cm and a height of 22 cm, with a Teflon tap, which was filled from bottom to top with the following materials: first a piece of glass wool, then glass beads to one third of the total height of the column and finally either in the first case the dried soil (10.000 ± 0.001 g), whether or not surmounted by hemp felt disks, or in the second case one or two hemp felt disks only (without soil).

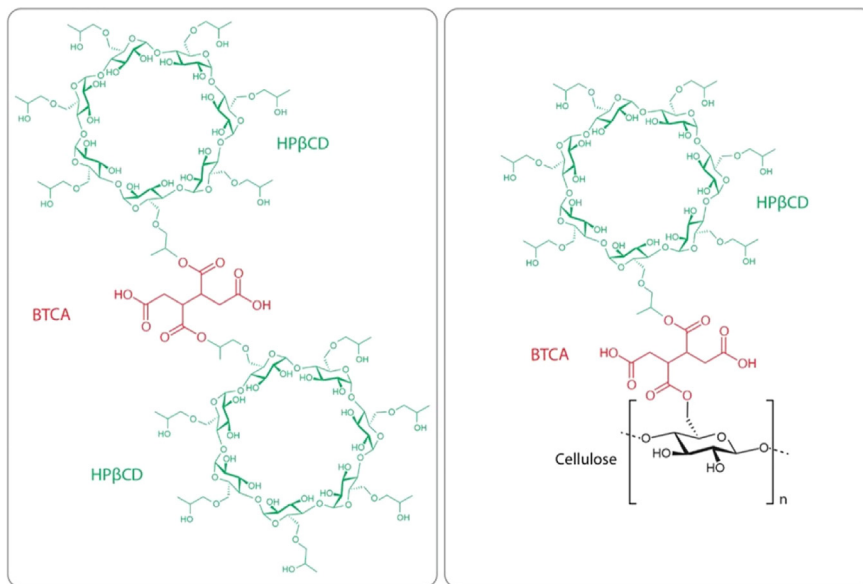
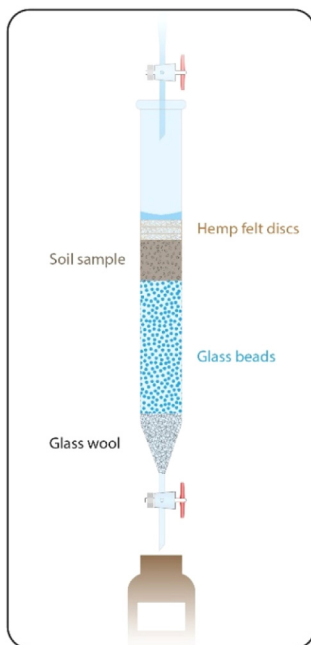


Fig. 1. Possible reactions between 1,2,3,4-butanetetracarboxylic acid (BTCA) and anhydro glucose units of (left) two hydroxypropyl-β-cyclodextrin (HPβCD) molecules and (right) one HPβCD molecule and one cellulose chain.



Scheme 2. Illustration of the percolation setup (first case).

Fifty milliliters of aqueous difenoconazole solution at a concentration of 1 or 15 mg L⁻¹ in 0.01 M CaCl₂ were introduced dropwise via a glass burette (percolation time = 2 h). Control experiments (without soil and hemp felt) were also carried out in the same conditions, to check that the device itself did not adsorb the fungicide.

Difenoconazole removal is expressed as the percentage abatement, defined as the ratio of the amount of pollutant absorbed to the initial amount of pollutant (C_0). Experiments were performed in triplicate.

2.5. Desorption test

Desorption was studied by percolation, with hemp felt disks or soils alone and with the combination of hemp felt disks and soil after the adsorption study. Fifty milliliters of 0.01 M CaCl₂, without difenoconazole, dropwise, was added to the wet hemp, soil or hemp/soil combination (lixiviation time = 2 h). This procedure was carried out once, or three times. Experiments were performed in triplicate. Difenoconazole desorption is expressed as a percentage of the previously adsorbed pesticide.

2.6. Extraction in aqueous solution

An aliquot of 10 mL of aqueous difenoconazole solution (initial solutions and solutions obtained in adsorption or desorption tests) was transferred into a 250 mL closed amber glass flask and 20 mL acetonitrile was added (Acetonitrile Distol[®], Fisher Scientific, Illkirch, France). The solutions were stirred with a magnetic stirrer for 10 min. Then, 3 g sodium chloride (purity: 99.5%) was added (Sigma Aldrich, Saint Quentin Fallavier, France) and the agitation was maintained for 10 min again. The organic supernatant layers were recovered with a separating funnel and transferred to a volumetric flask, for adjustment of the final volume to 25 mL. Volumes of 475 µL were collected and transferred to a vial syringeless filter (Mini-Uniprep 12 × 32 PTFE 0.45 µm silicone/PTFE clear housing, Interchim, Montluçon, France) containing 25 µL of deuterated internal standard, tebuconazole d₆, at a concentration of 2 mg L⁻¹ (Sigma Aldrich, Saint Quentin Fallavier, France).

2.7. Analysis

Sample extracts were analyzed with a GC-MS/MS apparatus (Agilent, Massy, France), consisting of a 7890A GC system, a 7000 GC triple quadrupole mass spectrometer, and an 80 GC CombiPAL autosampler. One microliter of the extract was injected at a temperature of 300 °C, in splitless mode, under a constant flow of He (purity 99.9999%) at 1.5 mL min⁻¹, followed by a purge flow to split vent after 0.5 min. We used a (5% phenyl)-methylpolysiloxane HP5MS column (30 m × 0.25 mm I.D., 0.25 µm film thickness, Agilent 19091J-433). The total run time was 42.27 min. The temperatures of the transfer line, ion source and quadrupoles were 300, 300, and 150 °C, respectively. An electron-impact ion source at 70 eV was required. Nitrogen, with a purity of 99.9%, was produced with a NiGenLC-MS40-1Claind generator (Gengaz, Wasquehal, France) and used as the collision gas, at a flow rate of 1.5 mL min⁻¹. The optimized mass parameters in multiple reaction monitoring mode were previously described by Crini et al. (2017).

2.8. Modeling

Difenoconazole adsorption data were analyzed with Henry, Freundlich and Langmuir models (Romita et al., 2019).

The linear Henry equation is described by Eq. (1) (Conde-Cid et al., 2019):

$$q_e = K_D C_e \quad (1)$$

where q_e (µg g⁻¹) is the amount of difenoconazole retained by the soil, C_e (µg L⁻¹) is the difenoconazole equilibrium concentration and K_D (L g⁻¹) is the coefficient of distribution.

The Freundlich isotherm equation (Eq. (2)) can be log-linearized, as reported in Eq. (3):

$$q_e = K_F C_e^{1/n} \quad (2)$$

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \quad (3)$$

where K_F [(µg g⁻¹)(L µg⁻¹)^{1/n}] is the Freundlich adsorption equilibrium constant representing the adsorption capacity constant. The n parameter is also a constant, representing adsorption intensity: $n < 1$ means that the process is unfavored, at $n = 1$ it is linear and at $n > 1$ it is favored.

The Langmuir adsorption theory is described by the following equation (Eq. (4)), which can be linearized (Eq. (5)):

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \quad (4)$$

$$\frac{C_e}{q_e} = \frac{1}{q_m} C_e + \frac{1}{q_m K_L} \quad (5)$$

where K_L (L µg⁻¹) is the Langmuir equilibrium constant related to the adsorption energy and q_m is the maximum amount adsorbed per unit mass of adsorbent.

The soil organic carbon-adsorption coefficient K_{OC} , used to control the adsorption capacity of the soil, was determined from the K_D values, with Eq. (6) (Mosquera-Vivas et al., 2018):

$$K_{OC} = \frac{K_D \cdot 100}{\%OM} \quad (6)$$

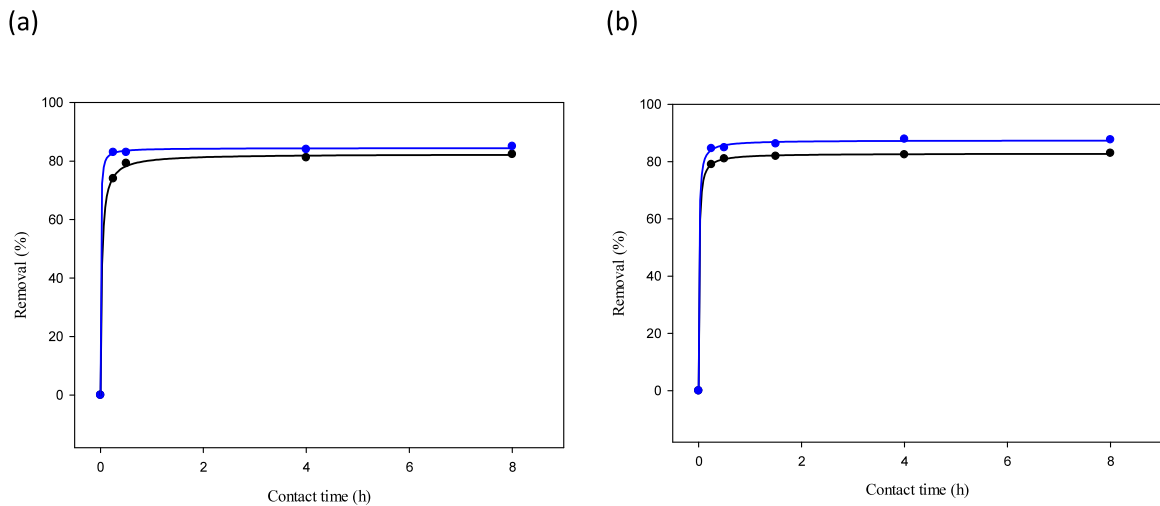


Fig. 2. Effect of contact time on difenoconazole adsorption (in %) on *Douce Terre* (DTP, blue curve) and *Champ Marie* (CMP, black curve) soils (conditions: volume 50 mL; soil 10 g; temperature 25 °C; $n = 3$; difenoconazole concentration C_0 1 mg L⁻¹ (a) and 15 mg L⁻¹ (b)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

where the units of K_D and K_{OC} are L g⁻¹. Higher K_{OC} values indicate stronger adsorption of the pesticide and, thus, lower levels of pesticide mobility.

The GUS index developed by Gustafson in 1989 (Gustafson, 1989) was also calculated, as an empirical model based on the persistence of pesticides (half-life DT_{50} or $t_{1/2}$) and K_{OC} . The relationship between these two parameters is as follows (Eq. (7)):

$$\text{GUS index} = \log(t_{1/2}) \times [4 - \log(K_{OC})] \quad (7)$$

where K_{OC} is expressed in mL g⁻¹ and $t_{1/2}$ in days.

The potential for movement toward groundwater was classified on the basis of GUS index, as follows: extremely low below 0, low between 0 and 1.8, moderate in the range 1.8–2.8 and high above 2.8.

3. Results and discussion

3.1. Kinetics of soil adsorption in batch experiments

Fig. 2 shows the removal of difenoconazole adsorbed in both *Douce Terre* (DTP) and *Champ Marie* (CMP) soils against contact time (t) for concentrations of 1 and 15 mg L⁻¹. Whatever the soil or fungicide concentration, the kinetic results indicated that adsorption was very fast, indeed, it was almost instantaneous ($t < 0.5$ h). Similar results were published by Paszko and Jankowska (2018), who modeled the effect of propiconazole adsorption in Polish luvisol profiles and by Siek and Paszko (2019), who assessed tebuconazole adsorption in mineral soil profiles. In less than two hours, the amount of fungicide adsorbed attended to a constant value, with no more fungicide retained by the soil (abatement > 80%), indicating strong interactions between difenoconazole molecules and binding sites in the soil. The number of binding sites for difenoconazole in the soil was not a limiting factor for adsorption, because the percentage removal was highest for the highest difenoconazole concentration (means of 84% (DTP) and 79% (CMP), versus 86% (DTP) and 81% (CMP), for 1 and 15 mg L⁻¹, respectively). Abatement was always slightly higher in the DTP soil than in the CMP soil, probably due to the slightly higher organic matter content of the DTP soil, as pointed out by Arias-Estévez et al. (2008) and by Lechenault-Bergerot et al. (2019).

3.2. Adsorption isotherms in batch experiments

Difenoconazole adsorption data were analyzed with Henry, Freundlich and Langmuir models (Romita et al., 2019). The Langmuir model was not suitable for either soil ($\frac{C_e}{q_e}$ was not a linear function of C_e , $r^2 < 0.76$), indicating that a monolayer adsorption model was not realistic. Other adsorption parameters, from Henry and Freundlich models, are presented in Table 2. The K_D values obtained with the linear Henry model were of the same order of magnitude as those reported by Mosquera-Vivas et al. (2018) for the adsorption of tebuconazole on Colombian agricultural soils. Determination coefficients (r^2) were good, especially for the Freundlich model, reflecting a favorable multi-layer adsorption of difenoconazole. This result suggests that π - π interactions occur between the aromatic rings of several molecules of difenoconazole. The K_D

Table 2

Comparison of distribution coefficient (Henry model, K_D), Freundlich isotherm parameters (K_F , n), K_{OC} values and GUS index (mean values, $n = 3$) for *Douce Terre* (DTP) and *Champ Marie* (CMP) soils ($r^2 =$ determination coefficient).

	K_D (L g ⁻¹)	r^2	K_F^a	n	r^2	K_{OC} (mL g ⁻¹)	GUS-Index
DTP	0.0493	0.860	1.383	1.859	0.917	1277	2.23
CMP	0.0320	0.899	1.323	1.967	0.950	911	2.60

^aExpressed in ($\mu\text{g g}^{-1}$)(L μg^{-1})^{1/n}.

Table 3

Mean removal (%) of difenoconazole on raw (RH) and modified (MH) hemp felt (1 disk for 1RH and 1MH, 2 disks for 2RH and 2MH) and *Douce Terre* (DTP) and *Champ Marie* (CMP) soils topped by two hemp disks; conditions: percolation (contact time = 2 h); volume 50 mL; soil 10 g; temperature 25 °C; $n = 3$; difenoconazole concentration C_0 1 mg L⁻¹.

	1RH	2RH	1MH	2MH
Hemp alone	27.7	33.6	43	74
Hemp + DTP	/	99.5	/	99.8
Hemp + CMP	/	100	/	99.9

and K_F values were higher for the DTP soil than for the CMP soil. This order is consistent with the findings of Hammi et al. (2019), who observed that the clay fraction present in the soils studied was irrelevant to pentaconazole adsorption (in our case, 7.73% versus 10.66% for DTP and CMP, respectively, Table 1), and highlighted the predominant role of organic matter (OM) (in our case, 3.86% versus 3.51% for DTP and CMP, respectively, Table 1). The DTP soil had a K_{OC} value (Eq. (6)) 1.4 times higher than that of the CMP soil. Difenoconazole was therefore less mobile in the DTP soil. We checked that this was, indeed, the case, by calculating the GUS index (Eq. (7)). The reported half-life of triazoles in the soil differs between studies. For example, Wu et al. (2018) reported values of 6.8 to 32.5 days, whereas Guo et al. (2010) reported values of 9.6 to 68.4 days, and values as high as 1600 days have been proposed in some marketing reports. Taking the half-life of difenoconazole as 315 days, as reported by the European Food Safety Authority (2011), DTP and CMP can both be considered to be soils associated with a moderate risk of difenoconazole leaching into groundwater (GUS index <2.8, Table 2). Maznah et al. (2018) study reported a higher GUS index (4.61) for hexaconazole, classified as a leacher, for cultivated soil under Malaysian tropical conditions and referred to other studies reporting that the triazole groups of propiconazole, triticonazole and penconazole gave rise to GUS index values of 2.04, 3.08 and 3.50, respectively.

3.3. Percolation experiments

Firstly, the adsorption of difenoconazole on soils alone and on a single piece of hemp felt alone was studied (RH or MH). The experiments were conducted at two difenoconazole concentrations (1 and 15 mg L⁻¹), for a percolation time of 2 h, similar to the batch time used. The results are reported in Fig. 3. For soils, percolation gave slightly better abatements than the batch method (83%–93% versus 81%–86% for the batch method). However, the DTP and CMP soils yielded different patterns of results for the percolation and batch experiments. Indeed, for percolation, whatever the concentration of difenoconazole, the CMP soil retained more fungicide than the DTP soil, probably due to the greater draining power of sand, the proportion of which was higher in the DTP soil (Table 1). The use of a higher concentration of fungicide did not limit the adsorption of difenoconazole to soils in experiments performed by the batch method, but such limitation did appear to occur in percolation experiments. This finding can be explained by a lack of prolonged contact of the soil binding sites with the difenoconazole molecules, which pass through the soil rapidly (absence of continuous exchange during the entire flow). For hemp felts, regardless of the nature of the material (raw or modified), adsorption capacity decreased with increasing difenoconazole concentration, but abatement was higher with MH than with RH felt (39% versus 25%, mean values for the two concentrations tested). The modified material was, therefore, able to retain more difenoconazole molecules, suggesting that difenoconazole/HP β CD inclusion complexes may have formed in this material (Crini et al., 2017).

Secondly, the adsorption of difenoconazole on two pieces of hemp felt and combinations of two piece of hemp felt plus soil, was studied at a difenoconazole concentration of 1 mg L⁻¹, for a percolation time of 2 h, to compare the results with those of the previous experiment with only one hemp disk. The results are reported in Table 3. The superimposition of two RH disks increased difenoconazole elimination by +21% relative to a single disk, and significantly higher levels of elimination (+72%) were achieved with two disks of modified hemp. In the soil systems topped with 2 hemp disks, in which all the difenoconazole was retained (abatement of ~ 100%), one third of the total amount eliminated was stored in the raw hemp, whereas the modified hemp trapped more than two thirds. This finding further demonstrates the potential of modified hemp for eliminating difenoconazole through the formation of host-guest interactions via cyclodextrins grafted onto the hemp.

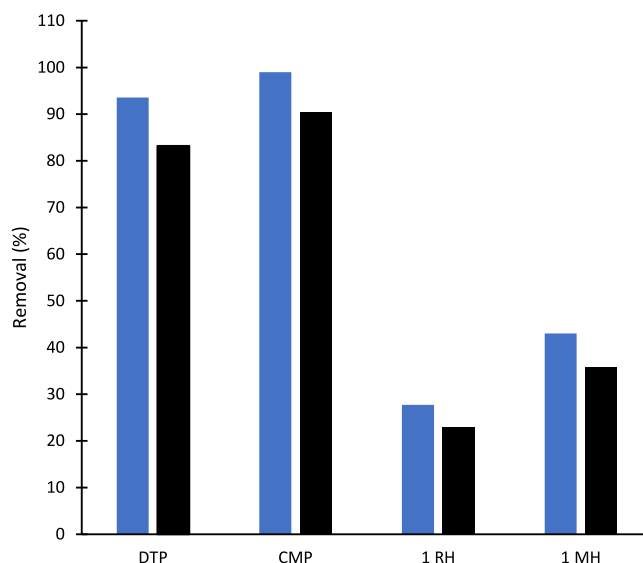


Fig. 3. Mean removal (%) of difenoconazole from *Douce Terre* (DTP) and *Champ Marie* (CMP) soils, one disk of raw (1RH) and modified (1MH) hemp felt; conditions: percolation (contact time = 2 h); volume 50 mL; soil 10 g; temperature 25 °C; $n = 3$; difenoconazole concentration C_0 1 mg L⁻¹ (blue bars) and 15 mg L⁻¹ (black bars). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Leaching experiments

Fig. 4 shows the percentage difenoconazole desorbed from soils (1 wash) and one piece of hemp felt (1 or 3 washes) for samples previously used in adsorption experiments. The total amount of difenoconazole desorbed depended on the amount previously added. For soils, the percentage desorption was low, and the values obtained were consistent with those reported by Conde-Cid et al. (2019) for propiconazole desorption on 10 acid sandy-loam soils with different organic matter contents. Desorption increased with the amount of difenoconazole initially fixed (1%–3% versus 9%–12% for C_0 initial solutions of difenoconazole at concentrations of 1 and 15 mg L⁻¹ respectively). For RH and MH felts, the opposite phenomenon was observed. However, whatever the initial concentration of fungicide and the type of felt, most of the fixed difenoconazole was removed in the first wash (61 to 75% of the total value for the three successive washes). The first wash with 0.01M CaCl₂, following the percolation of a 1 mg L⁻¹ difenoconazole solution, removed just under 60% of the amount fixed on one piece of RH felt, and just over 60% of the amount fixed on one piece of MH felt (Fig. 4), but similar leaching experiments on two pieces of hemp felt gave more contrasting results: again, about 60% was removed from the RH felt, but only 22% was removed from the MH felt (results not shown). This finding indicates that the interactions between difenoconazole and hemp felt were stronger for MH than for RH, due to the presence of cyclodextrin molecules (formation of more stable inclusion complexes with 2 pieces of MH than with one piece of this material). Washing one hemp disk with RO water (simulating rainwater) rather than CaCl₂ solution reduced fungicide leaching by a factor of 2 for RH and 4 for MH. This can be explained by the difference in ionic strength of the washing solution used, again revealing the high stability of the difenoconazole–CD complexes formed on MH, whereas the interactions established between difenoconazole and RH were weaker.

The leaching of the fungicide in 0.01M CaCl₂ from combinations of two pieces of hemp felt plus soil previously exposed to the percolation of a 1 mg L⁻¹ difenoconazole solution was extremely low (range: 0.24 to 3.31%, results not shown). Together, all these results suggest that, under the action of leaching, the difenoconazole molecules trapped on the hemp felt (the first compartment to be crossed) were able to migrate into the soil and remained strongly bound to it.

4. Conclusions

We describe here the adsorption of difenoconazole to two soils from a cereal farm. In the batch method, fungicide retention, which was consistent with the Freundlich model, was dependent on the amount of organic matter, with larger amounts of organic carbon in the soil associated with higher rates of adsorption. In the percolation procedure, adsorption decreased with increasing sand content in the soil. However, due to the high retention capacity of difenoconazole in both soils (maximum observed in this study: 677 μg of 750 μg, adsorbed by 10 g of the dried soil for CMP in the percolation method), we considered, for the first time, covering the soil with a natural felt with proven adsorbent properties, to prevent the migration into the soil of difenoconazole molecules sprayed on the plants for antifungal treatment. In a hemp plus soil system exposed to the percolation of a 1 mg L⁻¹ difenoconazole solution, the best results were obtained with a

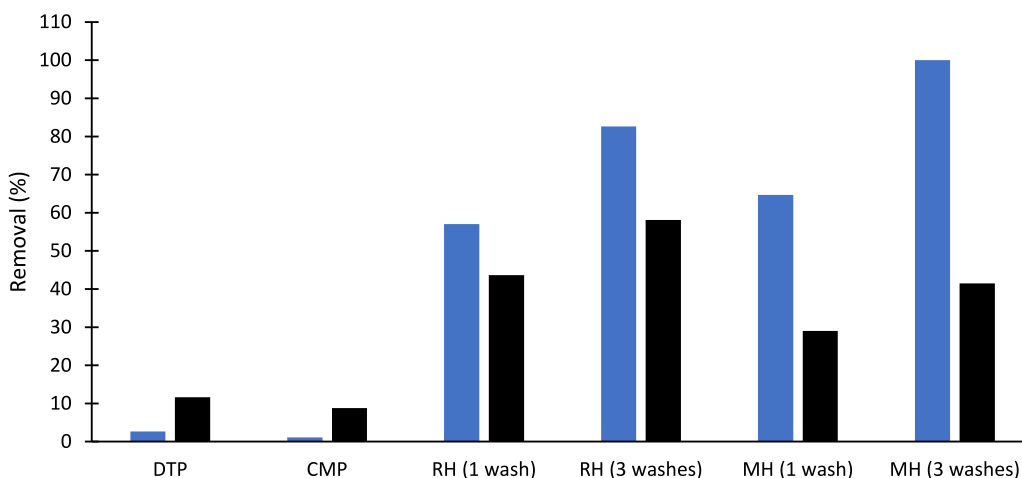


Fig. 4. Mean desorption (%) of difenoconazole on *Douce Terre* (DTP) and *Champ Marie* (CMP) soils, RH and MH disks (1 disk, 1 or 3 washes); conditions: percolation (contact time = 2 h); volume 50 mL; CaCl_2 0.01 M after adsorption of difenoconazole solutions C_0 at 1 mg L^{-1} (blue bar chart) and 15 mg L^{-1} (black bar chart) in the initial solution; soil 10 g; temperature $25 \text{ }^\circ\text{C}$; $n = 3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

double thickness of modified hemp felt on top of the soil: only $13 \text{ }\mu\text{g}$ of the $50 \text{ }\mu\text{g}$ of difenoconazole applied was stored in the soil, versus $49.5 \text{ }\mu\text{g}$ in the absence of felt. This material was, thus, considered effective, as it retained $37 \text{ }\mu\text{g}$ of difenoconazole, limiting the diffusion of this molecule deep into the soil by trapping it on the felt through the formation of inclusion complexes with the cyclodextrins grafted onto the material. This limiting of difenoconazole residues in soils should have the benefit of limiting the indirect effect of triazole fungicides on soil fungi and, thus, the development of resistance. However, as the desorption of difenoconazole adsorbed onto these materials was not null (the lowest levels of leaching from modified hemp were achieved with RO water), it would be appropriate, in field conditions, to deploy the adsorbent material on the soil during crop spraying to trap excess fungicide, and then to remove it, before the next rainfall.

CRedit authorship contribution statement

Chloé Godeau: Investigation, Formal analysis, Validation, Writing. **Nadia Morin-Crini:** Supervision, Funding acquisition, Validation, Review. **Jean-Noël Staelens:** Resources, Review. **Bernard Martel:** Resources, Review. **Steffi Rocchi:** Supervision, Funding acquisition, Review. **Gilles Chanet:** Resources, Review. **Marc Fourmentin:** Visualization, Review. **Grégoire Crini:** Conceptualization, Validation, Review.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5. Travaux en lien avec la thèse

D'autres travaux auxquels j'ai largement participé, en lien direct avec les problématiques présentées dans ce travail, ont été effectués et sont présentés ci-après.

Titre du chapitre :

Emergence of a pathogenic fungus resistant to triazole antifungal drugs.

Présentation succincte :

Dans cette revue, la problématique de la résistance aux triazolées chez *A. fumigatus* est discutée. Un état des lieux des connaissances concernant l'évolution de cette résistance tant sur le plan clinique qu'environnemental est présenté. Les paramètres qui influencent ce phénomène au niveau environnemental sont mis en avant et décrits comme des « hotspot » de la résistance.

Chapter 3

Emergence of a Pathogenic Fungus Resistant to Triazole Antifungal Drugs



Steffi Rocchi , Chloé Godeau, Grégorio Crini ,
and Eveline Snelders 

Abstract Over the past 20 years, emerging antifungal resistance has been observed in the human pathogen, *Aspergillus fumigatus*, and is responsible for serious infections. Resistance in this pathogenic fungus is mainly due to the unintended impact of triazole fungicides widely used in the environment to protect crops and materials from destroying fungi. *A. fumigatus* is naturally present in the environment and will thus be widely exposed to fungicides, which, through selection pressure, will favour strains that have become resistant. These fungicidal compounds have a chemical structure similar to that of medical compounds, thus rendering them inactive for the treatment of an infection in humans with a resistant strain. *A. fumigatus* does not cause infections in crops yet fungicidal compounds with activity against this widely present fungus select for an unintended cross-resistance. Therefore, it is important to understand the phenomena leading to resistance in the environment.

In this chapter, we first reviewed resistance mechanisms that have been described, both occurring under medical treatments and those rather attributed to environmental fungicidal use. We have detailed its spread from its probable origin in Europe to its current global distribution. Then, a focus on environmental contributing factors leading to resistance was done and in particular the cross-disciplinary use of azoles. We defined what can be considered as a hotspot of resistance selection and discussed

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the possible abiotic and biotics factors in soils implicated in the resistance emergence. Finally, we have considered various possibilities to stop the increase of this phenomenon: limit the uncontrolled spread of fungicides in the environment on the one hand and to reduce the exposure of fungicides on the other hand.

Keywords *Aspergillus fumigatus* · Antifungal resistance · Azoles · Agricultural fungicide use · Fungal life-cycle · Evolution

3.1 Introduction

The problem of resistance to biocides, and more particularly to antimicrobials, concerns all infectious organisms: bacteria, viruses, fungi and parasites. Resistance can occur naturally, but abuse and/or misuse of antimicrobials leads to resistance. The most well-known case is bacterial resistance, where the misuse of antibiotics in animal and human health has led to multidrug-resistance. In contrast, multidrug-resistant pathogenic fungi are much less well-known, even though it represents a considerable threat to the control of plant, animal and human diseases. Over the past 20 years, emerging antifungal resistance in human fungal pathogens has been observed but remains neglected in the overall context of antimicrobial resistance and is not currently the subject of most official surveillance programmes or addressed in for example any of the World Health Organisation programs.

Antimicrobial resistance threatens the heart of modern medicine and the long-term sustainability of an effective global public health response to the constant threat of infectious diseases. The World Health Organization adopted a global plan of action against antimicrobial resistance to maintain the capacity to treat and prevent infectious diseases as long as possible, through effective drugs, responsibly used and accessible to all those who need them. The Global Antimicrobial Resistance Surveillance System (GLASS) was launched in 2015 by the World Health Organization to support a standardized approach to global antimicrobial resistance data collection, analysis and reporting to support decision-making, motivate local, national and regional actions, and provide the evidence base for action and advocacy (Tornimbene et al. 2018). Antibiotic resistance is now recognized as a public health issue with concrete implemented actions: surveillance/prevention programs and directives such as prohibiting the use of certain antibiotics as additives in feeding stuffs. On the contrary, for 20 years, an emerging antifungal resistance has been observed but remains neglected in the overall antimicrobial resistance context. Three fungi of concern were however mentioned in the report of the second Global Antimicrobial Resistance Surveillance System meeting (5–16 December 2016, Geneva, Switzerland), namely *Candida*, *Aspergillus*, and fungi causing mucormycosis (GLASS 2016).

One particular fungus of concern for human health is *Aspergillus fumigatus* (Fig. 3.1). This ubiquitous fungus is responsible for a wide spectrum of pulmonary pathologies, ranging from chronic or immuno-allergic forms to severe invasive

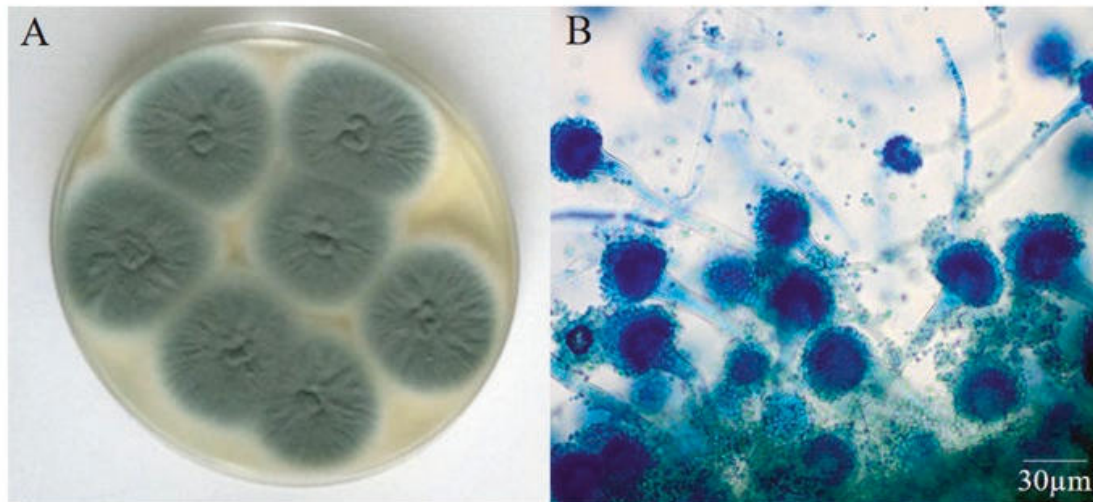


Fig. 3.1 Macroscopic (a) and microscopic (b) observations of *Aspergillus fumigatus*

infection with high mortality rates. In fact, *A. fumigatus* is the major etiological agent of opportunistic fungal infection in immunocompromised patients. Invasive aspergillosis, the most severe manifestation of *Aspergillus* disease, is difficult to diagnose and manage, has a mortality rate that can reach 65%, and is limited in treatment options (Verweij et al. 2009; Van der Linden et al. 2011).

Antifungal treatments for humans are limited to four classes of compounds: polyenes, echinocandins, pyrimidine analogue and triazoles. The development of triazole antifungal drugs, particularly voriconazole, in the early 2000s, has greatly improved invasive aspergillosis survival rate (Schwartz et al. 2005; Kourkoumpetis et al. 2012). Two other more recently available triazole molecules play an important role in the management of these diseases: isavuconazole, a first-line therapy for treating invasive aspergillosis, similar to voriconazole, and posaconazole, mostly used as a prophylactic treatment (Ullmann et al. 2018). However, the clinical advances made possible using medical triazoles could be threatened by the emergence of triazole-resistant *A. fumigatus* strains with a 20% lower survival rate in patients with culture-positive voriconazole-resistant invasive aspergillosis compared to azole-susceptible infections (Lestrade et al. 2019; Resendiz-Sharpe et al. 2019).

Antifungal resistance in *A. fumigatus* is however not widely considered to be an urgent public health problem for the following three reasons:

1. Fungal diseases are less known and there is a general lack of awareness despite their ubiquity and with global mortality rates exceeding those for malaria and breast cancer (Fisher et al. 2018);
2. Emerging antifungal resistance affects a number of patients that may seem limited, but there is a substantial underdiagnoses of fungal infections preventing accurate estimates. Also, during the congress of the European Society of Respiratory Diseases (September 17, 2018, Paris, France) it was recalled that aspergillosis is in fact a public health issue, with three million affected people worldwide;

3. An increasing part of the antifungal resistance is not due to misuse of antifungals in medicine but more and more evidence shows that it is due to the heavy use of fungicides in environment. Most agricultural research is focussed on phytopathogenic fungi and not human pathogenic fungi with a ubiquitous presence in our environment. Therefore, the antifungal cross-resistance development needs a multidisciplinary approach to maintain a durable use of triazoles in both agriculture as well as medicine. This is however easier said than done with the economic interests at stake and the necessity of intensive food production in our society. Therefore, substantial funding for interdisciplinary research on antifungal resistance is essential but unfortunately greatly under rewarded.

Triazoles molecules are used to treat human fungal diseases with available molecules that have activity against *Aspergillus* spp. such as itraconazole, voriconazole, posaconazole and isavuconazole. But triazoles are also used in animal health as well as fungicidal application in the environment for crop protection, with over 31 available molecules. In the past, it has been shown that using similar molecule structures for human health as well as animal health can select for multidrug cross-resistant bacteria. The use of large quantities of antibiotics in livestock for the purpose of growth enhancement is believed to contribute greatly to the emergence and spread of antibiotics resistant bacteria (Rohr et al. 2019). Similar to the massive use of antibiotics that has led to more and more frequent resistance in bacteria, the large use of fungicides has now led to antifungal resistance in fungi, for application of both crops and human health.

The time has therefore come to mobilize and better understand the sources of antifungal cross-resistance. At the European level, the European Centre for Disease Prevention and Control (ECDC) published a full report in 2013 that provides an overview of the threat situation (Kleinkauf et al. 2013). Researchers were encouraged to conduct studies by implementing large-scale surveillance of antifungal resistance to better identify populations and areas at risk and develop means of prevention. This need for monitoring has also been reported by international experts (International Society for Human & Animal Mycology, ISHAM, Resendiz-Sharpe et al. 2018) and by some agencies with, for example, a report on airborne antimicrobial resistance written by the UK Environment Agency and published in February 2020 (Jones 2020).

The emerging resistance problem in all its complexity, as well as an inventory of the phenomenon and the possible solutions are described in the following paragraphs.

3.2 Triazoles: Mode of Action and Mechanisms of Resistance

Azoles including imidazoles and triazoles are characterized by the presence of an azole nucleus, a pentavalent ring containing two (imidazoles) or three (triazoles) nitrogen atoms. In human and animal medicine historically, there are three

generations of azoles: First generation azoles are imidazoles: clotrimazole, enilconazole, parconazole and miconazole. The first three molecules are available in veterinary medicine, clotrimazole and miconazole in cosmetics. The second generation is ketoconazole, marketed for humans and animals. Finally, the triazoles with itraconazole, fluconazole, voriconazole, posaconazole and isavuconazole are the third generation. Fungicide products are however not only used in medicine. Triazoles are largely used in agriculture to protect flower, cereal and wine crops from phytopathogenic molds such as *Botrytis* and *Fusarium* but are also employed in the processing of vegetable crops and to preserve a variety of materials, such as wood (Verweij et al. 2009; Gisi 2014; Berger et al. 2017).

Triazoles affect an important step of the ergosterol biosynthetic pathway, a predominant sterol and essential membrane component. They bind at 14- α -lanosterol demethylase encoded by the *cyp51A* gene and stop the conversion of lanosterol to ergosterol, resulting in the accumulation of 14- α methyl sterols in the fungal cell. Thus, triazoles disrupt cell transport and fungal membrane structure (Verweij et al. 2009; Berger et al. 2017). Resistance to triazoles can be intrinsic, as is the case with some cryptic species of *A. fumigatus* belonging to the *Fumigati* section or *A. fumigatus* complex (Lamoth 2016). Intrinsic resistance is demonstrated for example for *Aspergillus lentulus*, *Aspergillus thermomutatus*, *Aspergillus udagawae* and *Aspergillus oerlinghausensis*. However, there is also acquired resistance which is the result of adaptation and selection processes. In fact, as for most “icide” products synthesized to counter biological pathogens (bactericides, fungicides, parasiticides) resistance can be selected for over time.

The use of azoles in agriculture leads to resistance selection, in crop pathogens such as *Penicillium digitatum* destroying citrus or *Mycosphaerella graminicola* prevalent on wheat crops (Leroux and Walker 2011; de Ramón-Carbonell and Sánchez-Torres 2020), but also in non-target organisms such as *A. fumigatus* that co-exist in the same environments. This is probably even more favoured for azoles that are single site inhibitors, and above targeting pathogens that have very important spores production and short generation times. Acquired resistance is defined when a fungal strain belonging to a species naturally sensitive to the antifungal agent, is able to tolerate significantly higher concentrations of this antifungal molecule than those that inhibit the *in vitro* growth of the majority of so-called susceptible strains of the same species. From a microbiological point of view, the acquired resistance results in an increase in minimal inhibitory concentration. In the clinical situation, this resistance can lead to therapeutic failure during a treatment under standard conditions including dose, frequency and administration.

The first descriptions of rare cases of triazole resistance in *A. fumigatus* have been reported in the 90's, but since 2000 more and more publications became available on triazole resistance. Two searches for publications were done in the PubMed database (2020, April 7) to assess the number of peer-reviewed publications per year on the following queries: (1) “*Aspergillus*” and “resistance”; (2) “*Aspergillus*” and “resistance” and “(tri)azole”. For the first search, 3171 publications were found

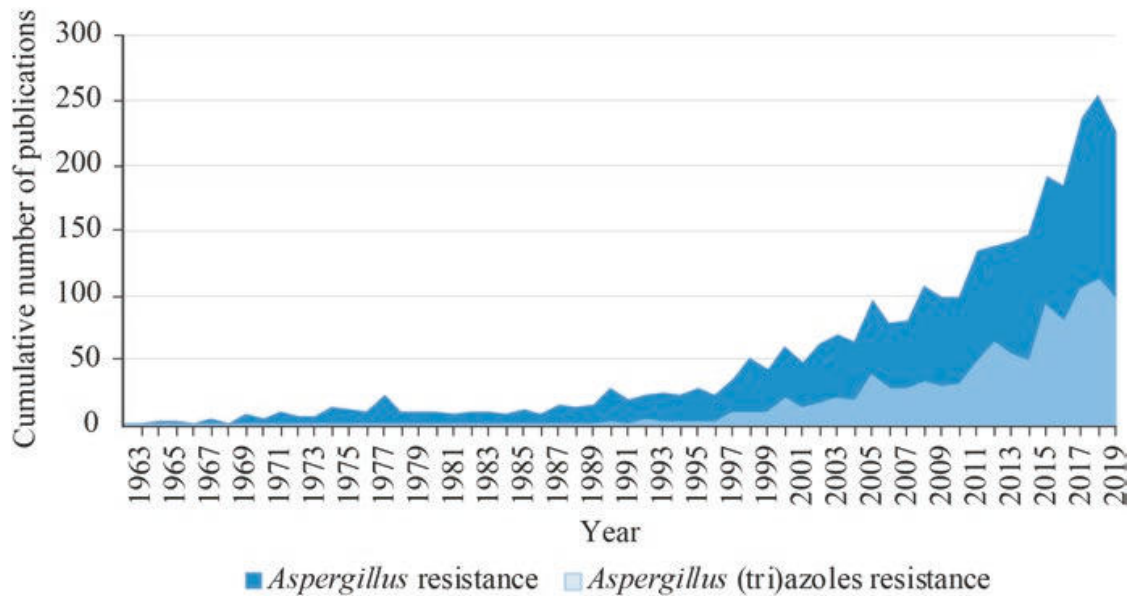


Fig. 3.2 Increasing number of publications on *Aspergillus* resistance and *Aspergillus* (tri)azole resistance

and 1084 for the second search. Results showing the increasing number of publications are presented in Fig. 3.2.

Mechanisms of triazole-resistance can be divided in two groups: *cyp51A* and non-*cyp51A*-mediated. In *A. fumigatus*, resistance mechanisms linked to mutations of the *cyp51A* gene (single nucleotide polymorphisms) and its promoter (tandem repeats) are the most commonly described. More than thirty single nucleotide polymorphisms have been identified in the *A. fumigatus cyp51A* gene, but whether they all have a significant impact on resistance has yet to be determined. In fact, single nucleotide polymorphisms could be classified in three categories (Fig. 3.3):

1. Those whose associated resistance could be explained by the analysis of the gene structure (modification of the active site for example) and by replacement of a wild type allele for the mutant allele and thereby conferring azole resistance;
2. Mutations that are associated with resistant phenotype but the mechanisms of which have not yet been studied;
3. Mutations that have only a small impact in phenotype (small increase in minimal inhibitory concentration) or that have been found in both susceptible and resistant strains and can therefore not be considered to be associated with azole resistance.

There are also mutations that do not confer resistance which are not presented in Fig. 3.3.

The most common mutated loci, reported to be mutation hotspots in triazole-resistant phenotypes are G54, M220 and G448. They are frequently found in patients with chronic aspergillosis who have been treated for long periods with triazole anti-fungals (Wiederhold and Patterson 2015; Meis et al. 2016; Garcia-Rubio et al. 2017) but also in the environment for G54 (Sharma et al. 2015; Tangwattanaachuleeporn et al. 2017) and M220 (Bader et al. 2015). These *cyp51A* gene single nucleotide

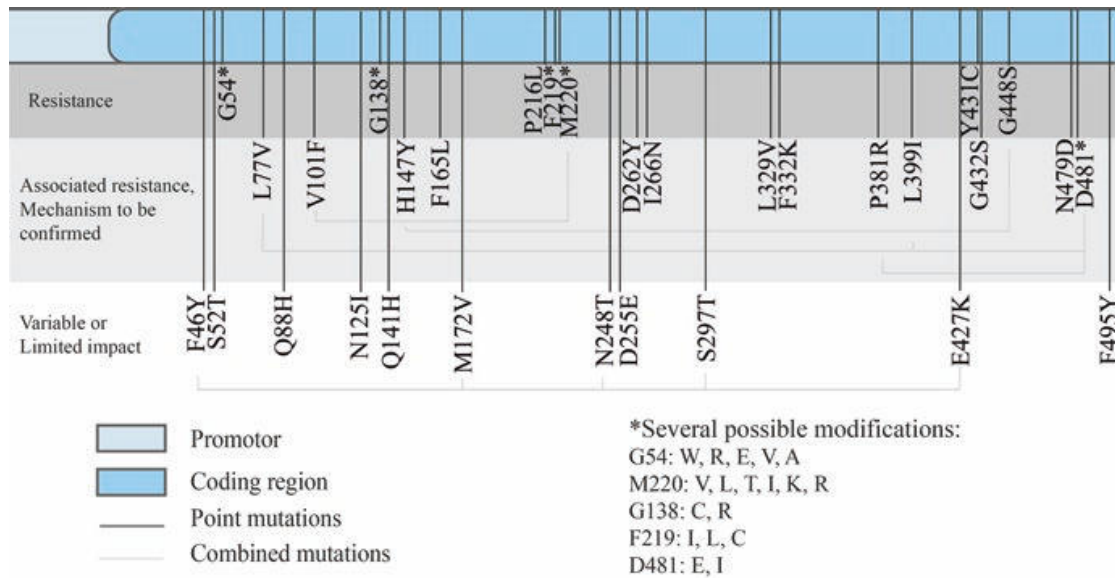


Fig. 3.3 Major single nucleotide polymorphisms found in *cyp51A* gene (position of mutations are represented by vertical lines in the coding region of *cyp51A* gene; the first letter is the amino acid on the wild gene, the number the position on the gene and the second letter the amino acid resulting from the mutated codon; the asterisk indicates that more than one amino acid modification is possible)

polymorphisms affect the protein's affinity for the azoles by modifying the binding site of azoles and restricting their entry. Table 3.1 summarizes the first descriptions of isolates with single nucleotide polymorphisms conferring azole resistance, by country and mutation.

Tandem repeats coupled or not with single nucleotide polymorphism(s), cause overexpression of the *cyp51A* gene and confer multi-triazole-resistant phenotypes. So far, six tandem repeat mutations have been described: TR₃₄, TR₄₆ (with the commonly reported mutations TR₃₄/L98H and TR₄₆/Y121F/T289A), TR₄₆³, TR₄₆⁴ (Zhang et al. 2017), TR₅₃ (Hodiamont et al. 2009; van der Linden et al. 2013; Alvarez-Moreno et al. 2017) and TR₁₂₀ (Hare et al. 2019). Table 3.2 summarizes the first descriptions of multi-triazoles-resistant isolates with mutations coupling both single nucleotide polymorphism(s) and tandem repeat, by country and mutation.

Other *cyp51A*-independent resistant mechanisms have been described and are also thought to be responsible for therapeutic failure (Wiederhold and Patterson 2015; Garcia-Rubio et al. 2017). *A. fumigatus* genome encodes for two paralogous genes of the P450 cytochrome, *cyp51A* and *cyp51B* (Mellado et al. 2001). Even if azole resistance in this fungus seems more depending on *cyp51A* mutations, *cyp51B* has already been described as resistance mediator in other species. The direct role of *cyp51B* ortholog on *A. fumigatus* azole resistance is not yet totally understood. No mutation on this gene has been described, but a significant overexpression of it has been reported in some resistant strains without *cyp51A* mutation. In this case, *cyp51B* seems to act as a replacement of *cyp51A* functions to ensure the membrane integrity (Kano et al. 2015).

Efflux pumps are a big mediator of resistance in many microbiological species. Efflux pumps are an energy-dependant resistance mechanism that consist in moving

Table 3.1 First descriptions of isolates with single nucleotide polymorphism(s) conferring resistance ordered by mutation and country (publications listed are those reporting the earliest isolate in time (year), not necessarily first published)

<i>cyp51A</i> mutations ^a	Continents	Country	Source of strain	Isolation year	References
G54 W/R/E/ V/A	Europe	UK	Clinical	1992	Howard et al. (2009)
		Spain	Clinical	2002	Diaz-Guerra et al. (2003)
		The Netherlands	Clinical	2007–2009	Van der Linden et al. (2011)
		Germany	Clinical	2011–2012	Bader et al. (2013)
			Environmental	2012	Bader et al. (2015)
		France	Clinical	2010	Burgel et al. (2012)
		Romania	Environmental	2013–2014	Sharma et al. (2015)
	Switzerland	Environmental	2015	Riat et al. (2018)	
	America	USA	Clinical	2003	Mann et al. (2003)
		Argentina	Clinical	2013	Leonardelli et al. (2017)
		Peru	Clinical	2016–2017	Bustamante et al. (2020)
	Asia	Japan	Clinical	1998–2001	Tashiro et al. (2012)
		China	Clinical	2002/2003	Chen et al. (2005)
		India	Clinical	2011–2014	Chowdhary et al. (2015)
			Environmental	2013–2014	Sharma et al. (2015)
	Thailand	Environmental	2014–2015	Tangwattanachuleeporn et al. (2017)	
	Oceania	Australia	Clinical	2011	Kidd et al. (2015)
Africa	Tanzania	Environmental	2013–2014	Sharma et al. (2015)	
M220 V/L/T/I/K	Europe	UK	Clinical	1992	Howard et al. (2009)
		France	Clinical	1995	Snelders et al. (2008)
		Sweden	Clinical	1998	
		The Netherlands	Clinical	2004	
		Spain	Clinical	2004	Mellado et al. (2004)
		Denmark	Clinical	2007	Mortensen et al. (2011)
		Germany	Clinical	2010	Fischer et al. (2014)
	Environmental		2012	Bader et al. (2015)	
	Asia	China	Clinical	2002	Chen et al. (2005)
	America	USA	Clinical	2001	Wiederhold et al. (2015)
Peru		Clinical	2016–2017	Bustamante et al. (2020)	
G448S	Europe	UK	Clinical	1992–2007	Howard et al. (2009)
		France	Clinical	2004	Bellete et al. (2010)
		Spain	Clinical	2011	Pelaez et al. (2012)
	Oceania	Australia	Clinical	2013	Kidd et al. (2015)
	Asia	Japan	Clinical	2013	Toyotome et al. (2016)
G138C	Europe	UK	Clinical	2004	Howard et al. (2006)
		The Netherlands	Clinical	1994–2008	Snelders et al. (2010)
	America	USA	Clinical	2012	Wiederhold et al. (2015)

(continued)

Table 3.1 (continued)

<i>cyp51A</i> mutations ^a	Continents	Country	Source of strain	Isolation year	References
F219 L/I/C	Europe	The Netherlands	Clinical	2008	Camps et al. (2012)
		Germany	Clinical	Unspecified	Bader et al. (2013)
	America	USA (Maryland)	Clinical	2014	Wiederhold et al. (2015)
P216L	Europe	The Netherlands	Clinical	2008	Camps et al. (2012)
		UK	Clinical	2005	Howard et al. (2009)
		France	Environmental	2016	Jeanvoine et al. (2017)
Y431C	Europe	UK	Clinical	2004	Albarrag et al. (2011)

^aFor mutations, the first letter is the amino acid on the wild gene, the number the position on the gene and the second letter the amino acid resulting from the mutated codon. When several letters are listed, more than one amino acid is possible

a toxic substance (here the antifungal treatment) out of the cell, preventing the anti-fungal from playing its role. In molds, two main types of efflux pumps are described: ATP binding cassette transporters and major facilitator superfamily transporters. ATP binding cassette transporters permit a unidirectional transport of molecules with hydrolyzing ATP as energy source whereas major facilitator superfamily transporters use proton exchange to translocate substrates. In *A. fumigatus*, 45 ATP binding cassette transporters and 275 major facilitator superfamily transporters are described and overexpression of genes (*AtrI*; *AtrF*; *Cdr1b*) encoding efflux pumps have already been observed as in environmental than in clinical strains (Hagiwara et al. 2016; Meneau et al. 2016; Berger et al. 2017). In other studies, the deletion of certain genes (*mdrA*, *Cdr1b*) has led to an increase triazole susceptibility (Meneau et al. 2016; Hagiwara et al. 2016).

HapE is a transcriptional factor, it's a part of a three subunits complex named CCAT binding complex. This complex is involved in the *cyp51A* gene expression as a repressor. A point mutation identified as P88L in *HapE* gene seems to affect the repressor effect of the complex. Once mutated, *HapE* induced a decreased affinity of the CCAT binding complex for its target, inducing a decreased of repressor effect and so an overexpression of *cyp51A* gene. This phenomenon is a theoretical implication in azole resistance since it has not been described in environmental or clinical strains but only in laboratory conditions (Camps et al. 2012; Gsaller et al. 2016).

3.3 Origin and Evolution of Environmental Azole Resistance

Since 1998, multi-triazole-resistance in *A. fumigatus* has rapidly spread in the Netherlands (Snelders et al. 2008) as well as globally (Table 3.2). Initially the rapidly spreading azole resistance phenotype was predominantly associated with two co-occurring mutations in the target gene of the azoles, *cyp51A*: (1) a tandem repeat

Table 3.2 First descriptions of isolates with tandem repeat (TR) and single nucleotide polymorphism(s) conferring multi-triazazole resistance ordered by mutation and country (publications listed are those reporting the earliest isolate in time (year), not necessarily first published)

<i>cyp5IA</i> mutations ^a	Continents	Country	Source of strain	Isolation year	References
TR₃₄/L98H also with S297T/F495I	Europe	The Netherlands	Clinical	1998	Snelders et al. (2008)
			Environmental	2002	Snelders et al. (2009)
		Italy	Clinical	1998	Lazzarini et al. (2016)
			Environmental	2011–2012	Prigitano et al. (2014)
		Turkey	Clinical	2000	Ozmerdiven et al. (2015)
		Norway	Clinical	2001	Snelders et al. (2008)
		Spain	Clinical	2003	Chowdhary et al. (2013)
		UK	Clinical	2007	Howard et al. (2010)
			Environmental	2009–2011	Bromley et al. (2014)
		Denmark	Clinical	2007	Howard et al. (2009)
			Environmental	2010	Mortensen et al. (2010)
		Portugal	Clinical	2010–2016	Pinto et al. (2018)
		France	Clinical	2010–2011	Burgel et al. (2012)
			Environmental	2012	Rocchi et al. (2014a)
		Germany	Clinical	2011	Fischer et al. (2014)
			Environmental	2012–2013	Bader et al. (2015)
		Belgium	Clinical	2011	Vermeulen et al. (2015)
Poland	Clinical	2011	Nawrot et al. (2018)		
Romania	Environmental	2013–2014	Sharma et al. (2015)		
Switzerland	Clinical	2015	Riat et al. (2018)		
		Environmental			

Asia	China	Clinical	2008	Lockhart et al. (2011)
		Environmental	2010–2015	Chen et al. (2016)
	India	Clinical	2008	Chowdhary et al. (2012a)
		Environmental	2012–2013	Chowdhary et al. (2014b)
	Iran	Clinical	2005	Seyedmousavi et al. (2013)
		Environmental	2013	Badali et al. (2013)
	Taiwan	Clinical	2011	Wu et al. (2015)
		Environmental	2014–2016	Wang et al. (2018)
	Kuwait	Environmental	Unspecified	Ahmad et al. (2014)
		Clinical	Unspecified	Ahmad et al. (2015)
	Thailand	Environmental	2014–2015	Tangwattanachuleeporn et al. (2017)
	Japan	Clinical	2016	Toyotome et al. (2017)
		Environmental	2016	Onishi et al. (2017)
America	Korea	Clinical	Unspecified	Lee et al. (2018)
	USA	Clinical	2008	Wiederhold et al. (2015)
		Environmental	2015	Hurst et al. (2017)
	Mexico	Clinical	2014–2017	Gonzalez-Lara et al. (2019)
	Colombia	Environmental	2015	Le Pape et al. (2016)
	Brazil	Clinical ^a	2015	Pontes et al. (2020)
	Peru	Clinical	2016–2017	Bustamante et al. (2020)
	Oceania	Clinical	2004	Kidd et al. (2015)
	New Zealand	Environmental	Unspecified	Korfanty et al. (2019)
	Tanzania	Environmental	2013–2014	Chowdhary et al. (2014b)
	Kenya	Environmental	Unspecified	Kemoi et al. (2018)

(continued)

Table 3.2 (continued)

<i>cyp51A</i> mutations ^a	Continents	Country	Source of strain	Isolation year	References
TR₄₆/Y121F/T289A	Europe	The Netherlands	Clinical	2009	van der Linden et al. (2013)
			Environmental	2013	
	Portugal	Portugal	Clinical	2010–2016	Pinto et al. (2018)
			Clinical	2011	Vermeulen et al. (2015)
	Germany	Germany	Clinical	2012	Fischer et al. (2014)
			Environmental	2013	Bader et al. (2015)
	Spain	Spain	Clinical	2014	Pelaez et al. (2015)
			Clinical	2014	Astvad et al. (2014)
	UK	UK	Clinical	2016	Moore et al. (2017)
			Environmental	2014–2016	Sewell et al. (2019a)
	France	France	Clinical	2014	Lavergne et al. (2015)
			Environmental	2014	Lavergne et al. (2017)
	Asia	India	Environmental	2012–2013	Chowdhary et al. (2013)
			Clinical	2013	Hagiwara et al. (2016)
	China	China	Clinical	2013	Chen et al. (2016)
			Environmental	2016	Ren et al. (2017)
Iran	Iran	Environmental	2017–2018	Ahangarkani et al. (2020)	
		Clinical	2008	Wiederhold et al. (2015)	
America	USA	Clinical	2009	Isla et al. (2018)	
		Environmental	2015	Le Pape et al. (2016)	
Africa	Tanzania	Environmental	2014	Chowdhary et al. (2014b)	
		Clinical	2008	Hodiamont et al. (2009)	
Europe	Colombia	Environmental	2015	Le Pape et al. (2016)	
		Clinical	2013	Hare et al. (2019)	
Europe	The Netherlands	Environmental	2012	Zhang et al. (2017)	
		Clinical	2012–2015		

^aThe index number corresponds to the number of base pairs (= motif) that are repeated in the promotor; the superscript number is the number of times the motif is repeated (nothing is indicated when it is only one more pattern repetition); the first letter is the amino acid on the wild gene, the number the position on the gene and the second letter the amino acid resulting from the mutated codon; when several letters are listed, more than one amino acid is possible

(TR) of 34 base pairs in the promoter region and (2) a leucine to histidine substitution in codon 98 (L98H) (TR₃₄/L98H). *A. fumigatus* harbouring these mutations significantly complicates patient management and recent studies show a 20% excess mortality in patients with azole-resistant invasive aspergillosis compared with azole-susceptible infection (van Paassen et al. 2016). Interestingly and importantly, in 2010 a new but similar mutation was described; TR₄₆/Y121F/T289A, that was recovered from patients and environmental samples like the TR₃₄/L98H (van der Linden et al. 2013) and has since then also been spreading on a global scale in both clinical as well as environmental *A. fumigatus* isolates.

Resistance of clinical *A. fumigatus* isolates has been reported at a low frequency in the past and is generally considered to be a rare phenomenon. Acquired resistance was only seen in *A. fumigatus* isolates cultured from rare cases of patients with aspergilloma during long-term treatment with azoles (Chen et al. 2005; Howard et al. 2006, 2009; Snelders et al. 2008). Evidence has however shown that the TR₃₄/L98H and TR₄₆/Y121F/T298A in *A. fumigatus* detected in humans are likely to be acquired from the environment rather than from selection within patients during azole therapy for four reasons:

1. Multi-azole-resistant isolates were found in azole-naïve patients with aspergillus disease (Verweij et al. 2007; Snelders et al. 2008) ruling out an azole resistance selection within the patient;
2. The tandem repeat azole resistance mechanism was previously described in azole-resistant phytopathogenic molds but not in clinical molds (Snelders et al. 2009);
3. Isolates with the specific TR₃₄ and TR₄₆ mutations are recovered from clinical samples as well as environmental samples (Snelders et al. 2009);
4. Medical-azole-resistant TR₃₄ and TR₄₆ isolates are cross-resistant to five agricultural azoles (Snelders et al. 2012). Environmental azoles are widely used as fungicides (including fruit preservation during storage, protecting wood, flower bulb dipping, and many more (Verweij 2017). However, they are not specifically directed against *A. fumigatus* but rather to protect crops from fungal pathogens such as *Botrytis* and *Fusarium*. Taken together, cross-resistance to medical azoles is likely to be an unintended side effect of the evolved resistance to environmental azole fungicides. Now, two possible routes for resistance development can be considered: through patient exposure; the “patient-route” and through exposure to azole compounds in the environment; the “environmental-route” (Fig. 3.4). On the left side in this figure, the patient-route of azole resistance development is shown. Patients at risk can be infected by an azole-susceptible isolate which only develops resistance after colonization and long-term azole treatment of the patient. On the right, the environmental-route of azole-resistance development is shown. *A. fumigatus* is airborne and present ubiquitously, however in the environment (Zhang et al. 2017) where *A. fumigatus* can grow and azole residues are present there can be selection and spread of resistant genotypes. Finally, patients can be infected by azole-resistant genotypes originating from the environment.

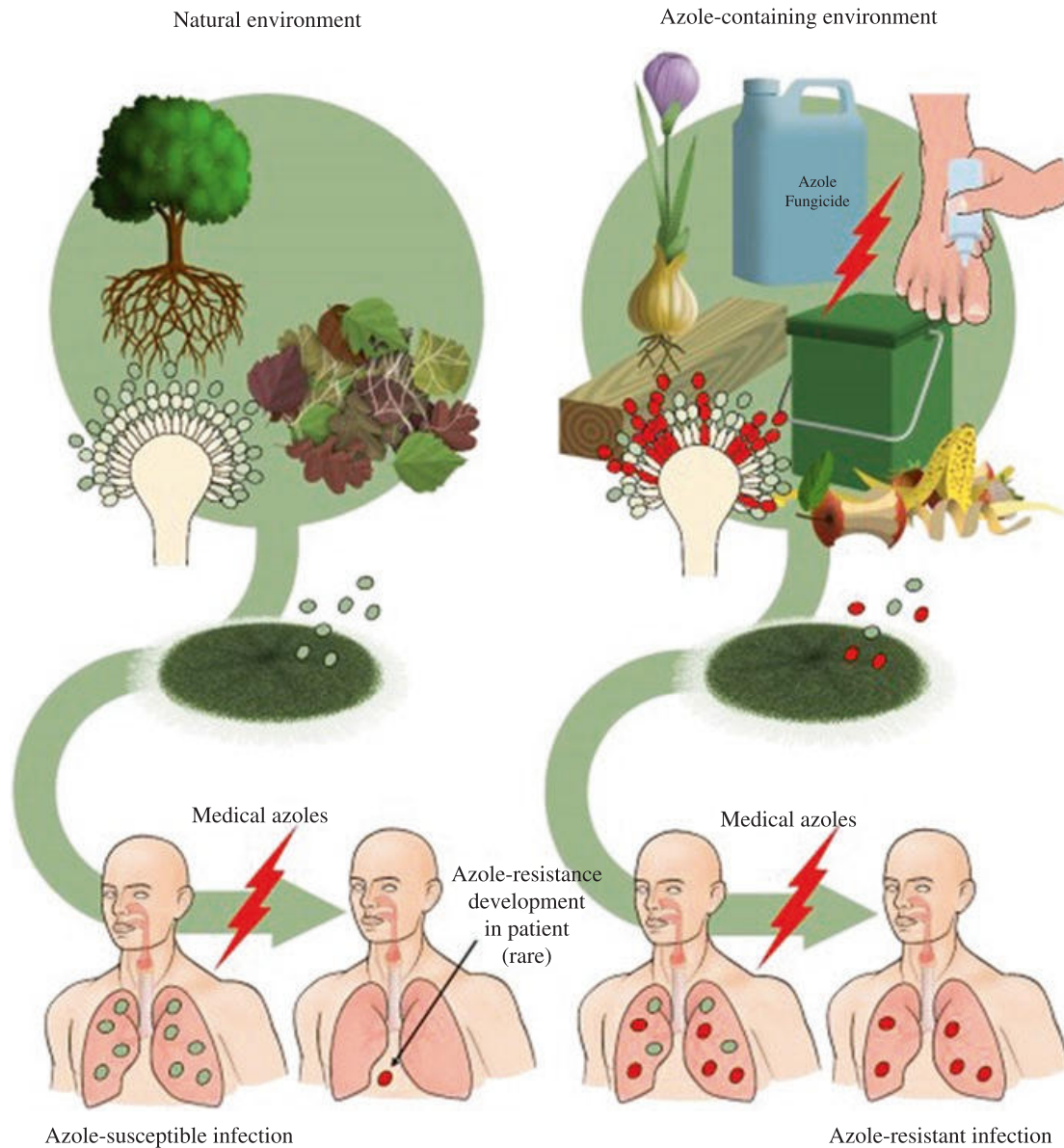


Fig. 3.4 Two routes of azole resistance development. (Source: Wageningen University & Research, The Netherlands)

The tandem repeat-genotypes of *A. fumigatus* have either spread or independently emerged on a global scale, however, newly emerged resistance mutations are generally thought to be associated with reduced growth rates in the absence of anti-fungals compared to wild type isolates. In order to persist in the absence of azoles and to not be outcompeted by sensitive isolates, resistant isolates need to mitigate this cost of resistance, and are thought to do so by acquiring second-site compensatory mutations. A fitness cost has however never been detected in clinical or environmental isolates. This can be explained because the compensation of fitness already took place or because there is no cost of fitness under standard laboratory conditions.

3.4 Global Spread of Azole Resistance

Today, the most affected country by tandem repeat azole resistance mechanism are the Netherlands, with a prevalence of more than 10% of resistance in clinical strains. Clinical and environmental cases of triazole resistance in *A. fumigatus* show inequalities in global distribution, with Europe at the centre of attention. High prevalence of triazole resistant *A. fumigatus* is observed in clinical practice and in the environment in the Netherlands but also in UK, Denmark, Germany and Eastern France. This prevalence varies between geographic regions and between hospitals, probably due to the differences in methods used to detect resistance and follow patients. However, there has recently been an emergence and extension of the phenomenon (also due to increased testing for antifungal resistance) to all countries in Europe, and to all continents, meaning that the phenomenon has reached worldwide prevalence.

A map showing the countries reporting resistance with TR₃₄ and TR₄₆ mutations, as it has been published before, did not seem informative to us since studies are not comparable in terms of sampling or techniques and often because the number of total strains isolated is not known. Thus, to generally illustrate global spread of tandem repeat azole resistance in *A. fumigatus*, we reported the number of papers published by year and by country in Fig. 3.5. We performed the following search on PubMed database: “TR34” or “TR46” and “*Aspergillus fumigatus*”. Results were exported in .xml format and data analysed with RStudio with “maps” and “XML”

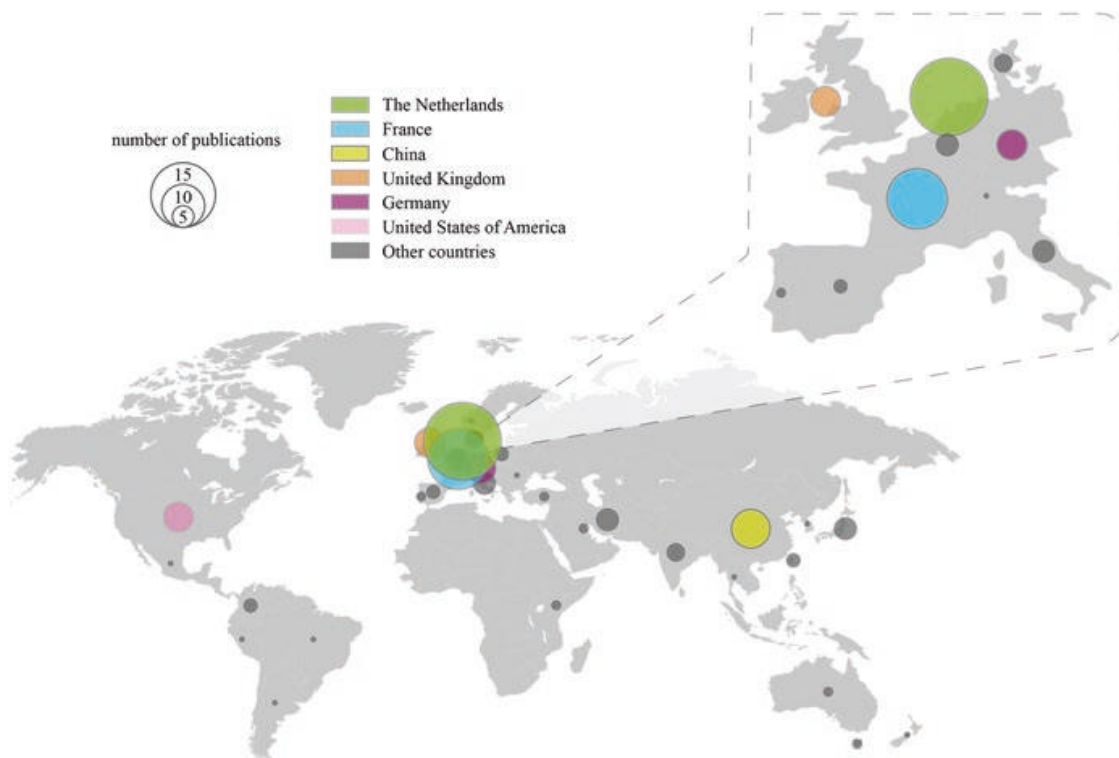


Fig. 3.5 Distribution by country of publications (source: PubMed database) reporting cases of resistant strains carrying tandem repeat mutations detected in clinical or environmental samples

packages. Reviews, original articles that develop diagnostic techniques on previously identified strains, as well as articles comparing several strains worldwide were excluded from the list. This resulting world map is a good illustration of disparities in resistance, but should be interpreted with caution, because papers does not reflect exact prevalence numbers and just because there's no publication does not mean the resistance is not there. One example is USA: in one of their publications on passive surveillance in 2015–2017, they reported 0.3% of resistant clinical isolates with TR₃₄ mutation (Berkow et al. 2018) and only one study with TR₃₄ mutation detected in the environment (Hurst et al. 2017). In the USA there are too few studies performed to have a representative picture of the situation in this vast country, especially since with the increasing use of azole fungicides, a potential increase in resistance can be expected to be observed similar to Europe (Lockhart et al. 2020).

In any case, tandem repeat azole resistance is increasingly present on all continents and it is expected to continue to spread. Previous studies in the Netherlands and UK have screened for environmental resistance of *A. fumigatus* isolates in plant waste material by using a single azole at a high concentration and thereby selectively enriching for highly resistant genotypes (Verweij 2017; Schoustra et al. 2019a). Rather easily, it is possible to detect environmental azole resistance, it is however unclear whether these tandem repeat type resistant isolates are genetically related or not. Are they arising frequently *de novo* in different environmental niches or have they only arisen a small number of times and subsequently spread to other geographic locations in a very efficient manner? It is also unclear, due to a lack of systematic surveillance programs, how many other tandem repeat resistance mechanisms potentially exist.

In parallel, with advances in modern medicine and cancer therapies, the number of people at risk is growing. In addition, the number of cases may affect a much broader category of patients than we think and not only immunocompromised patients. In 2018, the number of cases of invasive aspergillosis in patients with influenza has increased significantly. Triazole resistance is also an emerging concern for treating chronically colonized patients such as cystic fibrosis patients. Several European studies have been conducted and reported different prevalence of resistance depending on the country and the region, from 0 to 16% (Amorim et al. 2010; Mortensen et al. 2011; Burgel et al. 2012; Morio et al. 2012; Fischer et al. 2014; Prigitano et al. 2017; Guegan et al. 2018; Seufert et al. 2018; Godeau et al. 2019; Lavergne et al. 2019) (Fig. 3.6).

Initially, there seemed to be a clear separation between mutations called “clinically” selected and “environmentally” selected. The resistance mechanisms found in isolates that are thought to have become resistant through medical azole treatment consist of point mutations only, whereas the presence of a tandem repeat is an important mechanism found in plant pathogenic molds that developed resistance through exposure to azole fungicides. Therefore, the resistance mechanism seems to be indicative of the route of resistance development and therefore it implies that the TR₃₄/L98H and TR₄₆/Y121F/T298A have been selected in the environment rather than within patients. The tandem repeat resistant *A. fumigatus* isolates are also

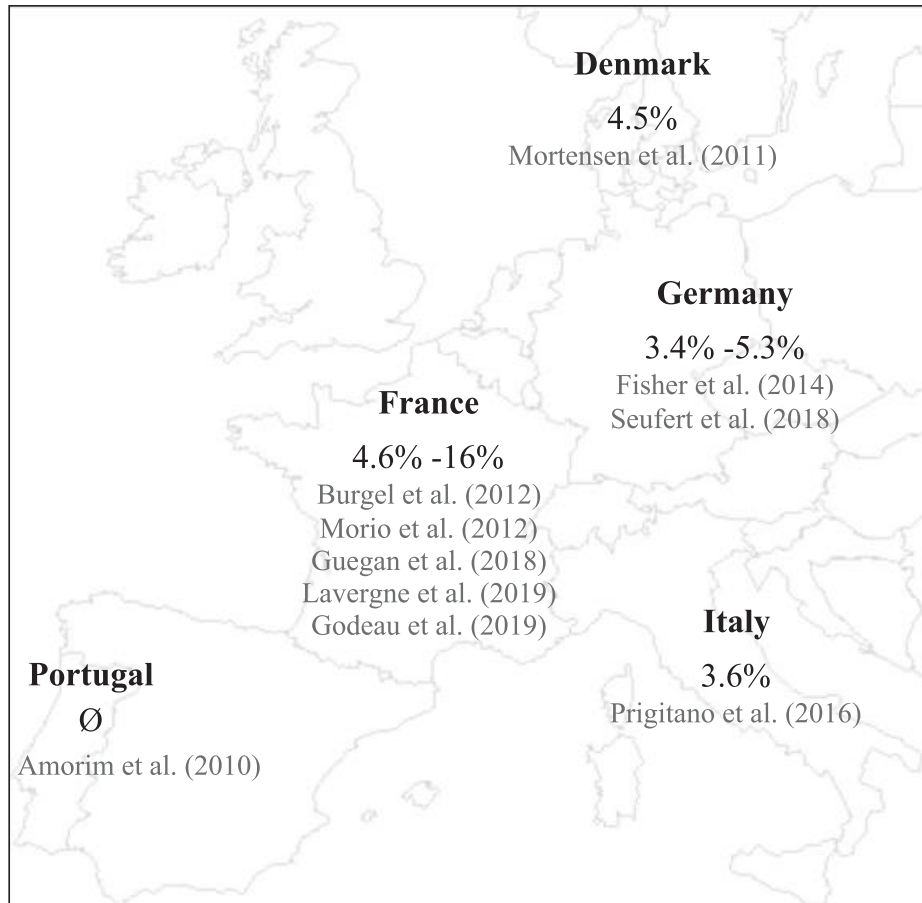


Fig. 3.6 European studies and prevalence of azole resistant *A. fumigatus* burden in cystic fibrosis patients (prevalence is given with the number of *A. fumigatus* positive culture)

widely distributed in the environment supporting this environmental-route of resistance development.

But, we soon also realized that the compartments may not be so distinct. For example, the G54 mutation initially described as “clinical” was also found in the environment in India, Tanzania, Romania, Germany (Bader et al. 2015; Sharma et al. 2015) (Table 3.1). A recent paper dealing with the TR₁₂₀ mutation in a fatal case of aspergillosis is also interesting: A wild type and a TR₁₂₀ isolate with the same genome (whole genome sequencing analysis) were detected in the same patient with azole long-term therapy (Hare et al. 2019). Thus, it questions the fact that only environmental exposure to fungicides can induce adaptations with tandem repeat mutations. The limits of the resistance acquisition pathways (patients vs environment) are not always distinct (Buil et al. 2019). It is therefore perhaps no longer necessary to speak of a compartment of emerging resistance but focus on environmental “conditions”, whether it be the pulmonary environment or the agricultural outdoor environment and the capacity of the fungus to thrive and adapt.

Clearly, multi-azole-resistant *A. fumigatus* isolates are spreading rapidly across the globe, but there is an alarming paucity of fundamental research into this

emerging problem. Therefore, a fundamental understanding of the causal factors of azole resistance selection is needed.

3.5 Contributing Factors in the Emergence of Azole Resistance

3.5.1 Cross-Disciplinary Use of Azoles

Impressive efforts have generally greatly increased living standards and conditions, which crucially includes intensified agriculture. However, with the intensification of agriculture, changes in land use that affects the ecological environment and the increase in human population density and concentration in urban areas, disease risk and epidemiology for plants, animals, and humans has similarly changed significantly. Fungal pathogens are a growing threat to human and animal health, ecosystems, food safety and the global economy, hence the importance of effectively controlling them. Triazole are used on a global scale for agriculture and horticulture, accounting for a quarter of all fungicides (Hollomon 2017), with 31 available molecules (Fisher et al. 2018). They are particularly used in Europe and Asia, where they represent one of the most commonly used pesticide classes. Forty percent of pesticides used in Europe are fungicides and more than a third of them are azole fungicides. This percentage could reach up to 23% in India and China (Chowdhary et al. 2013, 2015; Chen et al. 2016). Treatment with azoles fungicides would seem to be less important on the American continent where less than in 5% of the total cultivated crops azole fungicides are used (Verweij et al. 2009). However, azole fungicides are among the most predominant pesticides used in Colombia (Alvarez-Moreno et al. 2017) concluding that geographical differences of azole use are large.

A. fumigatus is a common saprobic fungus whose main habitat is decaying plant material. It also is the most prevalent airborne fungal pathogen causing fatal invasive infections in immunocompromised patients (Latgé 1999). Although *A. fumigatus* is not a phytopathogen, many environmental azoles were found to exhibit activity against *A. fumigatus* (Snelders et al. 2012). The fungicides are applied repeatedly over a long period of time and could thereby create a persistent pressure of azole compounds on saprophytic fungi. Several studies reported the presence of resistant strains (TR₃₄/L98H and TR₄₆/Y121F/T289A mutations) in industry, professional fields or other activities in link with triazole molecules. Due to the persistence of these molecules in the environment, resistant strains are not necessarily found in recently treated substrates. It takes at least 2 years after use for triazoles to be no longer detected in soils (Hollomon 2017). For example, in a study conducted in market gardens, a link has been identified between the presence azoles resistant *A. fumigatus* and the presence of difenoconazole in the soils of different farms, even 2 years after treatment of azoles (Rocchi et al. 2018). In a study in French

sawmills (Jeanvoine et al. 2017), azole-resistant strains were isolated in sawmills in contact with azole fungicide either by dipping in a tank, by occasional fungicide spraying, by importing foreign wood treated with fungicides or by storing treated wood coming from a sawmill using azoles. Thus, even if there is no fungicide treatment on site, the storage of treated wood (and probably its leaching by rain) could be sufficient to provide an environmental azole pressure and find resistant strains in soils.

Some of the studies depicted in Table 3.3 combine the research of resistant strains with a dosage and application of triazole molecules. In each of the professional sites surveyed, resistant strains were found in soils with low to moderate triazole concentrations (on the scale of azole concentrations that can be found in agricultural fields). The highest concentrations probably result in complete elimination of all viable fungal forms while the lowest concentrations exert a selection pressure high enough for fungi to grow and adapt; to select for antifungal resistance. In sawmills study, propiconazole concentration in substrates were up to 50.000 $\mu\text{g/g}$ of substrate and resistance was found for samples with 0.3–60 μg of propiconazole/g of substrate (Jeanvoine et al. 2017) but also less often in samples with greater quantity (one strain in soil with propiconazole concentration of 3.000 $\mu\text{g/g}$). In market gardens study (Rocchi et al. 2018), concentrations ranged from 4.6 to 414.5 ng of difenoconazole/g of soil and resistance were found until 90 ng of difenoconazole /g of soil. Studies show that fungicide concentrations can vary greatly at one site and application of fungicides cannot always be done in a homogenous manner; therefore, some pockets of very high concentrations will exist similarly for pockets with intermediate, low, or no fungicidal pressure all at one site.

3.5.2 Hotspots of Azole Resistance

Triazole fungicides have now been largely blamed for the emergence of resistance, but in some cases, even if triazoles are used for crop protection, only susceptible strains are isolated (Rocchi et al. 2018). This indicates that not all (agricultural) environments have a high pressure of azole resistance and constitute as a hotspot.

Research has shown that azole-resistant *A. fumigatus* can accumulate and thrive in plant-waste containing environmental azoles (Zhang et al. 2017; Schoustra et al. 2019a). These locations can be defined as a hotspot that is characterized by an environment that supports the growth and reproduction of *A. fumigatus* and where azole fungicides with anti-*Aspergillus* activity are present. During adaptive evolution in such a hotspot, mutants arise spontaneously, and through a process of natural selection, the adaptive mutant expands in the population until it either gets fixed or replaced by an even fitter variant that arises. Natural populations of *A. fumigatus* are likely to have a complex environmental structure containing multiple resistant genotypes. The exact genetic composition will depend on the selection pressures, the frequency of mutations, and the population size in the hotspot.

Table 3.3 Examples of studies with detection of tandem repeat mutations in environmental samples and if described, the link with fungicides use

Country	Mutations	Types of samples with resistant isolates (other samples without resistance are not mentioned)						References
		Bulb, seeds, flower beds/ fields	Compost	Culture fields	Green and wood chippings	Other	Details of samples	
The Netherlands	TR ₃₄	×	×				Fungicides used or residuals quantification	Snelders et al. (2009)
	TR ₃₄ TR ₄₆					×	Fungicides used for plant production. Possible residues present in organic matter compost	
	TR ₃₄ TR ₄₆		×				Air in hospital and dwellings	
UK	TR ₃₄ TR ₄₆	×			×		Quantifications: Prothioconazole-desthio, tebuconazole and thiabendazole. Concentrations ranging from 0.01 to 3.7 mg/kg	van der Linden et al. (2013) Personal communication
	TR ₃₄ TR ₄₆						Quantifications: Prothioconazole, prothioconazole-desthio, prochloraz, prochloraz-desimidazole-amino, difenoconazole, epoxiconazole, penconazole, propiconazole, azaconazole, tebuconazole. Concentrations ranged from 0.001 mg/kg to 6.4 mg/kg	
	TR ₃₄					×	Soil and air surrounding hospital	
	TR ₃₄ TR ₄₆	×				×	Flower beds outside hospitals, urban parks and remote forest	Dunne et al. (2017) Sewell et al. (2019a)

France	TR ₃₄				×				Cereals crops. Use of prothioconazole and epoxiconazole, amounts non available	Rocchi et al. (2014a)
	TR ₃₄					×			Sawmills. Quantifications: Propiconazole, tebuconazole, difenoconazole, epoxiconazole, bromuconazole. Resistance until 3000µg/g of propiconazole	Jeanvoine et al. (2017)
	TR ₃₄				×			×	Garden, indoor air from bedroom and bathroom	Lavergne et al. (2017)
	TR ₃₄ ^a TR ₄₆				×				Carrots, celery and cabbage crops. Quantifications: Propiconazole, tebuconazole, difenoconazole, epoxiconazole, bromuconazole. Resistance until 90 ng/g of difenoconazole	Rocchi et al. (2018)
	TR ₃₄							×	Electrostatic dust collector in dwellings	Dauchy et al. (2018)
	TR ₃₄							×	Tulip bulbs coming from the Netherlands, air and dust in hospital	Godeau et al. (2019)
Norway	TR ₃₄							×	Water	Snelders et al. (2008)
Denmark	TR ₃₄								Surroundings hospital, flower beds in park	Mortensen et al. (2010)
Italy	TR ₃₄							×	Cucurbit fields, apple orchards, rose pot composts. Use of azole fungicides in culture.	Prigitano et al. (2014)
	TR ₃₄							×	Orchards, market gardens, vineyards	Prigitano et al. (2019)
Romania	TR ₃₄							×	Not specified	Sharma et al. (2015)

(continued)

Table 3.3 (continued)

Country	Mutations	Types of samples with resistant isolates (other samples without resistance are not mentioned)						References
		Bulb, seeds, flower beds/ fields	Compost	Culture fields	Green and wood chippings	Other	Details of samples	
Colombia	TR ₃₄ TR ₄₆ TR ₅₃	×					Fungicides used or residuals quantification	Le Pape et al. (2016) Alvarez-Moreno et al. (2017) Alvarez-Moreno et al. (2019)
	TR ₃₄ TR ₄₆ TR ₅₃	×					Concentrations of penconazole (0.15 mg/kg), difenoconazole (0.41 mg/kg), tetraconazole (0.49 mg/kg) and tebuconazole (0.69 mg/kg)	
	TR ₃₄ TR ₄₆ TR ₅₃		×				Maize, potatoes, carrots, peas fields. Quantifications: Azaconazole, cyproconazole, difenoconazole, epoxiconazole, fluquinconazole, flusilazole, flutriafol, hexaconazole, penconazole, propiconazole, tebuconazole, tetraconazole, and triadimenol. No residuals.	
India	TR ₃₄	×				×	Air from hospital wards, flower beds of hospital garden, soils with bird droppings	Chowdhary et al. (2012b) Chowdhary et al. (2014a)
	TR ₃₄ TR ₄₆	×		×			Paddy/rice/red chilly fields, tea garden, beneath cotton trees Potato, fenugreek, red chili, aubergine, mustard crops and rose bed soils	

China	TR ₃₄ ^a TR ₄₆			x				Soils from market gardens, in greenhouses with <i>Luffia</i> and strawberry plants	Ren et al. (2017)
	TR ₃₄ ^a TR ₄₆			x				Strawberry, vegetables and rice plants. Quantifications: Difenoconazole, tebuconazole, epoxiconazole, prochloraz, imazalil, tricyclazole. Soils with resistance: Prochloraz until 0.05 mg/kg, difenoconazole until 0.0385 mg/kg, tebuconazole until 0.0805 mg/kg.	Chen et al. (2020)
Kuwait	TR ₃₄	x		x			x	Outdoor air and soil, seedlings or vegetables/ ornamental flowers	Ahmad et al. (2014)
	TR ₃₄						x	Outdoor air	Ahmad et al. (2015)
Iran	TR ₃₄	x						Surrounding the hospitals	Badali et al. (2013)
	TR ₄₆				x			Not specified	Ahangarkani et al. (2020)
Tanzania	TR ₃₄ TR ₄₆						x	Woody debris of tree trunk hollows, soil from hospital parking areas	Chowdhary et al. (2014b)
USA	TR ₃₄				x			Compost pile containing peanut plant harvest debris from fields treated with azoles	Hurst et al. (2017)
Kenya	TR ₃₄	x						Flower farming with extensive use of fungicides	Kemoi et al. (2018)

^aTR34/L98H/S297T/F495I

When taking in mind the frequency of mutation, it is necessary to take into account the lifecycle of *A. fumigatus*. The exact mechanisms and circumstances by which tandem repeat genotypes have evolved in *A. fumigatus* are unknown. Whole-genome sequencing of *A. fumigatus* isolates sampled worldwide has so far not been able to determine whether the most common resistance genotype emerged once with subsequent global spread or emerged multiple times (Abdolrasouli et al. 2015). This is largely due to a lack of understanding of the population structure in *A. fumigatus*, the extent and the nature of standing genetic variation, and its origin by mutation and recombination. Importantly, to date, there have been no systematic studies to determine mutation rates. Similarly, recombination in *A. fumigatus* is not well understood and there is no recombination map available.

Selection and recombination of mutations can take place during asexual, sexual and/or parasexual reproduction, both of which have been documented for *A. fumigatus*. The sexual cycle has been demonstrated in a laboratory set-up in 2009 but its natural niche has not been described yet, therefore it is unknown to what extent the sexual cycle, and accompanying recombination, contributes to the population structure (O’Gorman et al. 2009). Sexual spores or ascospores are able to survive a 70 °C heat shock of 1 h in contrast to asexual spores that do not survive. A recent report by Schoustra et al. (2019b) showed that plant waste material *A. fumigatus* growth after heat shock indicating the presence of sexual spores. It has also been shown that completion of the sexual part of the life-cycle of *A. fumigatus* could facilitate the emergence of the tandem repeat in the promotor region (Zhang et al. 2017). Given this recent evidence, it seems important that *A. fumigatus* can complete its full life cycle in the selection of the tandem repeat genotypes.

Finally looking at the population size, it has become clear that certain environments contain a much higher load of *A. fumigatus* spores than others. A recent study showed that in particular flower bulb waste, green waste material, and wood chip-pings had high concentrations of *A. fumigatus* in contrast to animal manure, grain, maize silage, fruit storage or exotic fruit waste (Verweij 2017; Schoustra et al. 2019a). Therefore, sites that facilitate abundant and prolonged growth can be considered as a hotspot for azole resistance selection.

Taking in mind the selection pressure by azoles, the frequency of mutations, and the population size of *A. fumigatus* certain sites where conditions are favourable can be considered as hotspots for azole resistance selection (Verweij 2017). These hotspots need to facilitate abundant and prolonged growth of *A. fumigatus*, so that the fungus can fulfil its full life cycle to exploit its full capacity for genetic variation. Conditions that support the growth of *A. fumigatus* are plant biomass temperatures between 12 °C and 65 °C, high humidity (85%–100%) and an acidity between pH 3.7 and 7.6. For selection of azole resistant genotypes, azoles need to be present that are active against *A. fumigatus* and present at sufficient levels of concentration (Verweij 2017). Three sites were selected as hotspots by a study from Verweij et al. (2017): compost from flower bulb waste, green compost and wood chippings but more sites can be considered under the above-mentioned conditions of a hotspot. Summarizing a hotspot for azole resistance selection can be defined as a site where *A. fumigatus* can grow, complete its lifecycle and where azoles are present.

3.5.3 *Abiotic and Biotic Factors Contributing to Azole Concentrations and Fungal Population*

The advice given to reduce resistance in plant pathogenic molds is to rotate treatments and apply a high-dose treatment rather than several low-dose treatments (Azevedo et al. 2015). Nevertheless, for the problem with *A. fumigatus*, the amounts in soils or others hotspots such as composts will eventually be in the right concentration range to select for resistance. In fact, fungicides exert selection pressure at concentrations lower than those needed to kill fungi. Thus, fungicide residues in soils, wastewater, slurry or tulip bulbs could be the drivers of antifungal resistance, regardless of the quantity sprayed at the start. The pathways in which these more or less persistent molecules are degraded or eliminated are therefore very important in this matter. The most important thing is probably the persistence of azole molecules in soils, with half-life estimated between 20 and 200 days (Hollomon 2017). The physicochemical and biological properties of the soil are known to regulate the processes involved in pesticide dissipation in general (adsorption, transfer and biodegradation), thus conditioning the exposure of living organisms in the soil and the ecotoxicological consequences resulting from this exposure. In soils, the selection of resistance probably results from interactions between growing practices (crop rotations, ploughing, use of fertilizers, types and quantities of fungicides) and soil characteristics.

Azole fungicides (hydrophobic pesticides) are mainly accumulated in the top soil layer (0–20 cm depending on the molecule) but it has already been reported that their behaviour depends on the quantity and quality of organic matter. For example, tebuconazole is considered slightly mobile and stay in first 10 cm of soil, with higher retention recorded in soils amended with organic materials (Čadková et al. 2013). The addition of organic matter or surfactants to soils could also reduce the migration of penconazole (Komarek et al. 2010). Percolations experiments under controlled conditions have shown that difenoconazole adsorption also depended on the organic content of the soil, with a retention percentage that increases with the organic matter content (Léchenault-Bergerot et al. 2019). At soil characteristics levels, mineral elements are also important factors. For example, sandy soil (or soil with low organic matter content) retains less water and therefore less diluted triazoles due to their hydrophobicity (Komarek et al. 2010). Thus, triazoles may not be retained in this type of soil long enough to promote the selection of resistant strains (less contact time). This assumption of interaction was raised during a study of market gardeners where even if difenoconazole was used on crops, no fungicide was detected in the soil and only susceptible strains are isolated (Rocchi et al. 2018): The soil was so sandy that everything was probably drained very quickly.

It is also assumed that a transformation of pesticides in soils can occur without knowledge of the resulting compounds effects. Thus, “aging effect” can be another area of investigation. It is defined as the redistribution of an element into less available chemical fractions, which reduces its mobility and bioavailability. Aging of

fungicides in soils can create stronger bonds between soil particles and fungicides with increased contact time that reduces the availability of pesticides for degradation and increases their half-life. The half-life of tetraconazole depends for example on the concentration at the time of treatment and soil texture ranging from 67 to 69 days to more than 1688 days (Sułowicz et al. 2016). But, global environmental factors such as climate would also be involved. Tetraconazole for example would tend to accumulate with successive annual precipitations (Sułowicz et al. 2016). Other fungicides like tebuconazole have half time between 40 and 170 days that would depend on the dose and average daily temperature (El Azhari et al. 2018). It is therefore likely that the nature of the soil plays a role in the dissipation of triazole residues and therefore indirectly in the development of resistance.

Agricultural work can also modify the soil structure (ploughing) or composition. For example, intensive agriculture can decrease the percentage of organic matter (Tilman et al. 2002) and the long-term use of fertilizers can also decrease pH (Bradley et al. 2006) with an impact of physicochemical and biological functioning of soils. It has been shown that vegetation cover (associated with high organic matter content) can reduce tebuconazole mobility (Dousset et al. 2010). The roots of plants will also modify the structural properties of the soil (compaction), draw water or secrete minerals, thus modifying very locally the bio-physical and chemical properties of the soil. There is a whole biological component (plant and microbial communities) to be considered.

The microbiological communities present will also have an impact on the soil, with perhaps a greater physical impact of fungal communities (filament networks) than bacteria. At the same time, the application of fungicides can affect the biomass of non-target organisms (others than *A. fumigatus*), change their biochemical, structural, functional activity, and genetic diversity. Depending on the treatment practices and molecules used, fungicides must have more or less marked toxicological effects, which will modify the biological communities present. Various controlled studies have shown, for example, that the impact of tebuconazole is even more negative when the soil has never been treated. For bacterial communities, a higher sensitivity of communities is found in soils with a treatment history, which can be explained by the lower diversity of communities (Sułowicz et al. 2016). It has also been shown that there is no effect of tebuconazole on the total abundance of bacterial communities, but rather on some taxa more than others. For example, beta and gamma proteobacteria increase in the most exposed soils. This can be explained by the fact that beta proteobacteria are capable of degrading organic compounds or that fungi exposed to fungicides reduce their ecological pressure on prokaryotic communities that may develop (El Azhari et al. 2018).

The fraction of bioavailable pesticides can also be visibly transformed by soil microorganisms. Environmental (bio)availability is therefore not homogeneous. Phenomena of solubility and mobility (availability) as well as absorption, metabolism, and bioaccumulation (bioavailability), related to the physico-chemical and biological structures of soils occur. Tebuconazole can be transformed by abiotic and biotic processes and give transformation products that can be recalcitrant and that are also involved in the transformation of lanosterol into ergosterol. Thirty-four

transformation products for tebuconazole have been identified and little is known about their ecotoxicity on soil microbial communities (El Azhari et al. 2018).

In addition, there are phenomena of competition and/or interactions between microorganisms, which are currently only minimally studied in *A. fumigatus*. In soils, it has been estimated that *Aspergillus* compete with 4000 eukaryotic and prokaryotic species per gram of soil (Montesinos 2003). *A. fumigatus* is able to develop strategies to avoid predation by amoebae: intracellular germination in the predator, synthesis of inhibition factors that lead to host cell lysis (Hillmann et al. 2015). These strategies are the same as those used to counter the immune system during lung infections (van de Veerdonk et al. 2017). Cooperation phenomena have also been shown, where *A. fumigatus* spores can be transported by *Paenibacillus* vortex bacteria (also present in the Rhizosphere) to avoid areas that would be contaminated with triazoles (Ingham et al. 2011).

Fungicide levels that allow resistance selection in fungal populations are therefore not related to fungicide use alone. And to think that giving general recommendations for agricultural soil treatments would be sufficient is certainly utopian. It is the properties (physico-chemical and biological) of environments in modulating fungicide availability, which control the fungicide selection pressure on fungal populations, if they are able to prosper with these conditions.

3.6 Solutions to Limit the Risk of Resistance

The complexity of durable azole use lies in the fact that it is difficult to recommend that the use of all triazoles has to be discontinued in the environment and reserved for human therapeutics. The main challenge of agriculture is to feed the entire population, which is threatened by, among other things, crop infections, largely dominated by fungi. Therefore, often, the only solution is preventive action yet considering that organic farming is not sufficiently productive (−30%). Concerning treatment options, triazoles are preferred in agriculture because they specifically target fungi. An immediate ban on triazoles in the environment would lead to excessive economic losses.

Due to their use, which currently seems essential for the protection of agricultural or wood products, azole fungicides can be found in the various environmental compartments (water, soil, sediment, plants, air) and accumulate in organisms. The proven toxicity of pesticides in general (fungicides, insecticides, weed killers) is currently a growing public concern and a source of debate in the scientific community. It is necessary to limit their spread in ecosystems. The European Union has the strictest pesticide authorization and control system in the world with legislation and measures in place to ensure a reduced, more accurate and safe use of pesticides in agriculture. With regard to triazole resistance, it is essential to better characterize exactly which fungicides lead to emerging resistance and in what conditions. In any cases, industrial and agricultural sectors must stop releasing large quantities of triazoles if this is the case. Recent work conducted in sawmills has shown that even if

the conditions for using treatment products (soaking time, drying) are respected, triazole residues are nevertheless released into the environment, around treatment tanks and near storage area (up to 45 mg of propiconazole per gram of substrate) (Jeanvoine et al. 2017).

3.6.1 Possible Environmental Remediation: A New Approach

A potential option to limit the emergence of resistance mechanisms in the environment would be to control the diffusion of triazole molecules in soils, before they accumulate in soils and end up in compost. Most of the time, those treatments are used to avoid the development of fungal infections on the aerial part of the plants. In those cases, there is no need for the fungicide to go through the soil, where they select resistant strains. Chemical components could be used to catch the fungicide before their penetration in the soil, and without affecting the treatment effectiveness on leaves.

Some conventional disposal methods can be used to remove this type of molecule (activated carbon or chemical oxidation, for example) from environmental compartments. However, despite their proven effectiveness, the latter have a number of disadvantages, including their high costs, and their non-ecological and energy-consuming aspects. Moreover, the significant production of waste is difficult to recycle, and the possibility of transferring technology from the laboratory scale to environmental sites is limited. It therefore appears necessary to propose new methods of eliminating these fungicidal molecules, which would be more chemically effective, greener and cost-effective.

Cyclodextrins are natural oligosaccharides obtained by enzymatic degradation of starch. These commercial molecules belong to the family of cage molecules due to their structure, which is composed of a hydrophobic cavity that can encapsulate other substances. These substances are also non-toxic, biodegradable, biocompatible, and produced at relatively low cost. Cyclodextrins find many applications from pharmaceuticals to cosmetics, food, textiles, agrochemicals, chromatography, biotechnology, catalysis, nanotechnology, and environment (Del Valle 2004; Crini et al. 2018). Chemical structures and dimensions of three native cyclodextrins, α -CD, β -CD and γ -CD, containing 6, 7 and 8 glucose units, respectively, are presented Fig. 3.7.

Their hydrophobic cavities make it possible to develop interactions as “host-guest” interaction type (Fig. 3.8). In medicine they are already commonly used as transporter or to increase solubility of triazole molecules (Peyton et al. 2015). In agriculture, some application has been described, especially in goal to gradually released a constant concentration of fungicide, which confirms their ability to encapsulate triazoles used in the environment (Balmas et al. 2006).

Applications in link with environment protection and soil and water decontamination are already described with organic and metallic molecules (Crini et al. 2018). Their potential application in this problem is by their ability to encapsulate azole

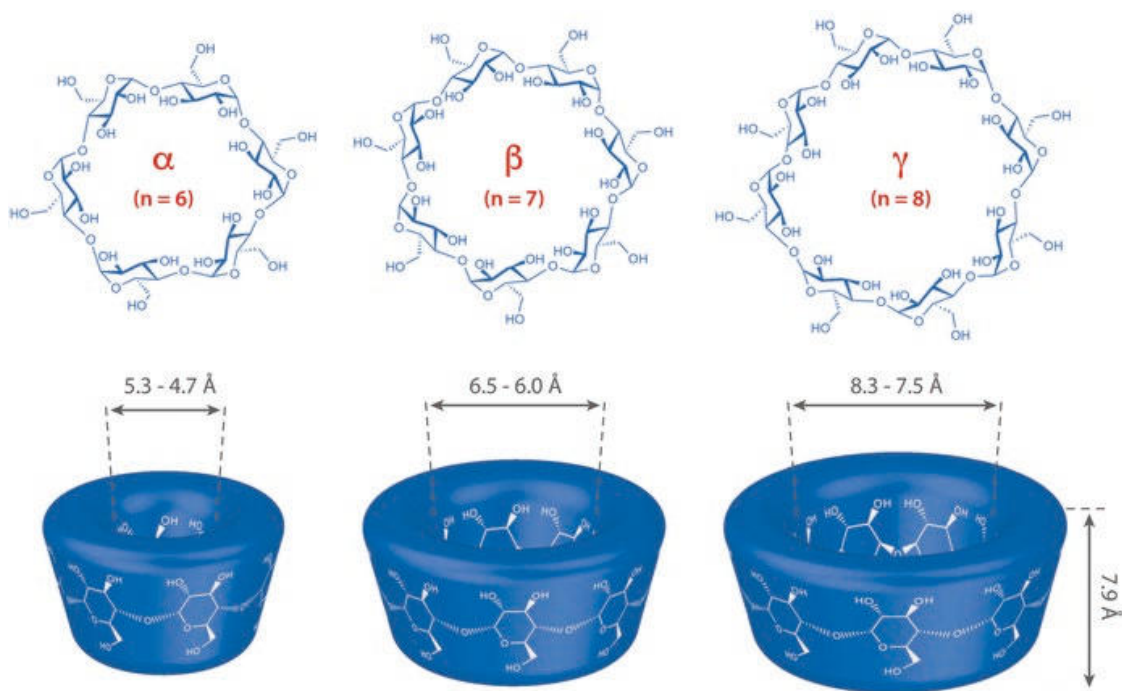


Fig. 3.7 Chemical structure and dimensions for the three native cyclodextrins: α -, β - and γ -cyclodextrin; (n = 6, 7 and 8 glucose units, respectively; source: with the kind courtesy of Marc Fourmentin, Dunkerque, France)

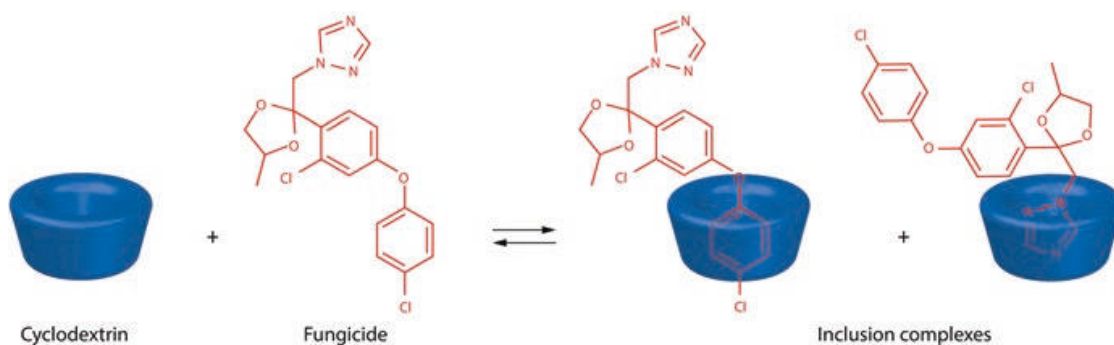


Fig. 3.8 Schematic representation of the inclusion phenomena between a cyclodextrin molecule (the host) and a solute (the guest) to form an inclusion complex (the example illustrates the two possibilities of interaction between difenoconazole fungicide and β -cyclodextrin; source: with the kind courtesy of Marc Fourmentin, Dunkerque, France)

molecules in the environment once they are, for example, grafted on a support which is currently under research. A moderate use of triazole molecules coupled with the development of that type of decontamination methods could permit to limit the dispersion of azole molecules and indirectly the phenomenon of resistance mechanisms selection. Recent *in vitro* studies, Rocchi et al. (2019) showed that in presence of cyclodextrins, triazole fungicides no longer had any action on *A. fumigatus* strains. Those results assumed that once trapped by cyclodextrins, triazole fungicides would probably have less impact leading to resistance. This underlines the interest of deepening the study on the interactions between cyclodextrins and

triazole fungicides, which represent an interesting solution to limit the resistance emergence.

3.6.2 Changing Hotspots into Coldspots

Not all sites where azoles are present selection of azole resistance *A. fumigatus* takes place, nor sites that show abundant growth of *A. fumigatus* show selection of azole resistance. A hotspot of azole resistance selection is characterized with abundant and prolonged growth of *A. fumigatus*, the ability of the fungus to undergo a complete lifecycle and growth of the fungus in the presence of azoles. By identification and defining hotspots for azole resistance selection the question arises if it is possible to change these sites from hotspots into coldspots. Since prohibiting the use of azole in the environment will be difficult to achieve, preventing the prolonged growth of *A. fumigatus* in the presence of azole can be much more feasible with possibly highly satisfactory effect. Further research will need to show what the common characteristics are for both hot- and coldspots as well as the effect of possible interventions taken at former hotspots to observe if they can effectively be changed into coldspots.

Limiting the length of storage of waste stockpiles could potentially be an interesting intervention resulting in a lower environmental pressure of azole resistant *A. fumigatus* isolates. By simply not giving *A. fumigatus* the time to grow to substantial concentrations and not allowing the fungus to undergo its full lifecycle can potentially have great effect on azole resistance selection. Future research will first have to determine the exact parameters that can define an azole resistance selection hotspot and then in turn investigate what appropriate measures can be taken to turn these locations into hotspots.

3.6.3 Identify Places at Risk and Protect Sensitive Populations

The spread of resistant strains from their place of selection to at-risk populations is poorly understood, particularly as the strains are able to migrate across vast distances of land and sea due to environmental and anthropogenic factors (Ashu et al. 2017). Several common genotypes were found within the same region and country and could be carried by the wind, if topography and climate permit it. In other cases, there are up to 7500 km between two individuals with identical genotype, which could be explained by winds or humans and marketed products movements. Thus, there could be local and long-distance dispersals (Sewell et al. 2019b).

For human fungal pathologies, it is likely that a certain number of people can be colonized by resistant strains present in their workplace or at home, and that the transition to infection occurs in the hospital during immunosuppression. For others, nosocomial infection with resistant strains present in the hospital environment is also possible, although no publication has yet proved this. Various risk factors for

invasive fungal infections during hospitalization of immunocompromised patients have already been mentioned for several years: weather conditions, construction work, plants, flowers, showers (Pagano et al. 2011). Given the increasing emergence of resistance, and because some resistant strains have been found at the entrance of intensive care haematology department (Godeau et al. 2019), there are probably simple steps that could be taken to further reduce the exposure of at-risk patients. For example, the bibliography points out that the presence of tulip bulbs is a source of soil contamination by resistant strains of *A. fumigatus* (Dunne et al. 2017; Godeau et al. 2019; Sewell et al. 2019a). These types of flowerbeds (but also composting soil) in area with intensive care units and departments with allogeneic hematopoietic stem cell transplantation should be forbidden, or alternative such as the planting of other types of flowers of controlled origin or of bulbs produced through organic farming methods must be proposed (Godeau et al. 2019). For example, the hospital in Besançon (Eastern France), which used to fill its flowerbeds with Dutch tulips, has changed its product reference and now orders tulips without azoles treatments from the same supplier.

In terms of patients' exposure outside hospitals, it is more complicated. It might seem that only patients in an environment using fungicides (work or housing surrounded by cultures) are at risk (Rocchi et al. 2014a; Jeanvoine et al. 2016), but exposure to resistant strains is much more complex. Indeed, resistant strains have also been isolated from urban housing, urban parks or forests far from potential hotspots (Dauchy et al. 2018; Sewell et al. 2019a). Therefore, since we do not know the possible extent of these strains, it could be important to follow at-risk patients' life courses and measure exposure at hospital, at home and at "at-risk" workplace. Some publications have moreover shown the presence of genetically related resistant isolates from gardens and dwellings of patients who have developed aspergillosis with resistant strains (van der Linden et al. 2013; Lavergne et al. 2017). Even without mentioning the problem of resistance, prevention in hospital and at home has been recognized as effective in apprehending the risk of invasive aspergillosis in immunocompromised patients (Reboux et al. 2014; Rocchi et al. 2014b). Following these works, a follow-up of immunocompromised patients with a home survey by an environmental counsellor to measure potential fungal sources is proposed to patients in the Burgundy-Franche-Comté region (Eastern France) undergoing induction chemotherapy or hospitalized for allogeneic hematopoietic stem cell transplantation. For sure, even if home fungal monitoring is implemented, it cannot always be possible to do something for invasive fungal infection prevention, above all if just some colonies of *A. fumigatus* are isolated. However, home visits represent a means of providing personalized prevention to patients and could elucidate sources mini hotspots. Incredible at-risk situations, such as immunocompromised patient living in a home decorated with plaited dried wheat in all rooms and associated with important *A. fumigatus* concentrations in air (Rocchi et al. 2014b), could not have been identified without the home visit, just with the general advice given to the patients by the doctor. At this time, resistance testing was not done. But, this kind of situation has to be mentioned to the clinician which can opt for a prophylactic treatment or give advices to patient to go in another dwelling temporarily, during at-risk periods.

3.7 Conclusion

To maintain the ability to control fungal infections with the effective use of antifungal compounds and taking into account human and ecosystem health, the fight against antifungal resistance must become a high priority. There are three priority actions with the following objectives: To better understand the conditions in which resistance develops, identify the characteristics of hotspots for azole resistance selection and link the spread of resistance from hotspots with patients. To improve techniques to better diagnose and cure patients from fungal infections; and finally to obtain a durable azole use for both environmental as well as medical purposes. A “One Health” approach (a concept combining human health, animal health and ecosystem health) is needed to understand the development and spread of these treatment-resistant infectious agents in order to ultimately protect the health of ecosystems (including wild and cultivated/cultivated/raised fauna and flora) and humans.

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Titre de l'article :



Molecular epidemiology of azole-resistant *Aspergillus fumigatus* in sawmills of eastern France by microsatellite genotyping.

Présentation succincte :

Un autre domaine d'utilisation des molécules triazolées est le traitement du bois. Afin d'évaluer la présence potentielle d'aR-Af dans l'environnement proche des zones de traitements, des prélèvements d'air et de sols ont été effectués dans 20 scieries de l'Est de la France. Sur ces sols, 23 aR-Af ont été retrouvés dans 18 des scieries étudiées. Leur présence a pu être liée à l'utilisation de molécules triazolées telles que le propiconazole et le tébuconazole et semble être dépendante de la dose de résidu de molécule triazolée retrouvée dans ces mêmes sols. Dans cette étude une analyse de génotypage par microsatellites a été réalisée sur 42 souches sensibles d'*Aspergillus fumigatus* et les 23 aR-Af retrouvés dans les scieries. Le constat a été établi que des génotypes communs ou distincts pouvaient être retrouvés au sein d'une même scierie suggérant que la théorie d'une expansion clonale d'aR-Af issus d'une souche commune ne permet pas n'est pas suffisante pour expliquer la distribution de ces génotypes.

Article

Molecular Epidemiology of Azole-Resistant *Aspergillus fumigatus* in Sawmills of Eastern France by Microsatellite Genotyping

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Abstract: Background: Wood chipping has been described as a potential hotspot for the selection of azole-resistant *Aspergillus fumigatus* (ARAf). We previously reported ARAf isolates in sawmills (Eastern France), most of which contained the TR₃₄/L98H mutation. **Methods:** To study genotypic relatedness, microsatellite genotyping (short tandem repeat for *A. fumigatus* (STRAf)) was performed on 41 azole-susceptible *A. fumigatus* (ASAf) and 23 ARAf isolated from 18 sawmills and two clinical *A. fumigatus* (sensitive and resistant) isolated from a sinus sample of a woodworker. **Results:** Fifty-four unique multilocus genotypes (MLGs) were described among the 66 isolates: 13/24 ARAf and 41/42 ASAf. Allelic diversity was higher for ASAf than for ARAf. Among the 24 ARAf, five isolates had their own MLGs. Thirteen ARAf (54%) belonged to the same group, composed of four close MLGs, defined using Bruvo's distance. Thirty-two of the 42 ASAf (76%) had their own MLGs and could not be grouped with the Bruvo's distance cutoff used (0.2). **Conclusion:** Thus, at a regional scale and in the particular environment of the wood industry, common but also different distinct genotypes, even in the same sawmill, were identified. This suggests that the hypothesis of ARAf clonal expansion from a common strain is probably insufficient to explain genotype emergence and distribution.

Keywords: *Aspergillus fumigatus*; azole-resistant; sawmill; microsatellite; multilocus genotype

1. Introduction

Aspergillus fumigatus is a saprophytic fungus that is widespread in the environment with an ecological niche of decaying vegetation and soil [1]. It is also a ubiquitously opportunistic pathogen responsible for aspergillosis, notably invasive aspergillosis (IA)—the most severe form of the disease [2]. Indeed, the IA mortality rate is high and can reach 65% [3].

Although azole antifungals improve the management of *Aspergillus*-disease, this clinical advance might be threatened by the emergence of azole-resistant *A. fumigatus* (ARAf) worldwide [4,5]. Over the past 20 years, there have been increasing reports of ARAf recovered on the five continents both in clinical and environmental samples [4]. The environmental route of resistance, based on the wide scale use of azole fungicides in the environment, plays a significant role [6]. Two main mechanisms of resistance that are probably of environmental origin, TR₃₄/L98H and TR₄₆/Y121F/T289A mutations, have commonly been described in strains from azole-naïve patients and from the environment [4,5].

Over the past few years, several environmental areas have been reported as potential hotspots for the selection of ARAf. In the Netherlands, composts containing azole residues constitute a potential hotspot for the emergence of new mutations conferring resistance [7]. Recently, wastes

originating from flower bulbs, green materials, and wood chippings have been reported to be three new hotspots containing azole fungicides with the highest proportion of ARAf, as compared to wheat cereals, animal manure, grain, maize silage, fruit storage or regional and exotic fruit waste [8]. ARAf have been identified in wood environments for some years now. In fact, ARAf carrying TR₃₄/L98H and TR₄₆/Y121F/T289A have been described in wood debris of tree trunk hollows in Tanzania and Romania [9]. We also previously described the presence of ARAf in the wood environment, namely sawmills of Eastern France, most of which carried the TR₃₄/L98H mutation [10]. In this previous study, the presence of ARAf carrying the TR₃₄/L98H mutation seemed to greatly depend on the azole fungicide formulation and the quantity in substrates [10].

Consequently, the azole fungicide selection pressure could occur in different environments and in different countries and seems to play an important role in the selection of ARAf. Although typing of *A. fumigatus* isolates could provide insights into the dynamics of azole resistance development, the origin and global diffusion of azole-resistant *A. fumigatus* with TR₃₄/L98H and TR₄₆/Y121F/T289A are still unclear [11,12]. Currently, short tandem repeat for *A. fumigatus* assay (STRAf), based on a nine microsatellite analysis, is accepted as the reference and robust typing method for this species [13,14].

The aim of this study was to genotype ARAf and azole-susceptible *A. fumigatus* (ASAf), previously isolated from sawmills of Eastern France, by using STRAf typing in order to study their genetic relatedness and to describe the potential clustering of isolates according to their susceptibility profile [10]. In addition, the clinical isolates (sensitive and TR₃₄/L98H) previously described in an immunocompetent woodworker with invasive sinus aspergillosis were compared to the sawmill isolates [15].

2. Materials and Methods

2.1. Collection of *A. fumigatus* Strains

A total of 64 environmental *A. fumigatus* strains, isolated from 18 French sawmills (Eastern France) between September 2014 and April 2016, were analyzed (Table 1). Among them, 23 ARAf isolates had previously been isolated by using two selective homemade malt extract agar (ThermoFisher, Waltham, MA, USA) media containing azole antifungals: itraconazole called “Maltitra” and voriconazole called “Maltvori” [10]. The 41 selected ASAf isolates matched according to the location of ARAf isolates for each sawmill. Two clinical isolates (one ARAf and one ASAf), isolated from sinus samples in an immunocompetent woodworker who developed an invasive sinus aspergillosis following facial injuries from a work-related accident, were also included (Table 1) [15].

All *A. fumigatus* isolates were identified to the species level by PCR amplification and sequencing a part of the highly conserved β -tubulin gene [10].

For all ARAf, minimal inhibitory concentrations (MIC) of four medical azoles (itraconazole, voriconazole, posaconazole and isavuconazole) and two azoles used in agriculture and sawmills (propiconazole and tebuconazole) were obtained by using the EUCAST (European Committee on Antimicrobial Susceptibility) microdilution method [16].

Amplification and sequencing of the *cyp51A* gene were performed as previously described [10]. Among the 23 environmental ARAf, 20 carried the TR₃₄/L98H mutation, two the TR₃₄/L98H/S297T/F495I mutation and one the P216L mutation [10]. The clinical ARAf, one of the two isolates recovered in the woodworker, also carried the TR₃₄/L98H mutation [15].

MIC of voriconazole and itraconazole for environmental and clinical ASAf isolates were measured by the EUCAST method [16].

Table 1. Characteristics of analyzed azole-resistant (AR*Af*) and azole-susceptible (AS*Af*) *A. fumigatus*.

Isolate Identification	Date of Isolation	Origin	Susceptible or Resistant and Cyp51A Mutation
A25	February 2015	Substrate, sawmill A	Resistant, TR ₃₄ /L98H
D2	January 2016	Substrate, sawmill D	Resistant, TR ₃₄ /L98H
D3	January 2016	Substrate, sawmill D	Resistant, TR ₃₄ /L98H
D4	January 2016	Substrate, sawmill D	Resistant, TR ₃₄ /L98H
D5	January 2016	Substrate, sawmill D	Resistant, TR ₃₄ /L98H/S297T/F495I
D6	January 2016	Substrate, sawmill D	Resistant, TR ₃₄ /L98H
D7	January 2016	Substrate, sawmill D	Resistant, TR ₃₄ /L98H
D8	January 2016	Substrate, sawmill D	Resistant, TR ₃₄ /L98H
F13	February 2016	Substrate, sawmill F	Resistant, TR ₃₄ /L98H
F14	February 2016	Substrate, sawmill F	Resistant, TR ₃₄ /L98H/S297T/F495I
G15	February 2016	Substrate, sawmill G	Resistant, TR ₃₄ /L98H
H16	February 2016	Substrate, sawmill H	Resistant, TR ₃₄ /L98H
K17	March 2016	Substrate, sawmill K	Resistant, TR ₃₄ /L98H
M18	March 2016	Substrate, sawmill M	Resistant, TR ₃₄ /L98H
O19	March 2016	Substrate, sawmill O	Resistant, TR ₃₄ /L98H
O20	March 2016	Substrate, sawmill O	Resistant, TR ₃₄ /L98H
P11	September 2014	Substrate, sawmill P	Resistant, TR ₃₄ /L98H
P12	September 2014	Substrate, sawmill P	Resistant, TR ₃₄ /L98H
Q10	January 2016	Substrate, sawmill R	Resistant, TR ₃₄ /L98H
R21	April 2016	Substrate, sawmill S	Resistant, TR ₃₄ /L98H
R22	April 2016	Substrate, sawmill S	Resistant, TR ₃₄ /L98H
S23	April 2016	Substrate, sawmill T	Resistant, TR ₃₄ /L98H
S24	April 2016	Substrate, sawmill T	Resistant, TR ₃₄ /L98H
Wood-worker	October 2013	Clinical	Resistant, TR ₃₄ /L98H
A175	February 2015	Substrate, sawmill A	Susceptible
A176	February 2015	Substrate, sawmill A	Susceptible
A177	February 2015	Substrate, sawmill A	Susceptible
A178	February 2015	Substrate, sawmill A	Susceptible
C1P	January 2016	Substrate, sawmill C	Susceptible
D156	January 2016	Substrate, sawmill D	Susceptible
D157	January 2016	Substrate, sawmill D	Susceptible
D158	January 2016	Substrate, sawmill D	Susceptible
D159	January 2016	Substrate, sawmill D	Susceptible
D161	January 2016	Substrate, sawmill D	Susceptible
D162	January 2016	Substrate, sawmill D	Susceptible
D9P	January 2016	Substrate, sawmill D	Susceptible
D10P	January 2016	Substrate, sawmill D	Susceptible
E21P	January 2016	Substrate, sawmill E	Susceptible
F19P	February 2016	Substrate, sawmill F	Susceptible
F20P	February 2016	Substrate, sawmill F	Susceptible
F163	February 2016	Substrate, sawmill F	Susceptible
G24P	February 2016	Substrate, sawmill G	Susceptible
H22P	February 2016	Substrate, sawmill H	Susceptible
H23P	February 2016	Substrate, sawmill H	Susceptible
H164	February 2016	Substrate, sawmill H	Susceptible
I15P	February 2016	Substrate, sawmill I	Susceptible
J16P	March 2016	Substrate, sawmill J	Susceptible
K2P	March 2016	Substrate, sawmill K	Susceptible
K165	March 2016	Substrate, sawmill K	Susceptible
L4P	March 2016	Substrate, sawmill L	Susceptible
M5P	March 2016	Substrate, sawmill M	Susceptible
N6P	March 2016	Substrate, sawmill N	Susceptible
O7P	March 2016	Substrate, sawmill O	Susceptible
O8P	March 2016	Substrate, sawmill O	Susceptible
O166	March 2016	Substrate, sawmill O	Susceptible
P173	September 2014	Substrate, sawmill P	Susceptible
P174	September 2014	Substrate, sawmill P	Susceptible
Q17P	January 2016	Substrate, sawmill R	Susceptible
R11P	April 2016	Substrate, sawmill S	Susceptible
R12P	April 2016	Substrate, sawmill S	Susceptible
R168	April 2016	Substrate, sawmill S	Susceptible
S13P	April 2016	Substrate, sawmill T	Susceptible
S14P	April 2016	Substrate, sawmill T	Susceptible
S169	April 2016	Substrate, sawmill T	Susceptible
S179	April 2016	Substrate, sawmill T	Susceptible
Wood-worker	July 2013	Clinical	Susceptible

2.2. Short Tandem Repeat for *A. fumigatus* (STRAf) Typing and Analysis

STRAf typing is based on the amplification of nine highly polymorphic microsatellite markers (STRAfs 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, 4C). These nine STRAf loci were amplified with three triplexed PCRs as previously described [13].

PCR products were diluted 10-fold with water for molecular biology and then analyzed on the Applied Biosystems 3130 Genetic Analyzer (ThermoFisher, Waltham, MA, USA) with GeneScan™ 400 HD ROX™ size standard according to the manufacturer’s instructions (ThermoFisher®, Waltham, MA, USA).

Amplicon sizes were determined with GeneMapper software (version 5, ThermoFisher, Waltham, MA, USA) and then transformed in repeat number.

STRAf analysis was then performed using RStudio software (version 3.2.2, Boston, MA, USA). Microsatellite genotype distances were calculated using Bruvo’s distance (cutoff value = 0.2) and a minimum spanning network was calculated via the bruvo.msn function on the poppr library [17]. Finally, allelic diversity was calculated for the nine STRAf loci for ARAf and ASAf isolates by using the Simpson index of diversity (*D*).

3. Results

A total of 54 STRAf unique multilocus genotypes (MLGs) were described among the 66 typed *A. fumigatus*: 13 for the 24 ARAf and 41 for the 42 ASAf. None of the ARAf isolates had MLGs close to those that of the ASAf isolates, and the sensitive clinical isolate identified in the woodworker before the resistant one revealed a completely different MLG.

The nine microsatellite markers including STRAf genotypes, allele count, repeat range, median repeat number and allelic diversity are summarized in Table 2. All loci were polymorphic with a number of alleles ranging from 3 (loci 3B and 4B) to 11 (locus 3A) and from 9 (locus 4B) to 19 (loci 3A and 3C) for ARAf and ASAf isolates, respectively. Loci 3A and 3C displayed the highest polymorphism and allelic diversity for both ARAf and ASAf isolates, whereas locus 4B was the least discriminating with the lowest number of alleles (Table 2). For all microsatellite markers, allelic diversity was higher for ASAf isolates (average *D* = 0.811) than for ARAf isolates (average *D* = 0.518) (Table 2).

Table 2. Polymorphism information for the nine microsatellite markers obtained after short tandem repeat for *A. fumigatus* (STRAf) typing of sawmill azole-resistant (ARAf) and azole-susceptible (ASAf) *A. fumigatus*.

Azole-Resistant <i>A. fumigatus</i> (ARAf, n = 24)					Azole-Susceptible <i>A. fumigatus</i> (ASAf, n = 42)				
Microsatellite Marker	N Alleles	Repeat Range	Median Size	Diversity (<i>D</i>)	Microsatellite Marker	N Alleles	Repeat Range	Median Size	Diversity (<i>D</i>)
2A	5	13–26	14	0.507	2A	11	10–27	18	0.768
2B	4	10–24	21	0.462	2B	9	12–25	19	0.766
2C	4	8–16	8	0.552	2C	11	8–20	12	0.868
3A	11	10–119	32	0.872	3A	19	10–49	26	0.909
3B	3	8–11	8	0.403	3B	10	8–22	9	0.771
3C	7	6–32	6	0.649	3C	19	6–45	18	0.924
4A	5	5–18	8	0.361	4A	13	7–26	9	0.774
4B	3	7–10	10	0.392	4B	8	5–26	9	0.746
4C	5	5–30	20	0.465	4C	9	5–36	7	0.770
Total alleles	47	NR	NR	NR	Total alleles	109	NR	NR	NR
Average <i>D</i>	NR	NR	NR	0.518	Average <i>D</i>	NR	NR	NR	0.811

N Alleles = Allele counts; *D* = Simpson index of diversity; NR = non relevant.

Among the 42 ASAf, 41 MLGs were identified, with two isolates from two different sawmills (A and D, 60 km away) sharing the same genotype (A177 and D159, Figure 1). Thirty-two MLGs (76%) were very distinct and could not be grouped with the Bruvo distance cutoff used. However, four groups of two ASAf were found: D161 and R12P, E21P and O166, S179 and C1P, F20P and F19P

(genotypes connected by lines in Figure 1). These *ASAf* were isolated from different sawmills, except for isolates F20P and F19P which were recovered in the same sawmill (F) at different locations.

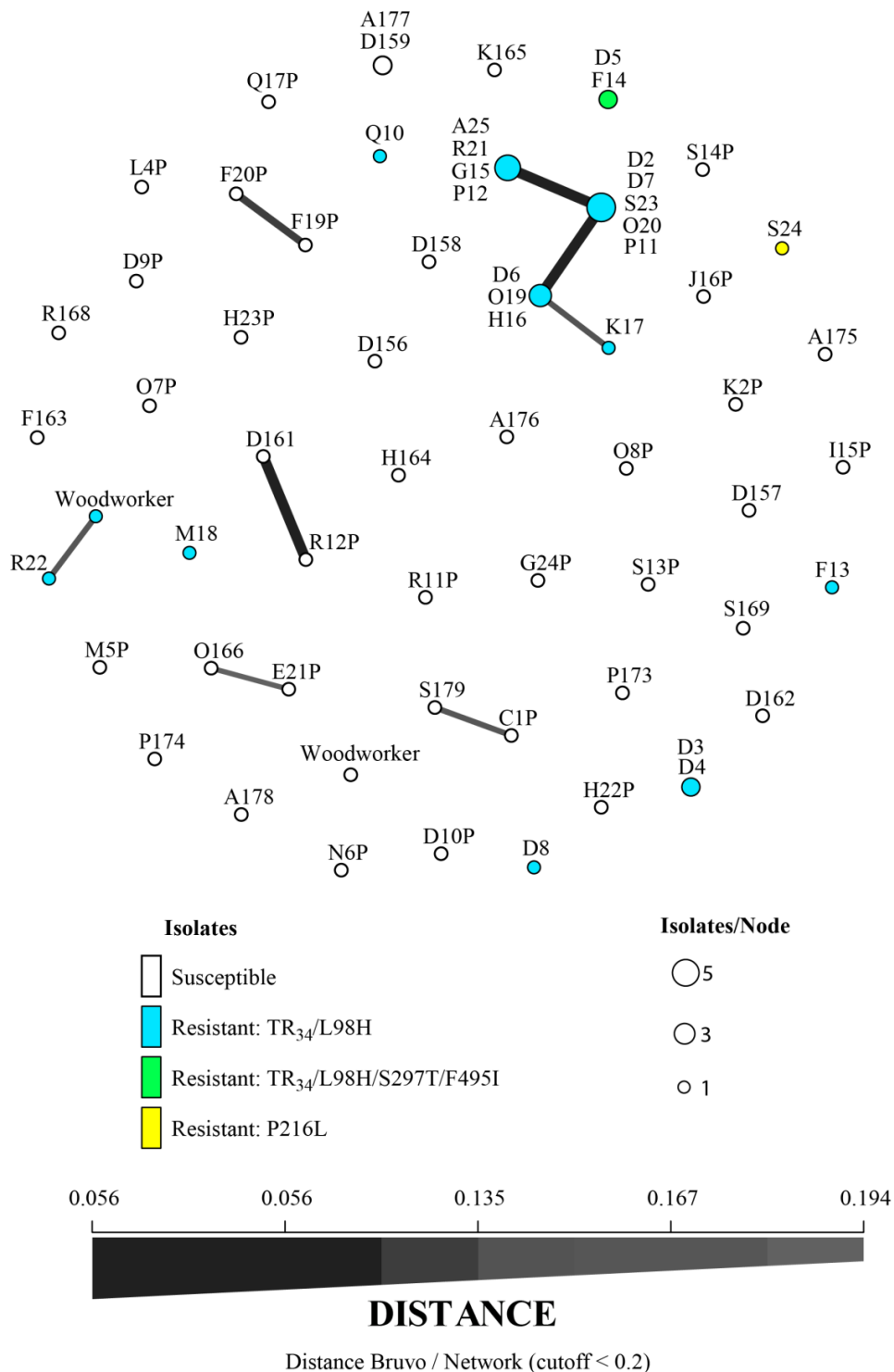


Figure 1. Minimum spanning network of nine microsatellite loci *STRAf* using Bruvo’s distance. Letters correspond to sawmill and the following numbers ± letters are genotype numbers. Each circle represents one multilocus genotype, the size of which is proportional to frequency. Different colors represent the different isolates (susceptible or resistant, and their mutations on the *Cyp51A* gene and its promoter). Link thickness is proportional to genotype similarity.

Among the 24 *ARAF*, 13 MLGs were characterized. Four isolates with the TR₃₄/L98H mutation (genotype numbers Q10, M18, D8 and F13, Figure 1) and one with the P216L mutation (genotype S24) had their own MLGs and could not be grouped with Bruvo's distance. The others were recovered for several isolates grouped in the same circles within the minimum spanning network (Figure 1).

For TR₃₄/L98H isolates, two MLGs that were genotypically distinct from others were identified: genotypes D3 and D4 with the TR₃₄/L98H mutation, found in the same soil sample, and D5 and F14 with the TR₃₄/L98H/S297T/F495I, taken from two geographically distant sawmills (170 km).

Moreover, two groups of MLGs were found with Bruvo's distance (Figure 1). The first group was composed of four MLGs, including 13/24 *ARAF* (54%, genotype numbers A25, R21, G15, P12 / D2, D7, S23, O20, P11 / D6, O19, H16 and K17). Among these four closely related STRAf genotypes, three differed by only one repetition of the microsatellite marker 3A; the fourth (K17 *ARAF*) differed from the three others by the number of repetitions of microsatellite markers 3A and 3C. These four genotypes included TR₃₄/L98H *ARAF* isolated from different sawmills that could be located as far as 200 km from each other; two of these genotypically similar *ARAF* (P11 and P12) were isolated in one sawmill that imported wood from Russia. The second group consisted of one clinical TR₃₄/L98H isolate responsible for invasive sinus aspergillosis in an immunocompetent woodworker and had a genotype close to a sawmill *ARAF* also carrying the TR₃₄/L98H mutation (genotype R22 in Figure 1). It differed by the number of repetitions on microsatellite markers 3A and 4A. However, the woodworker did not work in sawmill R.

Identical MLGs were thus found in different sawmills, but different MLGs were also found in one sawmill: five different MLGs were identified among the seven *ARAF* found in sawmill D. This sawmill shared one MLG (genotype D5, mutation TR₃₄/L98H/S297T/F495I) with another sawmill. Sawmill D shared MLGs with other sawmills and genotypes that closely resembled each other (genotypes D2, D7 and D6). It also had one MLG (genotypes D3 and D4) that was different from all other MLGs.

4. Discussion

In this study, we reported the presence of both common and different genotypes in *ARAF* and *ASAF* from sawmills of Eastern France by using STRAf typing. Our findings are similar to those of several other studies, as we reported that *ASAF* had a greater genotypic diversity than *ARAF* [14,18,19]. This could suggest a predominantly clonal expansion of *ARAF* in the environment. In fact, contrary to *ASAF*, more than half of *ARAF* (54%) belonged to the same group with the Bruvo's distance cutoff used and seemed to be genotypically close. These *ARAF* came from different sawmills that were geographically far from one another (up to 200 km) and for which a selection pressure by azole fungicide was reported. Direct or indirect contact with azole fungicide was shown, and azole fungicides were detected in substrate samples in these sawmills [10].

This study has some limitations. First, we describe here the genotypic structure of isolates coming from only one region in France and one type of environment. A broader study including genotyping of isolates from other regions, environments and countries would make it possible to know if the genotypes of *ARAF* and *ASAF* from Eastern France are shared by isolates in other regions of the world; this could help to improve our understanding of the emergence, diffusion and distribution of *ARAF*.

In addition, we used STRAf typing which is widely accepted as the reference typing method for *A. fumigatus* [14]. However, two of the nine microsatellite markers, 3A and 3C, show a lower level of stability which has to be taken into account when interpreting STRAf data during molecular epidemiological analysis [20]. This is illustrated by the greater polymorphisms for these two markers described in our study. Moreover, three genotypes differed by only one repetition on marker 3A and grouped isolates coming from the same sawmill or the same samples (P11 and P12). The difference between these three genotypes might be valid but we cannot exclude a bias in the genotyping method used. Despite the high discriminatory power of STRAf typing, it remains lower than that of whole genome sequencing (WGS). So, it would be interesting to determine whether or not *ARAF* with the

same genotype, revealed using STRAf typing, would reveal exactly the same genotype when using WGS. Likewise, exploring genotypic diversity with WGS could be useful.

It is important to emphasize that 13 STRAf genotypes were found among the 24 ARAf. Moreover, some ARAf had their own genotypes and were genotypically distinct from others despite sharing the same mechanism of resistance (mutation TR₃₄/L98H). It is interesting to note that this diversity was reported in one sawmill (D), without any particular characteristics when compared to other sawmills using a dip processing tank, and where five MLGs were reported among the seven ARAf genotyped. These findings contrast with those of some studies describing only one clone of TR₃₄/L98H ARAf strains [18,21]. The local diversity of the genotype reported here was also highlighted for ARAf isolates in market gardens of the same region in Eastern France. In this study, 22 STRAf genotypes among 44 analyzed ARAf and six groups of genotypically close ARAf were recorded. ARAf isolates could be more diverse in other countries such as Colombia where 19 MLGs were identified in 21 ARAf isolates in the environment [17,22]. Additionally, it has also been shown by STRAf typing that resistant isolates, coming from several countries, belonged to several groups. Similarly, some authors have reported a dispersed structure in ARAf with tandem repeat (TR) mutations [11,14]. Thus, it seems unlikely that the emergence of azole-resistant strains could be the work of a single clone.

The population structure of *A. fumigatus* is very complex and is likely to be due to several past and current events: historical differentiation, contemporary gene flow, sexual reproduction, recombination, and the localized azole fungicide selection that could drive expansion of ARAf genotypes [11,12]. It was recently reported that certain practices like composting (i.e., stockpiling plant waste) might be the key to resistance selection in *A. fumigatus* [8]. Some environments supporting the growth, sexual reproduction, genetic variation and containing residues of azole fungicides could cause these complex mutations to emerge, amplify and spread. So, ARAf may very well emerge in specific environments and spread to other countries due to natural factors such as wind, or anthropogenic factors such as human travel and commercial trade [11,23]. This gene flow could explain the fact that genotypically close ARAf, or those having the same genotype, were found in different countries [18,19,21].

A comparison of genotypes from sawmill ARAf with genotypes from other *A. fumigatus* isolates in a London database (<https://afumid.shinyapps.io/afumID/>, 4049 *A. fumigatus* isolates collected worldwide) showed that some sawmill ARAf (A25, R21, G15, P12 with the same MLG) presented the same genotype as a clinical Australian isolate (data not shown). Among them, isolate P12 is one of the two isolates found in one sawmill that did not treat wood, but rather imported foreign wood from Russia that had already been treated with fungicides (sawmill P). Curiously, these two ARAf shared the same MLG as other sawmills ARAf. According to these results, the gene flow could not be excluded here and it may be possible that these ARAf emerged in a favorable environment outside of France and then, when imported, spread to the Eastern France sawmills. Conversely, an MLG identified twice in a single sawmill (D) is not linked to any profile in the London database.

The distribution of sawmill ARAf and ASAf genotypes, with a lower diversity of ARAf isolates, also seems to concur with two hypotheses reported by other authors to explain the emergence and distribution of ARAf: the specific genetic background and predisposition of some clones to develop azole-resistance, and the better ability of ARAf isolates to accept the azole-resistant genes via mating and recombination [11,14]. ARAf with TR₃₄/L98H and TR₄₆/Y121F/T289A mutations may have a genetic component that is restricting the resistance genotype to certain strains [24]. It has also been reported that the genetic backgrounds of TR₃₄/L98H and TR₄₆/Y121F/T289A ARAf were less diversified than those of wild-type isolates [24]. This is compatible with selective gene sweeps accompanying the selection of beneficial mutations and the genetic adaptation of *A. fumigatus* which enable it to survive and reproduce in prevailing or new environments, such as those with azole fungicides [24,25]. Selective sweeps would reduce allelic diversity and one, or a limited number, of clones would predominate locally [23]. The lower allelic and STRAf genotype diversity reported for ARAf isolates from sawmills in our study make this hypothesis likely. So, according to their genetic background, some strains could adapt more easily than others to a given environment and could be more able to develop and persist

in an environment where the azole fungicide selection pressure is substantial. Moreover, the lower diversity of the ARAf, despite their different origin and the fact that they have been isolated in different sawmills, could suggest that ARAf belonging to the same group (with the Bruvo's distance cutoff used) are genotypically closed and probably evolved from each other.

In conclusion, in this study we reported both common and different genotypes on a regional scale. Despite having lower allelic diversity than ASAf isolates, some ARAf could not be grouped together with other sawmill ARAf isolates. The diversity of genotypes described here, at a local level, seems to support the multiple origins hypothesis, thus suggesting that the hypothesis of clonal expansion from a common strain is now probably obsolete and insufficient to explain ARAf emergence and distribution. The evolution of ARAf from different origins could also be involved.

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Titre de l'article :

Molecular epidemiology of Azole-resistant *Aspergillus fumigatus* in France shows patient and healthcare links to environmentally occurring genotypes.

Présentation succincte :

Une analyse des génotypes de 225 isolats d'*Aspergillus fumigatus* issus de différentes études menées dans la région Bourgogne-Franche-Comté regroupant des isolats retrouvés dans les prélèvements cliniques et des isolats retrouvés dans l'environnement a été effectuée en utilisant la technique des 9 microsatellites décrite par de Valk et al. (2005). Des isolats sensibles (n = 112) et résistants (n=113) ont été utilisés pour cette analyse. Parmi les isolats résistants, la plupart étaient porteurs de la mutation TR₃₄/L98H (80%). Des génotypes communs ont pu être mis en évidence entre des isolats provenant de différents types d'échantillons et sur différentes périodes (*e.g.* entre des isolats retrouvés chez des patients et des isolats retrouvés dans les couloirs du CHU). Une comparaison avec les génotypes disponibles issus d'autres études internationales a été effectuée et a pu mettre en évidence la présence d'isolats de notre région très proche d'un isolat décrit en Australie par exemple. A l'inverse, d'autres génotypes n'avaient jamais été décrits et semblaient être endémiques de la région.



Molecular Epidemiology of Azole-Resistant *Aspergillus fumigatus* in France Shows Patient and Healthcare Links to Environmentally Occurring Genotypes

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Resistance of the human pathogenic fungus *Aspergillus fumigatus* to antifungal agents is on the rise. However, links between patient infections, their potential acquisition from local environmental sources, and links to global diversity remain cryptic. Here, we used genotyping analyses using nine microsatellites in *A. fumigatus*, in order to study patterns of diversity in France. In this study, we genotyped 225 local *A. fumigatus* isolates, 112 azole susceptible and 113 azole resistant, collected from the Bourgogne-Franche-Comté region (Eastern France) and sampled from both clinical ($n = 34$) and environmental ($n = 191$) sources. Azole-resistant clinical isolates ($n = 29$) were recovered mainly from cystic fibrosis patients and environmental isolates ($n = 84$) from market gardens and sawmills. In common with previous studies, the TR₃₄/L98H allele predominated and comprised 80% of resistant isolates. The genotypes obtained for these local TR₃₄/L98H isolates were integrated into a broader analysis including all genotypes for which data are available worldwide. We found that dominant local TR₃₄/L98H genotypes were isolated in different sample types at different dates (different patients and types of environments) with hospital air and patient's isolates linked. Therefore, we are not able to rule out the possibility of some nosocomial transmission. We also found genotypes in these same environments to be highly diverse, emphasizing the highly mixed nature of *A. fumigatus* populations. Identical clonal genotypes were found to occur both in the French Eastern region and in the rest of the world (notably Australia), while others have not yet been observed and could be specific to our region. Our study demonstrates the need to integrate patient, healthcare, and environmental sampling with global databases in order to contextualize the local-scale epidemiology of antifungal resistant aspergillosis.

Keywords: azole resistant, *Aspergillus fumigatus*, TR₃₄/L98H, genetic relatedness, microsatellites

INTRODUCTION

Fungal pathogens pose a growing threat to the health of humans, animals, ecosystems, food security, and the global economy, making their effective control necessary (Fisher et al., 2012). Azoles fungicides, with more than 30 compounds available (Fisher et al., 2018), are widely used in agriculture to protect cereal, vegetables, and vines from phytopathogenic fungi, as well as in the cultivation of ornamental plants and to preserve materials such as timber. These fungicides are used worldwide, particularly in Europe and Asia, where they represent one of the most commonly used classes of pesticides (Chowdhary et al., 2015; Chen et al., 2016). Due to their broad-spectrum action across the fungal kingdom, the azoles are also widely used as first-line drugs in medicine (human and animals) for the treatment of superficial and invasive fungal infections. Within this context of widespread use of antifungals across different compartments, the occurrence of multi-resistant pathogenic fungi represents an emerging threat to the control of plant, animal, and human diseases (Fisher et al., 2018). Recently reviewed (Verweij et al., 2020), substantial progress has been made towards building the argument that ecological “hotspots” occur where biotic and abiotic conditions converge to allow the growth of the thermophilic *Aspergillus fumigatus* in contact with sub-MIC concentrations of agricultural triazoles, generating conditions that are suitable for adaptation to drug pressure. Specifically, highly related genotypes of azole-resistant *A. fumigatus* have now been described in clinical and environmental samples, suggesting that humans are increasingly exposed to drug-resistant aerosolized *A. fumigatus* spores with broad public health consequences (Sewell et al., 2019; Rhodes et al., 2021).

Studying the population genetics and evolution of pathogens can help further our understanding of the evolution and spread of antimicrobial resistance, including tracing potential sources of inocula using molecular epidemiology. Specifically, for azole-resistant *A. fumigatus* (ARAF), it is important to understand whether genotypes are randomly distributed or cluster geographically, and whether a common source of colonization may exist (van der Torre et al., 2021). In 2012, we described a first case of invasive aspergillosis caused by TR₃₄/L98H ARAF in a farmer (Rocchi et al., 2014). In 2013, we described a second case, in a woodworker, who developed sinus aspergillosis due to TR₃₄/L98H *A. fumigatus* (Jeanvoine et al., 2016). Motivated by these findings, the mycology team of Besançon (Eastern France) undertook a systematic screening of *A. fumigatus* to assess the burden of azole resistance across both clinical and environmental samples. In 2017, we observed an increase in the number of cystic fibrosis (CF) patients colonized with ARAF, with 16% of patients with *A. fumigatus*-positive cultures observed carrying the TR₃₄/L98H resistance allele (Godeau et al., 2019) whereas previously, only one patient had this type of isolate in 2015. This percentage of resistance mirrors that reported by a British team (Abdolrasouli et al., 2018) and is among the highest occurrence in CF patients observed in European studies (Amorim et al., 2010; Mortensen et al., 2011; Burgel et al., 2012; Morio et al., 2012; Fischer et al., 2014; Prigitano et al., 2017; Guegan et al., 2018; Seufert et al., 2018; Lavergne et al., 2019).

Since 2017, a number of ARAF bearing the TR₃₄/L98H allele were detected in the air of Besançon hospital following fungal aerocontamination monitoring. Undertaken weekly using seven air sample locations in general hospital corridors, our surveillance has detected ARAF periodically between 2017 and 2019 (Rocchi et al., 2021). In parallel, two environmental studies were conducted in agricultural fields where azoles had been sprayed, in sawmills (Jeanvoine et al., 2017) and market gardens (Rocchi et al., 2018).

Across our team, we have collected 1,100 strains of the *Aspergilli* complex (*A. fumigatus* and cryptic species) isolated in our region (rural landscape in Eastern France). Here, we assess the genetic relatedness among a retrospective sample of 225 susceptible and resistant *A. fumigatus* from our collection for both clinical and environmental samples by comparing them to data (worldwide scale) held within pre-existing genotype databases.

MATERIALS AND METHODS

Current Systematic Screening in Besançon Hospital

For both clinical and environmental samples, *A. fumigatus* were first isolated on malt agar media supplemented by itraconazole (2 mg L⁻¹) or voriconazole (1 mg L⁻¹) (van der Linden et al., 2013; Bader et al., 2015) at 37°C for clinical ones and 48°C for environmental ones. Subsequently, resistance was measured by concentration gradient Etest strip technique (Etest, bioMérieux, France) and/or checked by European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution method (Arendrup et al., 2013). In case of azole-resistant isolate, a 495-bp section of the *beta-tubulin* gene (Glass and Donaldson, 1995) and the *cyp51A* gene and its promoter were amplified and sequenced (Mellado et al., 2001; Alanio et al., 2011).

PCRs were carried out in a 50- μ l volume, containing 25 μ l of GoTaq[®] G2 Hot Start Green Master Mix, 2 μ l of each set of primers (10 μ M, **Table 1**), 50–100 ng of genomic DNA, and water (molecular biology quality) to 50 μ l. Amplification was performed in a C1000 Touch[™] thermal cycler (BIO-RAD) with 2 min at 95°C, and 34 cycles of 15 s at 95°C, 15 s at 61°C (for *beta-tubulin* primer sets), or 15 s at 55°C (for *cyp51A* primer sets), 10 s at 72°C, and one final cycle of 5 min at 72°C. The PCR products were analyzed by electrophoresis on 1% agarose gels, visualized with SYBR[™] Safe DNA Gel Stain (ThermoFisher Scientific), and then purified (NucleoSpin[®] Gel and PCR clean-up, Macherey-Nagel) to Sanger sequencing (Applied Biosystems 3130 Genetic Analyzer).

Selection of Isolates for Genetic Relatedness Analysis

All ARAF obtained from April 2012 to December 2018, 113 isolates in total, were included in the genetic relatedness analysis. Azole-susceptible isolates (ASAF), isolated on azole or azole-free media, were retrospectively selected (see below) from the available collection according to the origin of resistant ones

TABLE 1 | Sequences of primers used for *beta-tubulin* and *cyp51A* and its promoter.

		Sequences	
<i>beta-tubulin</i>		Bt2a	5'-GGTAACCAATCGGTGCTGCTTTC-3'
		Bt2b	5'-ACCCTCAGTGTAGTGACCCTTGGC-3'
<i>cyp51A</i> and its promoter	Set 1	PA5	5'-TCTCTGCACGCAAGAAGAAC-3'
		PA7	5'-TCATATGTTGCTCAGCGG-3'
	Set 2	AF306F	5'-CACTGCAACTCTAATCCTCG-3'
		AF855R	5'-TAACGCAGACTGAGTCAAGC-3'
	Set 3	AF766F	5'-TTCGGATCGGACGTGGTGTGA-3'
		AF1330R	5'-CGCTGATGACGAAGACGAA-3'
	Set 4	AF1179F	5'-TGACGGTGACAAGGACTCTC-3'
		AF1709R	5'-ACAACCTCGTCTCTCTCTG-3'
	Set 5	AF1426F	5'-AGTCTTCTCCGCTCCAGTA-3'
		AF2025R	5'-ACACCTATTCCGATCACACC-3'

and to have groups with a comparable number of isolates between the different environments. For clinical isolates, especially those from CF patients, sensitivity to azoles was assayed using Etest strip technique and confirmed by EUCAST method for only one colony growing on medium with azole from sputum until 2018. For environmental analysis, sensitivity of all strains growing on medium with azole were assayed with EUCAST method and we inoculated the samples in parallel on azole free media (DG18). Due to the large number of *A. fumigatus* isolated on DG18, we selected only one isolate for every 10 growing on media to test their sensitivity. Isolates growing on medium with azoles but with EUCAST MIC > 2 µg/ml were stored as susceptible strains.

The majority of environmental resistant ARAf isolates came from market gardens ($n=43$), sawmills ($n=24$), and air of hospital ($n=12$). For sawmills and market gardens, we chose ASAf to match the location of ARAf isolates for each professional field (preferably within the same sample when possible, or in the same field or building for sawmills or, if not possible, on the same farm). In total, we analyzed a total of 63 isolates from market gardens (43 ARAf and 20 ASAf) and 65 from sawmills (24 ARAf and 42 ASAf).

With respect to isolates from hospital air sampling, as the detection of ARAf did not always coincide with an increase in the total number of *A. fumigatus* in hospital corridors, we selected 45 ASAf in the following way: ASAf isolated from a contamination peak in June 2017, localized in one hospitalization ward (after a heating system malfunction), with a selection of 24 ASAf in six rooms and bathrooms of the department. ASAf were isolated during a general contamination peak in December 2018 (68 colony forming units/m³ of air) in the general corridors of the hospital where we selected 12 ASAf. Six ASAf were isolated in June 2018, on the same day as a TR₃₄/L98H ARAf and three ASAf were isolated the following week.

In total, we analyzed 112 ASAf coming from 5 clinical and 107 environmental samples and 113 ARAf coming from 29 clinical and 84 environmental samples (Figure 1).

Genotyping Method

Microsatellite genotyping by short tandem repeats for *A. fumigatus* (STRAf) assay (de Valk et al., 2005) was performed to assess the genetic relatedness between 225 susceptible and resistant *A. fumigatus*.

We modified the method developed by de Valk et al. (2005) by changing the fluorochrome TET (tetrachlorofluorescein) to NED (2,7',8'-benzo-5'-fluoro-2',4,7-trichloro-5-carboxyfluorescein). Diluted PCR products were combined with GeneScan™ 400 HD ROX™ size standard (ThermoFisher, Waltham, MA, USA) for fragment detection by capillary electrophoresis (Applied Biosystems 3130 Genetic Analyzer device, ThermoFisher, Waltham, MA, USA).

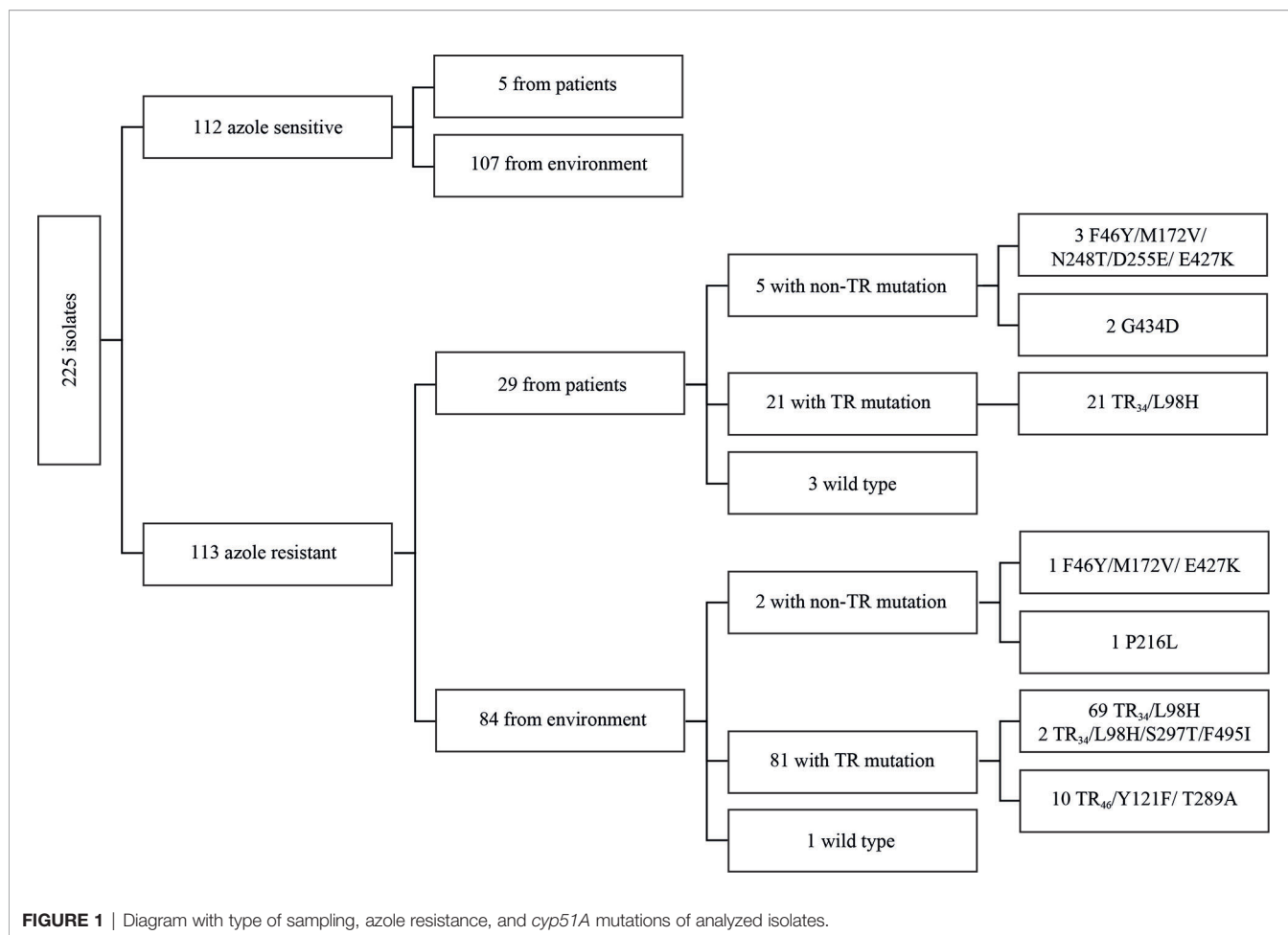
Peaks were visualized with Microsatellite analysis application on ThermoFisher cloud dashboard (<https://apps.thermofisher.com/apps/spa/#/dashboard>). For each marker, the correct peak was selected and transformed into a repeat number based on the Af293 STRAf genotype. The number of repeats was inferred from the available genome for this strain by an automated script using R Studio software.

Data Analysis

Microsatellite genotype distances were calculated using Bruvo's distance (Bruvo et al., 2004). The analyses were done in three steps. Firstly, all susceptible and resistant isolates from the mycology team of Besançon were used. Genotypic richness was assessed by the total number of unique multilocus genotypes (MLGs) or expected MLGs (eMLGs), which approximates the number of genotypes that would be expected at the largest, shared sample size based on rarefaction. Diversity (Simpson's, Shannon-Wiener, or Stoddart and Taylor's Indexes) and evenness were also calculated for each type of isolate (azole susceptible and resistant) with the R package poppr (Kamvar et al., 2015).

Secondly, only local TR₃₄/L98H isolates were analyzed in order to see the proximity according to their origin. Thirdly, we compared our local TR₃₄/L98H isolates to those with available data worldwide. For this, we used a global dataset housed within the online AfumID application (<https://afumid.shinyapps.io/afumID/>) (Sewell et al., 2019). We thus included a further 194 isolates that had previously been isolated and genotyped from 16 countries. In order to ensure harmonization, the number of alleles was recalibrated between the two databases using the reference strain Af293.

Data were represented by circular genetic tree build by a Neighbor-Joining method clustering from a matrix of bruvo distance (Bruvo et al., 2004) using ape library (Paradis and



Schliep, 2019) and interactive tree of life (iTOL) software (<https://itol.embl.de/>) and by minimum spanning networks, calculated *via* the bruvo.msn function on the poppr library (Kamvar et al., 2015).

RESULTS

Cyp51A Gene Mutations

Among the 113 ARAf that were isolated, 90 isolates contained the TR₃₄/L98 *cyp51A* allele, 2 TR₃₄/L98/S297T/F495I, 10 TR₄₆/Y121F/T289A, and 7 non-TR allele (F46Y/M172V/N248T/D255E/E427K, F46Y/M172V/E427K, P216L, or G434D). Four ARAf (1E010, 1E011, and 1E081 from clinical samples and 1E085 from an environmental sample, **Figure 2** and **Supplementary Table data**) did not contain any polymorphisms in the *cyp51A* gene.

Genotyping Results: Besançon Local Scale

The total number of MLGs was higher in the sensitive group than in the resistant group with 102 MLGs found in the 112 ASAf isolates and 53 MLGs observed among the 113 barcoded ARAf.

When comparing the two most abundant groups (ASAf and TR₃₄/L98H ARAf), it was apparent that genotypic richness, represented by the total number of MLG or expected MLG (eMLG), was lower in the TR₃₄/L98H ARAf group (**Table 2**). Regardless of the index used (Simpson's, Shannon–Wiener, or Stoddart and Taylor's Indexes), the genotype diversity was also lower in the TR₃₄/L98H ARAf group. The evenness measure (**Table 2**) indicates that the MLGs observed in the ASAf population are closer to equal abundance (which would be equal to 1) than those in the TR₃₄/L98H ARAf population. Indeed, when we look at the distribution of MLGs for each group, there are many more unique ASAf profiles than ARAf profiles.

When one MLG was shared by isolates of ASAf, it was only shared by two isolates and from a similar source (clinical or environmental). Only one MLG was found both in a clinical isolate (1E005, CF patient June 2017) and in an environmental isolate (2E042, hospital air June 2018).

One MLG was shared by sensitive and resistant *A. fumigatus* isolated from one CF patient: one susceptible (1E007) and two resistant (2E022 and 1E008) isolates. These three strains had F46Y/M172V/N248T/D255E/E427K polymorphism in *cyp51A*.

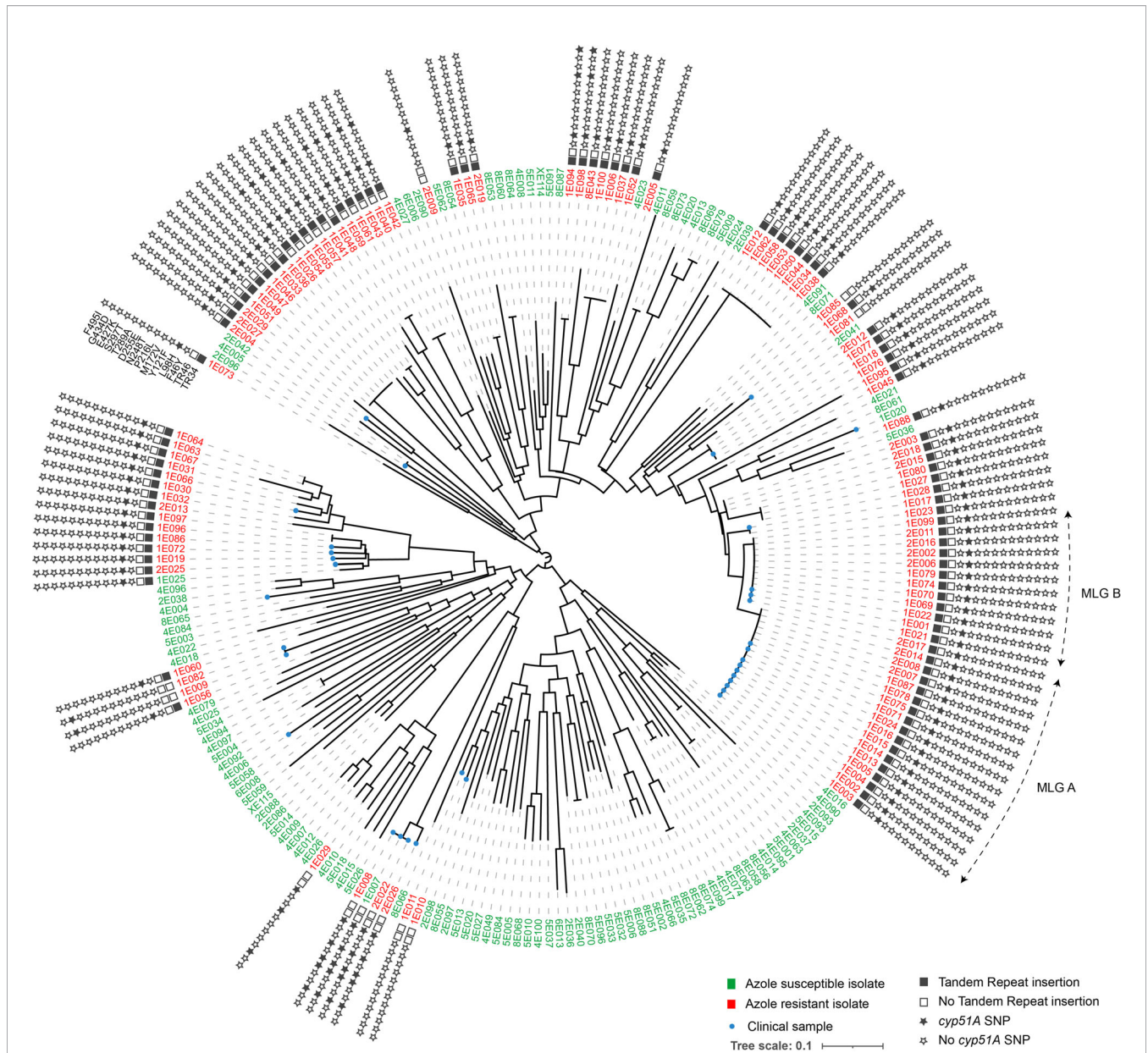


FIGURE 2 | Circular dendrogram illustrating relationship among 113 azole-resistant (with details of tandem repeat and *cyp51A* SNP) and 112 azole-susceptible *Aspergillus fumigatus*, isolated from clinical or environmental samples. Genetic relationships are based on short tandem repeat at nine loci, using Bruvo’s distance and Neighbor-Joining clustering. The two most common multilocus genotypes (MLG) are denoted by the letters A and B. Numbers are ID isolates. The stars provide information on the TR or SNP present on the *cyp51A* gene.

TABLE 2 | Genotypic richness, diversity, and evenness information for azole-susceptible and azole-resistant TR₃₄/L98H isolates.

Population	N	MLG	eMLG	λ	H	G	E.5
Azole-susceptible isolates	112	102	9.93	0.989	4.59	95.03	0.960
Azole-resistant TR ₃₄ /L98H isolates	90	37	7.80	0.923	3.07	12.98	0.583

N, Number of individuals observed; MLG, Number of multilocus genotypes observed; eMLG, The number of expected MLG at the smallest sample size based on rarefaction; λ, Simpson’s Index; H, Shannon–Wiener Index; G, Stoddart and Taylor’s Index; E.5, Evenness measure.

TABLE 3 | Multilocus genotypes (MLG) shared by isolates with TR₃₄/L98H allele.

MLG	Origin of sample	Type of patient or environment	Date of isolation	ID	
MLG A (17 isolates)	Clinic	Cystic fibrosis patient	09/09/2015 ^a	1E002	
			13/12/2016 ^a	1E003	
			10/01/2017 ^b	1E014	
				1E015	
			12/01/2017	1E016	
			17/01/2017	1E004	
			11/07/2017	1E013	
			12/07/2017	1E005	
			14/09/2017 ^a	1E071	
			10/11/2017 ^a	1E075	
	Environment	Hospital (air)	11/06/2015	1E024	
			17/11/2017	1E078	
			10/04/2018	1E087	
			Sawmill (soil)	24/11/2014	2E007
				16/02/2015	2E017
				09/02/2016	2E008
			06/04/2016	2E014	
MLG B (12 isolates)	Clinic	Hematology	27/04/2012	1E021	
			11/05/2012	1E022	
			04/10/2016 ^a	1E001	
	Environment	Hospital (air)	17/10/2017	1E074	
			21/11/2017	1E079	
			24/11/2014	2E006	
		Sawmill (soil)	14/01/2016 ^c	1E099	
				2E002	
			31/03/2016	2E016	
			21/04/2016	2E011	
			Market garden (soil)	10/03/2017 ^c	1E069
					1E070
MLG C (8 isolates)	Environment	Market garden (soil)	07/03/2017 ^d	1E012	
				1E034	
				1E038	
				1E044	
				1E050	
				1E053	
				1E058	
				1E062	
MLG D (6 isolates)	Environment	Market garden (soil)	07/03/2017 ^d	1E033	
				1E036	
				1E046	
				1E047	
				1E049	
				1E051	
MLG E (4 isolates)	Environment	Sawmill (soil)	14/01/2016	1E100	
			07/03/2017 ^c	1E006	
		Market garden (soil)		1E037	
				1E052	

Date of isolation: Day/Month/Year.

^aSame cystic fibrosis chronically colonized APBA patient.

^bTwo different cystic fibrosis patients.

^cIn the same sawmill or market garden but different soil samples.

^dSame market garden, in six different soil samples coming from two different sites found 1.5 km apart.

For ARAf, five MLGs corresponding to TR₃₄/L98H allele were shared by several isolates (Table 3). Two main MLGs (A and B in Figure 2), differing only by a single repeat at the STRAf 3A loci and corresponding to 17 and 12 isolates, respectively, were shared by isolates recovered from both clinical and environmental samples. In addition to being isolated both in the clinic and in the environment, these isolates were also widely found in different environments across our region and also from different categories of patients or date of sampling (Figure 3 and Table 3).

Two highly dissimilar MLGs of eight and six isolates (MLG C and D, Figure 3) were also found in the same market garden, in six different soil samples coming from two different sites where difenoconazole was used to treat vegetable fields. These fields were found 1.5 km apart. In this market garden, we also recovered 14 different MLGs, with one MLG (MLG E, Figure 3) shared with one sawmill 40 km away (all green MLG in Figure 3, except MLG B and 1E073 MLG, which were found in another market garden).

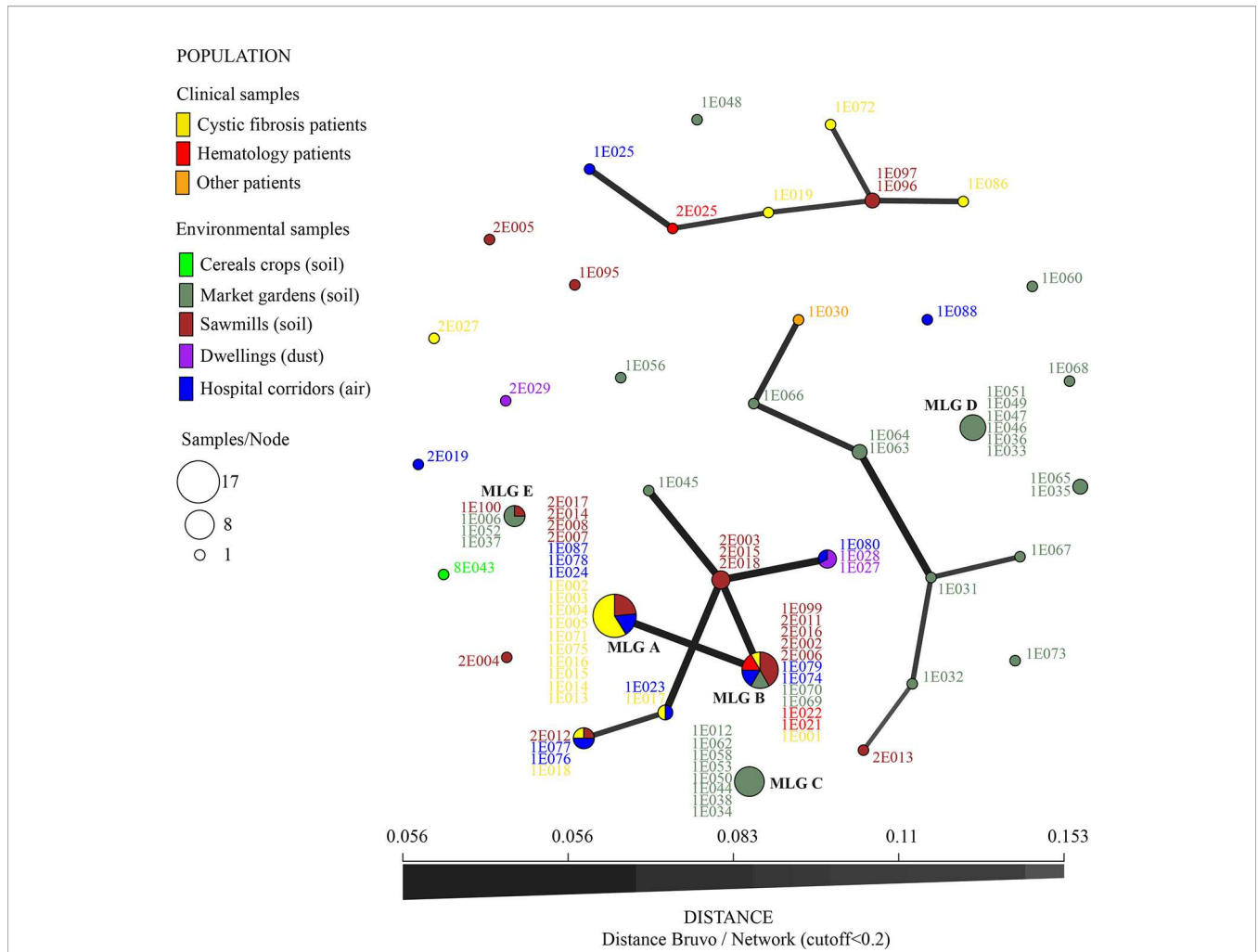


FIGURE 3 | Minimum spanning network for TR34/L98H isolates (nine microsatellite loci STRaf using Bruvo’s distance, with distance cutoff < 0.2). Colors represent the origin of sampling and numbers are ID isolates. Link thickness is proportional to genotype similarity. The most common multilocus genotypes (MLG) are denoted by the letters A, B, C, D, and E.

Genotyping Results for TR₃₄/L98H Isolates: Global Scale

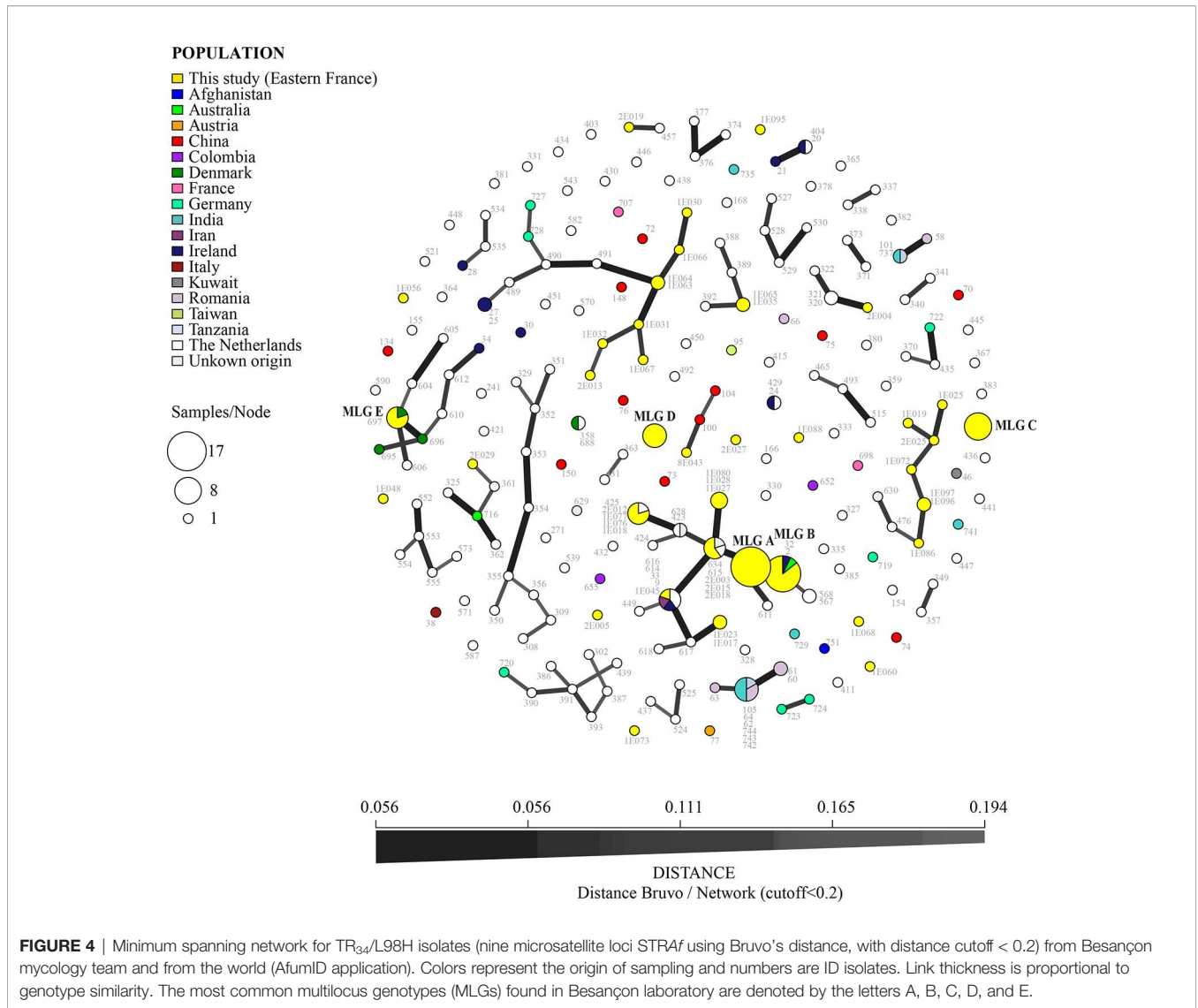
A subset of the dominant MLGs in the Franche-Comté area were commonly shared with isolates found worldwide: MLG B (found in CF patients, hospital air, sawmills, and market gardens) was also detected in Australia and Ireland and MLG E (detected in market gardens and sawmills) was also isolated in Denmark (Figure 4). In comparison, MLG A, C, and D, while common in our dataset, were not observed in the global dataset, suggesting that they are geographically spatially constrained.

Other MLGs found in Eastern France were also closely related to those occurring more widely including those found in the Netherlands (links between the MLG on the Figure 4). One MLG was shared by four isolates in Besançon (a sawmill, twice in hospital air and once in a CF patient) and one isolate in the Netherlands. Another MLG is common with Ireland and Iran isolates and one is common with the Netherlands and another

country (not known in the AfumID application). Nine MLG corresponding to nine isolates (five from market gardens, two from sawmills, one from hospital air, and one from CF patient) appear to be completely different (no link in Figure 4 with fixed cutoff) and were only found in the Besançon collection of isolates

DISCUSSION

In our study, we describe linked patient sampling, healthcare bioaerosol monitoring, and environmental sampling of *A. fumigatus* in order to explore potential links using molecular epidemiology. STRaf genotyping that we undertook demonstrated spatial heterogeneity, with some clones occurring far more widely than others. These data highlight the importance of incorporating spatial scale towards understanding of the local population genetic structure of *A. fumigatus* and the epidemiology ARAf.



Currently, the modes of dissemination, the occurrence of resistant genotypes in the environment, and the likelihood of exposure to at-risk patients remain poorly known. When applied locally, genotyping methods can shed light into the source of infection with various studies showing possible nosocomial acquisition of *A. fumigatus* and potentially ARAf (van der Torre et al., 2021). Three studies have suggested that dissemination from patient to patient occurs, as well as from patient to the healthcare environment (Pegues et al., 2002; Lemaire et al., 2018; Engel et al., 2019). For animals, the dynamics of resistant animal populations in livestock in relation to the environment (except for poultry) are poorly described and are unknown in wildlife. In both cases, it is likely that exposure to ARAf genotypes is higher for individuals in environment that are frequently exposed to fungicides and/or also to organic matter (leaf litter, compost, and cultivated soils rich in organic matter). Clearly, studies to assess the extent that resistant genotypes are stratified by region and environment will lend

nuanced understanding to the ecological processes that are thought to generate ARAf.

In this study, as in others, we observe a reduced genetic diversity of TR₃₄/L98H isolates (the most common resistance mechanism) compared to susceptible strains, suggesting a more recent emergence of this genotype (Camps et al., 2012). This allele is suggested to have emerged around 1997, perhaps in the Netherlands and dispersed across Europe, although other regions of origin are possible (Snelders et al., 2012; Abdolrasouli et al., 2015; Bader et al., 2015; Garcia-Rubio et al., 2018). Currently, TR₃₄/L98H is increasingly described in other global settings and the occurrence of TR₃₄/L98H genotypes does not seem to be limited to Europe. Aerial dispersal is most likely a major contributor to the long-distance migration of airborne *A. fumigatus* conidia owing to the widespread detection of aerosolized *A. fumigatus* spores (Shelton et al., 2020), which can also be moved in traded products (Dunne et al., 2017).

Indeed, identical genotypes were described in several centers and countries, sometimes separated by thousands of kilometres (Chowdhary et al., 2012; Sewell et al., 2019). In 2012, a study of environmental isolates from Iran reported a TR₃₄/L98H MLG closely related to Dutch and Indian isolates (Badali et al., 2013). A recent Japanese publication described TR₃₄/L98H isolates clustering with strains from the Netherlands and France (Tsuchido et al., 2019).

In our study, one of the two dominant MLGs (MLG B) isolated in different environments in the Besançon region and isolated from different patients over several years was identical to an MLG in Australia and Ireland. Owing to the highly diverse nature of the STRAf barcoding loci, the likelihood by these MLGs being identical by chance rather than by descent is vanishingly unlikely (Sewell et al., 2019). Conversely, we have also found MLGs that have so far not been found in other countries. This is particularly the case for MLG A, which has high frequency in varied environments (sawmills and hospital air) as well as in different patients over several years. In Taiwan, a team has observed a potential global spread of TR₃₄/L98H isolates but also a local genetic clustering of some clinical and environmental TR₃₄/L98H (Wang et al., 2018). Other works have also reported local clusters, as in China with clinical and environmental TR₃₄/L98H/S297T/F495I isolates (Deng et al., 2017; Chen et al., 2018). The two isolates carrying this mutation found in the French sawmill do not have the same STRAf profiles as those described in these works. In Germany, local factors have been evoked as the reason for the increased prevalence of specific genotypes (Bader et al., 2015). Resistance could therefore also result from the appearance of different clones located in different countries or regions when there is sufficient selection pressure. This could explain the greater diversity of genotypes found in vegetable crops in the market gardens, for example.

In our study, we have shown that ARAf occurs in the air of hospital and, in this case, could be acquired by patients during episodes of immunosuppression. In our hospital, resistant isolates were found in the general corridors, not in the hematology departments, thus reducing the risk of acquiring invasive aspergillosis caused by a resistant genotype. However, we described MLGs found in CF patients and in environments such as hospital air, and also with hospital visits coinciding with the isolation of certain resistant genotypes; this is the case, for example, for MLGs A and B. However, the wide distribution of these MLGs, both locally for MLG A (different environments) and globally for MLG B (different environments and in other countries), prevents us from conclusively asserting nosocomial acquisition of these isolates in CF patients. This is because, on a probabilistic basis, there is a non-negligible likelihood that these dominant genotypes may have been acquired through exposures outside of the hospital. However, a single MLG found only in Besançon was isolated from hospital air (1E023) and from sputum of a CF patient (1E017) 1.5 months apart. Even if we do not have the analytical power to definitively prove this, the potential for nosocomial contamination of the healthcare environment cannot currently be excluded.

Microsatellite genotyping is widely used in eukaryotes. In *A. fumigatus*, it has been used to identify the potential origins of resistant genotypes, and how genotypes are shared between patients, geographical regions, countries, and continents

(Korfanty et al., 2019). Genotyping analyses with the nine-microsatellite-based method in *A. fumigatus* (de Valk et al., 2005) provide high discriminatory power and reproducibility (Klaassen and Osharov, 2007). For our local isolate comparisons, we can be sure that MLG A and B of the Besançon region isolates are different, as they were analyzed with the same methodology (PCR, fragment analysis, and copy number transformation). However, when we compare the profiles of isolates analyzed in different laboratories, how can we be sure that two isolates with only one microsatellite difference, for example, are indeed different or that the difference is due to interlaboratory variations? MLG A (only local isolates) and MLG B (local and found in Australia and Ireland) only differ by one repeat on a microsatellite, STRAf 3A, which is the loci showing the greatest diversity, as has already been shown for agricultural strains (Ashu et al., 2018). It is therefore important to secure or find ways to harmonize methods of analysis on a large scale so that results can be reliably compared, and ensure in our case that it is indeed the MLG A that is local and that the MLG B has a global distribution. Interlaboratory controls should also be considered with, in particular, the implementation of standardization of sizing data by using allelic ladders (de Valk et al., 2009). Moreover, microsatellite genotyping does not provide sufficiently fine-grained information on the degree of proximity of two strains with two MLG, for example, whereas whole genome sequencing (WGS) can (Hagiwara et al., 2014; Rhodes et al., 2021). High-throughput WGS techniques are now poised to surpass other typing methods because of the high resolution of the data generated and the decreasing costs as performance increases. Better genetic knowledge of resistant strains through genomic approaches will help define the population structure of *A. fumigatus* and inform the future evolutionary trajectory of resistant phenotypes.

While WGS techniques have a higher analysis resolution than other genotyping techniques, the STRAf method, coupled with mutation screening and even mating type characterization, appears to be a good compromise to rapidly and simply screen relatively large numbers of isolates that have resulted from surveillance programs (van der Torre et al., 2021). Then, as in our case, where a “super clone” emerges from the analysis, it would be judicious to use WGS analysis to refine analyses in order to further explore patient–environmental links. Going forward, there is a need to continue to study the environmental conditions (both in host and in the environment) that facilitate the development, selection, and spread of resistance genotypes with large-scale multidisciplinary collaborations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization: SR and MF. Molecular analysis: SR, CG, and AL. Data analysis, SR, TS, and BV. Writing—original draft

preparation: SR. Writing, review, and editing: SR, MF, TS, CG, BV, and LM. Funding acquisition: SR and LM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2021.729476/full#supplementary-material>

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6. Discussion et Conclusion générale

Le bilan des résultats pour les différents domaines étudiés, cultures céréalières, maraîchères ou plantes ornementales, est présenté dans le Tableau 13.

Concernant les études des communautés ciblées sur *A. fumigatus*, le type de cultures du domaine étudié (différentes céréales, fruits, légumes, fleurs...) et les quantités de molécules triazolées appliquées (nombre de traitement annuel ; concentration appliquée lors des traitements) peuvent influencer la microbiologie des sols. Ces conclusions expliqueraient les différences observées en termes de nombre d'*A. fumigatus* détectés. Les sols des cultures maraîchères étaient en effet plus de 4 fois plus riches en isolats d'*A. fumigatus* que les sols de cultures céréalières. A noter que certains milieux de culture utilisés en microbiologie pour favoriser la reproduction sexuée des moisissures sont particulièrement riches en légumes (le milieu V8 notamment), ce qui laisse à penser que certaines cultures en place dans les exploitations maraîchères, pourraient potentiellement favoriser le développement d'*A. fumigatus* (Vanbreuseghem *et al.*, 1978). Des analyses des communautés par qPCR ont par ailleurs montré que les sols issus des pots de fleurs contenaient en moyenne 5000 fois plus d'ADN d'*A. fumigatus* que les sols des cultures céréalières. Le terreau utilisé pour ces plantations semble donc être un terrain particulièrement propice au développement de ces espèces fongiques.

L'évaluation de la contamination des sols en triazolés a montré que les sols des cultures céréalières contenaient tous des résidus de tébuconazole avec une médiane située à 7,05 ng.g⁻¹. Les parcelles qui n'avaient pas été traitées l'année des prélèvements et les parcelles en agriculture biologique présentaient, elles aussi, des résidus détectables, signe de la grande persistance de ces molécules et d'une potentielle diffusion provenant des parcelles voisines. En comparaison, 88% des sols étudiés issus des cultures maraîchères contenaient quant à eux du difénoconazole mais dans des valeurs allant jusqu'à 414 ng.g⁻¹, ce qui semble dépendant de la formulation et/ou molécule appliquée et du nombre de traitements (plus important chez les maraîchers).

Concernant la présence des *A. fumigatus* résistants aux triazolés, elle semble être fonction de l'échelle considérée et des quantités et de la nature des molécules triazolées appliquées. Les fleurs dans les massifs de plantes ornementales constituent, parmi les systèmes étudiés dans ce travail de thèse, la plus importante niche de souches résistantes. Cet environnement est considéré, en comparaison des différents autres domaines, comme un « hotspot » de résistance, ce qui est cohérent avec les observations issues de la littérature (Doughty *et al.*, 2021). La

provenance des bulbes et les traitements qu'ils ont reçus avant leur exportation, paraissent déterminants pour expliquer la présence de résistance dans les pots de fleurs, puisque ces bulbes sont la source de diffusion de ces isolats dans les sols et plus globalement dans l'environnement. Dans cette étude les bulbes provenaient des Pays-Bas, où la résistance aux triazolés chez *A. fumigatus* dans l'environnement est largement décrite, ces bulbes de fleurs ont d'ailleurs déjà été décrit comme pouvant être une source de cette diffusion de résistance (Dunne *et al.*, 2017). Pour appuyer cette observation, le remplacement l'année N+1, des bulbes de fleurs traités aux triazolés, par des bulbes de fleurs issus de l'agriculture biologique, sans changement de sol, a permis de considérablement diminuer le niveau de résistance observé dans ces pots (passage de 71% de souches résistantes dans les sols ayant contenu des bulbes traités aux triazolés à 3% de souches résistantes dans ces mêmes sols contenant des bulbes issus de l'agriculture biologique), signe que ces bulbes semblent être la source directe de cette résistance.

Une différence en termes de proportion d'isolats résistants a également été observée entre les cultures céréalières (0% d'aR-Af) et les cultures maraichères (6% d'aR-Af). Cet écart pourrait en partie être expliqué par les réglementations en termes de traitements, les cultures maraichères peuvent être traitées jusqu'à 4 fois par an avec des formulations à base de triazolés d'après l'Anses, là où le nombre de traitement sur les cultures céréalière est limité à 2 par an. Cet écart, ainsi que l'influence du type de culture sur la présence des isolats fongiques dans les sols peuvent en partie expliquer les différences observées en termes de résistance entre les cultures maraichères et céréalières. Ce constat souligne l'importance de l'effet dose concernant l'impact non intentionnel des résidus de fongicides dans les sols sur *A. fumigatus*, conséquence de l'émergence de résistance. La présence d'isolats d'*A. fumigatus* résistants a d'ailleurs déjà été décrite dans les champs agricoles en Asie (Chen *et al.*, 2020, Cao *et al.*, 2021). Cependant les normes applicables aux conditions de traitement sont différentes en fonction des pays et ce phénomène n'a jamais été décrit à cette échelle dans des pays européens.

Concernant les causes de la résistance, les analyses effectuées sur les isolats résistants retrouvés dans les cultures maraichères et dans les pots de fleurs ont montré que la mutation TR₃₄/L98H sur le gène *Cyp51A*, le mécanisme de résistance le plus décrit dans les isolats environnementaux (Sharma *et al.*, 2015), est le plus largement retrouvé dans les isolats issus des sols étudiés (90,74%). Une autre mutation déjà décrite dans l'environnement (Chowdhary *et al.*, 2013), TR₄₆/Y121F/T289A, a également été retrouvée (8,33% des isolats résistants) dans les cultures maraichères de notre région. L'isolat résistant restant (0,93%) ne présentait aucune mutation dans le gène *Cyp51A*. D'autres mécanismes, comme la surexpression de pompes d'efflux par exemple, peuvent être la source de cette résistance (Meneau *et al.*, 2016).

Etude	Types de prélèvements	Nombre de prélèvements	Surface de culture (ha)	Nature du sol	Molécule appliquée	Sols présentant des résidus de triazolés	Résidus triazolés (ng.g ⁻¹)	Isolats section <i>Fumigati</i>			
								DG18	Mitra	Mvori	Isolats résistants
Grandes cultures	Surface (<5 cm)	220	1,5-22,6 \bar{x} = 9,6	Principalement argile limoneuse	Tébuconazole Metconazole Prothioconazole	22/22 testés (100%)	5,5-19	106	37	23	0
Maraichers	Surface (<5 cm) Et tarière	180	3-12 \bar{x} = 5,7	Principalement sableuse	Difénoconazole	23/26 testés (88%)	4-414	580	30	163	44 (6%)
Plantes ornementales	Surface (<5cm)	85	/	Terreau	Bulbes traités aux azolés	/	/	/	45	45	64 (71%)

Tableau 13 : Bilan des résultats observés dans les différents domaines étudiés.

La contamination des sols par les triazolés, notamment sur les cultures en pleine terre, implique des dommages écosystémiques au-delà de l'impact que l'on peut observer sur les populations d'*A. fumigatus*. Leur diffusion dans les sols, l'eau et l'air entraîne un risque d'exposition pour d'autres organismes non cibles, des microorganismes du sol notamment mais également des micromammifères, des oiseaux voire l'Homme (Souders *et al.*, 2019 ; Béranger *et al.*, 2018).

Trouver des moyens pour limiter la diffusion de ces molécules dans l'environnement est nécessaire. Dans les domaines où l'utilisation des (tri)azolés est considérée indispensable pour maintenir des rendements de production, le développement systématique de procédés de production sans ces molécules n'est pas aisé. Concernant la résistance chez *A. fumigatus*, éviter le contact permanent entre les souches fongiques et les (tri)azolés dans les sols constitue le meilleur moyen de limiter la pression de sélection.

L'utilisation des cyclodextrines pourrait être particulièrement intéressante dans cette problématique. Des tests *in vitro* et des méthodes de modélisation moléculaire ont été utilisés pour confirmer qu'une inclusion potentielle des (tri)azolés au sein de ces molécules cages était possible. Ce complexe d'inclusion bloque l'action fongistatique de la molécule (tri)azolée, probablement en empêchant son import dans la cellule fongique. De fait, le risque de pression de sélection est diminué. Les utilisations environnementales de ces molécules cages se développent progressivement, notamment dans des démarches de décontamination des eaux, mais elles sont plus rares pour limiter les transferts de contaminants (dont les pesticides) dans les sols, contrairement à d'autres matériaux adsorbants tels que les charbons actifs (Landy *et al.*, 2012 ; Yehya *et al.*, 2017 ; Cova *et al.*, 2021). D'un point de vue pratique, leur utilisation afin de limiter le transfert des fongicides vers les sols pourrait être envisagée en les greffant sur un tapis adsorbant dans lequel elles seraient piégées. Cette « barrière » sur laquelle elles seraient immobilisées pourrait être un matériau de type feutrine de chanvre. En effet, des tests *in vitro* sur le chanvre modifié ont montré que les capacités d'adsorption de ces feutrines étaient de l'ordre de 74% de rétention d'une solution de difénoconazole contre 33% pour le chanvre brut (Godeau *et al.*, 2021).

L'application de ces feutrines adsorbantes « vertes » serait envisageable dans certains domaines où la présence à la fois de résidus de triazolés et de souches d'*A. fumigatus* résistantes est avérée. Ils pourraient ainsi être par exemple envisagés dans les scieries, pour limiter l'écoulement des solutions de traitement à base de triazolés vers les sols, durant l'étape d'égouttage des planches de bois après trempage dans les cuves (Jeanvoine *et al.*, 2017).

Pour conclure, le développement de résistance aux triazolés chez *A.fumigatus* est un problème global qui nécessite que l'on comprenne le fonctionnement de cette moisissure au-delà de la sphère clinique. De nombreuses études démontrent le fonctionnement de cette moisissure dans les poumons, et son adaptation à l'hôte dans les cas d'infection (Osherov, 2012 ; Gago *et al.*, 2018 ; Kirkland *et al.*, 2021 ; Fuller *et al.*, 2022). Mais les données disponibles sur l'écologie même de cette espèce sont relativement faibles. Trop peu de données sont disponibles, hors cas cliniques, concernant l'écologie et l'habitat des pathogènes fongiques (*A. fumigatus* et mucorales notamment) alors que les résultats présentés ici permettent de conclure qu'il existe des différences en termes de population fongique selon les types de culture notamment. Développer des études scientifiques dans le but de mieux comprendre quelles sont les conditions et processus qui favorisent le développement de ces populations fongiques dans les sols est indispensable afin de mieux comprendre, et donc de mieux faire face à l'émergence de ces résistances.

En ce qui concerne la contamination environnementale aux triazolés, l'emploi de feutres de chanvre associées à une utilisation raisonnée de ces molécules peuvent permettre en partie d'éviter l'apparition d'un problème de santé publique. Bloquer la diffusion des fongicides triazolés dans les sols est une approche prometteuse pour limiter la pression de sélection qu'ils induisent sur les souches fongiques.

7. Perspectives

Etude des populations d'*A. fumigatus* en fonction des conditions environnementales :

Concernant les isolats d'*A. fumigatus* retrouvés dans ce travail de thèse, qu'ils soient résistants ou sensibles, un séquençage des gènes connus comme étant impliqués dans la résistance (*Cyp51A* ; *Cyp51B* ; *HapE* ; *AtrI* ...) pourrait permettre de détecter des mutations. D'éventuelles mutations qui ne permettent pas forcément de développer de la résistance à elles seules pourraient être identifiées et mises en lien avec l'émergence de la résistance *via* la reproduction de cette espèce par exemple.

Un génotypage par microsatellites sur l'ensemble des isolats résistants pourrait potentiellement permettre d'identifier des clusters, et d'évaluer la diffusion de ces derniers à différentes échelles, à l'intérieur d'une zone, entre les différentes zones, à l'échelle nationale et mondiale (Rocchi *et al.*, 2021). Faire des liens entre les populations résistantes d'*A. fumigatus* est une nécessité pour mieux comprendre leur diffusion, particulièrement dans un contexte où la proximité génétique entre les souches résistantes retrouvées dans l'environnement et celles responsables d'infection chez les patients est avérée (Rhodes *et al.*, 2022).

Afin de mieux comprendre l'écologie des populations d'*A. fumigatus* dans les sols, un suivi des communautés fongiques sur les mêmes parcelles dans le temps semble indispensable, parallèlement au suivi des traitements appliqués. Des analyses de métagénomique sur les sols, pourraient permettre de faciliter ces observations (Sota *et al.*, 2014). Bien que cette technique ne permette pas actuellement d'avoir la précision nécessaire pour identifier les communautés au niveau de l'espèce, elle donne une vue d'ensemble des populations présentes et pourrait permettre d'établir un suivi selon les conditions environnementales qui s'appliquent (Sumbula *et al.*, 2022).

Etude de l'inclusion entre les cyclodextrines et les molécules triazolées :

Afin de confirmer l'inclusion entre les cyclodextrines et les molécules triazolées mise en évidence par modélisation moléculaire, des analyses de spectroscopie de résonance magnétique nucléaire (RMN) doivent être réalisées. A partir de cette technique, les constantes d'associations des différentes molécules pourraient être déterminées, ce qui permettrait d'évaluer l'impact de la structure géométrique de chaque molécule sur la capacité d'inclusion (Zhao *et al.*, 2016). Les données spectroscopiques sur les (tri)azolés sont rares dans la

bibliographie. Une technique particulière pourrait être la RMN DOSY (*Diffusion Ordered Spectroscopy*). Cette séquence RMN est actuellement très utilisée pour l'étude de la formation des complexes d'inclusion des cyclodextrines vis-à-vis de molécules pharmaceutiques et alimentaires, permettant la détermination de la constante d'association des complexes d'inclusion des molécules ayant des variations de déplacement chimique très faibles (Ferrazza *et al.*, 2014 ; Chatzigiannis *et al.*, 2021). Même dans le cas d'interaction faible entre la cyclodextrine et la molécule invitée (ce qui pourrait être le cas pour les (tri)azolés), cette technique serait un outil de choix pour déterminer la constante d'association d'un complexe.

Ainsi il apparaît nécessaire de poursuivre les travaux de recherche dans ce domaine en se focalisant sur différents aspects et lever certains verrous :

- ✓ **SUIVRE** les mutations éventuelles des gènes connus comme impliqués dans des mécanismes de résistance

Sur les souches d'*A. fumigatus* sensibles et résistantes
Au moyen de séquençages des gènes décrits comme impliqués dans la résistance

- ✓ **MESURER** la diffusion de souches d'*Aspergillus fumigatus* résistantes à l'échelle locale et globale

Sur les souches résistantes d'*A. fumigatus*
Au moyen d'un génotypage par microsatellites

- ✓ **COMPRENDRE** l'écologie des populations d'*A. fumigatus* en réalisant un suivi sur plusieurs années des mêmes zones de prélèvements

Sur des sols caractérisés dont les données concernant les traitements notamment sont connues
Au moyen d'analyses de métagénomique et de qPCR ciblées

- ✓ **CONFIRMER** l'inclusion des triazolés dans les molécules de cyclodextrines

Sur des complexes cyclodextrines/triazolés
Au moyen de la spectroscopie par résonance magnétique nucléaire

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Annexe : matériel et méthodes

Prélèvements environnementaux

Prélèvements de sols

Dans les cultures

Dix prélèvements de sols ont été réalisés en surface (5 cm de profondeur) dans chaque parcelle. Pour chacune des parcelles, un transect a été réalisé sur toute la longueur. Les prélèvements ont été effectués avec du matériel stérile, et conditionnés dans des contenants adéquats, soit des sachets de prélèvement stériles pour l'analyse chimique (lyophilisation préalable des échantillons) soit des pots stériles pour l'analyse microbiologique sur sol frais.

Dans les pots/parterres de fleurs

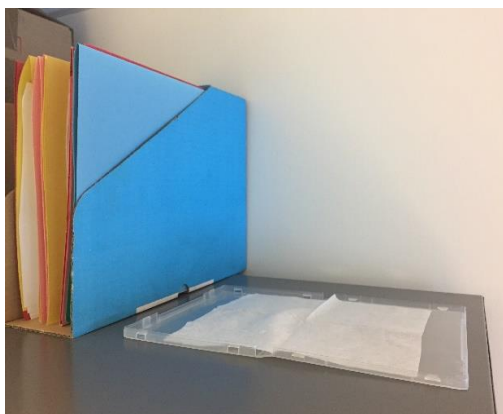
Quatre zones de prélèvements à l'intérieur de l'enceinte de l'hôpital et dans l'environnement proche ont été déterminées et annotées A, B, C et D. Des prélèvements de surface ont été réalisés pour les parterres de fleurs et pour les pots contenant des arbustes. Pour les pots contenant des tulipes, les prélèvements ont été réalisés à environ 5 cm de profondeur à proximité du bulbe. Deux prélèvements par pots ont été réalisés pour ceux contenant des tulipes, un prélèvement unique a été réalisé pour les autres pots contenant les arbustes.

Prélèvements de poussières au CHU de Besançon

Des capteurs électrostatiques de poussières peuvent être utilisés pour évaluer la nature des communautés fongiques présentes dans un environnement intérieur (Scherer *et al.*, 2014). Ce procédé repose sur l'utilisation de lingettes stériles disposées à l'intérieur d'un boîtier qui vont collecter de façon passive les poussières (Figure 33 A). Ces capteurs ont été déposés dans le service de réanimation médicale, tous les mois en 2019.

Cette technique a également été adaptée à l'environnement extérieur, en plaçant 2 de ces lingettes stériles sur un dispositif mobile (Figure 33 B).

(A)



(B)



Figure 33 : Photos des dispositifs de prélèvement de poussières (A) girouette placée sur le toit ; (B) capteur à poussière.

L'une des lingettes a été analysée de façon hebdomadaire ; une analyse mensuelle a été réalisée sur la deuxième.

Techniques d'analyses des sols

Mesure du pH et de la matière organique du sol

La mesure du pH se fait sur un sol lyophilisé puis tamisé à 2 millimètres. L'équivalent de 10 mL de sol a été mesuré dans une éprouvette graduée puis placé sous agitation dans 50 mL d'eau ultrapure pendant 20 minutes. Le mélange sol/eau ultrapure a reposé pendant 1 nuit avant que le pH ne soit mesuré grâce à un pH-mètre.

La mesure de la matière organique se fait ici par calcination, sur un sol lyophilisé puis tamisé à 2 millimètres. Dix grammes de sol ont été placés au four pendant 4 heures à 550°C. La masse du sol incinéré nous permet d'évaluer le pourcentage de matière organique selon la formule :

$$\%MO = \frac{\text{poids du sol sec} - \text{poids du sol incinéré}}{\text{poids du sol sec}} \times 100$$

Détermination de la structure du sol par granulométrie

La mesure de la taille des particules présentes dans le sol a été réalisée par granulométrie selon une technique couverte par l'ISO13320. Cette analyse a été réalisée au moyen d'un granulomètre laser, LS320 Beckman Coulter qui comprend 132 détecteurs, dont la gamme d'analyse s'étend de 0,04 à 2000 μm . L'analyse a été réalisée sur un sol séché et tamisé à 2 mm. L'échantillon a ensuite été mis en présence d'un défloculant, 10 mL d'une solution de sodium d'hexametaphosphate entre 5 et 10 g.L^{-1} avant d'être placé dans un bain à ultrasons pendant 3 minutes. La mesure a été faite après un temps de repos minimum de 2 heures ; une fraction représentative de l'échantillon a été placée dans la cellule de mesure de l'appareil. La séquence d'analyse automatisée a suivi un cycle en 5 étapes :

- Mesure du bruit de fond électronique pendant 60 secondes
- Alignement des détecteurs
- Mesure du bruit de fond pendant 60 secondes
- Analyse pendant 90 secondes
- Rinçage du circuit

Chaque particule dévie le faisceau laser de 750 nm présent dans l'appareil selon son volume donc selon sa dimension. Les résultats de diffraction du faisceau obtenu ont été transformés en utilisant un modèle optique et mathématique suivant la théorie globale de Mie (de Boer *et al.*, 1987 ; Blott *et al.*, 2004) permettant de déterminer une distribution des particules de l'échantillon en fonction du volume. Ces proportions ont ensuite servi à déterminer le triangle de texture de chaque échantillon.

Des échantillons étalons ont permis de confirmer la fiabilité des mesures :

- Coulter LS Control GB (500 μm)
- Coulter Latron G15 (15 μM)
- Coulter Latron 300LS (300 nm)
- Multimodal Particule Size Standars (Thermo scientific): 81 nm, 203 nm et 508 nm

Technique d'extraction et de dosage des triazolés

Extraction des triazolés et analyse quantitative par GC-MS/MS

La mesure se fait sur 10 g de sol lyophilisé et tamisé à 2 mm. Une extraction QuEChERS a été réalisée sur les échantillons : le sol sec a été remis en suspension dans 5 mL d'eau ultrapure avec 3 g de chlorure de sodium. Il a ensuite été vortexé pendant 1 minute avant ajout de 20 mL d'acétonitrile. Puis le mélange a été vortexé une nouvelle fois 3 minutes avant séparation des phases par décantation.

L'extrait a été concentré au moyen d'un évaporateur rotatif (BUTCHI®). Une fois l'évaporation du solvant terminée le volume obtenu a été ajusté à 1 mL au moyen d'une fiole jaugée. L'échantillon est ensuite placé dans un vial filtrant dans le but d'éliminer les résidus de sel. Un étalon interne (constitué de tébuconazole D6) est ajouté avant la mesure.

Les échantillons ont été analysés par GC-MS/MS (Agilent, Massy, France) composé d'un système GC 7890A, d'un spectromètre de masse triple quadripôle 7000 GC et d'un passeur d'échantillons CombiPAL GC 80. Un volume correspondant à 1 µL de l'échantillon a été injecté dans l'appareil à une température de 300°C sous un flux constant d'hélium (pureté 99,9999%) à 1,5 mL.min⁻¹ suivi d'un flux de purge après 0,5 minute. La colonne utilisée était une colonne HP5MS (5% phényl)-méthylpolysiloxane (30 m × 0,25 mm de diamètre interne avec une épaisseur de film de 0,25 µm, Agilent 19091J-433). La durée totale de la mesure était de 42,27 minutes. Les températures de la ligne de transfert, de la source d'ions et des quadripôles étaient respectivement de 300, 300 et 150°C. Une source d'ions à impact électronique à 70 eV était nécessaire. Les différents paramètres de mesure des triazolés en GC-MS/MS sont présentés dans le Tableau 14.

Tableau 14 : Paramètres optimisés de la mesure des triazoles étudiés en GC-MS/MS

	Ion précurseur	Ion produit	Energie de collision (en V)	Temps de rétention (en min)	Limite de quantification (en ng.g⁻¹)
Propiconazole	173	146	18	26,361 ; 27,066	9,0
	173	109	30		
Tébuconazole	125	99	20	27,353	2,7
	125	89	20		
Epoconazole	192	138	12	26,648 ; 27,918	2,5
	192	111	32		

Bromuconazole	173	145	26	28,312 ; 29,016	7,6
	173	109	30		
Difénoconazole	265	202	36	34,366 ; 34,485	13,8
	265	139	40		

Le dosage du metconazole et du prothioconazole qui n'avait pas été développé en interne au laboratoire a été sous-traité au groupe EUROFINS.

Minéralisation et dosage des métaux par ICP-AES

Les concentrations en éléments totaux dans les sols ont été déterminées par spectrométrie d'émission atomique à plasma à couplage inductif (ICP-AES, Radial ICAP 6500 Model, Thermo Fisher Scientific, Courtaboeuf, France) via la minéralisation de 0,5 g de sol lyophilisé puis tamisé à 2 mm avec 2 mL d'acide nitrique et 5 mL d'acide chlorhydrique dans des tubes fermés placés dans un système de digestion en bloc (DigiPREP, SCP Sciences, Courtaboeuf, France). Un mode de chauffage progressif a été utilisé pour atteindre une température finale de 100°C (durée totale de 265 min). Ensuite, de l'eau ultrapure (système Millipore Milli-Q Integral 3, Molsheim, France) a été ajoutée pour obtenir un volume final de 50 mL. Enfin, une filtration à 1 µm a été effectuée. La liste des éléments qui ont été mesurés ainsi que les limites de détection et de quantification qui correspondent à chacun d'eux sont présentées dans le Tableau 15.

Tableau 15 : Liste des éléments dosés en ICP-AES et limites de détection et de quantification correspondantes.

Elément dosable	Limite de détection (µg.g⁻¹)	Limite de quantification (µg.g⁻¹)
Aluminium	0,53	1,78
Arsenic	0,38	1,27
Bore	0,12	0,39
Calcium	0,45	1,50
Cadmium	0,07	0,23
Cobalt	0,08	0,26
Chrome	0,10	0,34
Cuivre	0,44	1,47
Fer	0,10	0,33
Mercure	0,16	0,52
Potassium	1,51	5,03
Magnésium	1,31	4,36
Manganèse	0,02	0,06
Sodium	0,50	1,66
Nickel	0,11	0,36
Phosphore	0,68	2,27
Plomb	0,42	1,38

Soufre	2,30	7,66
Antimoine	0,55	1,85
Sélénium	0,68	2,28
Silicium	0,44	1,45
Etain	0,28	0,92
Strontium	0,01	0,02
Titane	0,05	0,17
Zinc	0,02	0,07

Techniques de microbiologie

Mise en culture des Aspergillus fumigatus

Pour les prélèvements de terre, 2 grammes de chaque prélèvement ont été re-suspendus dans 10 mL d'une solution de Tween 80 à 0,1%. Le mélange a été agité pendant 1 minute puis 100 µL ont étéensemencés sur différents milieux de culture :

- Milieu Dichloran-Glycérol (DG18) comme milieu de référence ;
- Milieux malt supplémentés des deux antifongiques médicaux itraconazole et voriconazole : Mitra (Malt supplémenté en itraconazole à 2 mg.L⁻¹) et Mvori (Malt supplémenté en voriconazole à 1 mg.L⁻¹) ;

Les boites ont ensuite été placées à 48°C pendant 48 heures, température sélectionnant préférentiellement les *A. fumigatus* ou bien à 37°C afin de cibler les espèces susceptibles d'être pathogènes pour l'Homme.

Les lingettes des différents capteurs à poussière ont été rincées à l'aide d'une solution de Tween 80 à 0,1% (Merck, Darmstadt, Germany), agitées 10 minutes dans un stomacher (MIX2 AES laboratoire, France). Les liquides de rinçage ont été récupérés, puis 100 µL ont étéensemencés sur milieu Dichloran-Glycérol (DG18) incubé ensuite à 25°C, ainsi que sur milieu DG18, Mitra et Mvori incubés à 37°C et 48°C.

Après la durée nécessaire à la pousse fongique, une identification est effectuée au moyen d'observations macroscopiques (couleur, taille, aspect des colonies) et microscopiques afin de distinguer les isolats d'intérêt.

Test de sensibilité par méthode EUCAST

La technique EUCAST (European Committee on Antimicrobial Susceptibility Testing) est la technique de référence pour mesurer la sensibilité des souches fongiques à un antifongique (Guinea *et al.*, 2022). Elle fournit la valeur de la concentration minimale inhibitrice (CMI).

Un milieu RPMI 1640 (Sigma Aldrich, Saint Louis, USA) liquide a été réalisé puis stérilisé avant utilisation. Les souches de section *Fumigati* à tester ont été repiquées puis placées à l'étuve durant 3 jours à 37°C. Des solutions de spores ont été réalisées dans du Tween 80 à 0,1% et calibrées à l'aide d'un densitomètre (Densimat Biomérieux) à 0,5 McFarland.

Les antifongiques testés, itraconazole et voriconazole (resuspendus dans du DMSO), ont été préparés en suivant une dilution de raison 2 afin d'obtenir une gamme de concentration de 16 mg.L⁻¹ pour le premier puits à une concentration de 0,03125 mg.L⁻¹ pour le dernier puits couvrant en tout 10 points de gamme.

Chaque souche a été testée en parallèle de 2 souches de référence, l'une sensible (souche CBS 10/355) et l'autre résistante (souche n°70041901) dont la CMI est connue pour les antifongiques testés, avec des CMI inférieures ou égales à 1 mg.L⁻¹ pour chaque antifongique testé pour la souche sensible et des CMI supérieures à 2 mg.L⁻¹ pour la souche résistante. La technique est faite en plaque 96 puits. Cent microlitres de chaque concentration des différents antifongiques ont été placés dans les puits 1 à 10, puis sont inoculés par 100 µL des solutions de spores calibrées comme indiqué précédemment. Pour la validation de l'expérience un témoin de pousse qui ne contient pas de solution d'antifongique et un témoin négatif qui ne contient que le milieu RPMI et de l'eau ont été réalisés (Figure 34). Leur validation permet de confirmer les bonnes conditions de l'expérience. La lecture des plaques a été faite visuellement 24 heures après le dépôt par 2 lecteurs différents.

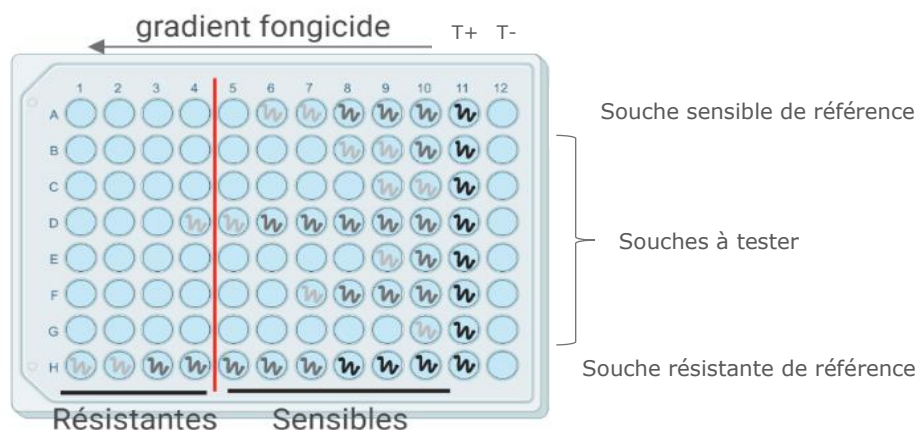


Figure 34 : Schéma d'une plaque EUCAST. Réalisé avec Biorender

Les souches testées sont ensuite réparties en 2 groupes selon des seuils déterminés par l'EUCAST (EUCAST, 2020) :

- Souches sensibles qui ont une CMI inférieure ou égale à 1 mg.L⁻¹ ;
- Souches résistantes avec des CMI strictement supérieures à 1 mg.L⁻¹ ;

Afin de limiter le risque de ne pas détecter la résistance, les isolats présentant des CMI supérieures à 1 mg.L⁻¹ sont considérés comme résistants, mais une CMI égale à 2 mg.L⁻¹ est considérée comme appartenant au domaine d'incertitude technique, ce qui nécessite une vérification en biologie moléculaire.

Techniques de biologie moléculaire

Extraction d'ADN

Les ADN d'une sélection de souches déterminées comme étant résistantes au test de sensibilité ont été extraits grâce à un kit DNeasy[®] plant mini kit (Qiagen[®]). L'ADN a ensuite été dosé au moyen d'un NanoDrop[™].

*Identification génétique d'*Aspergillus fumigatus**

Des amorces codant pour le gène de la *βtubuline* (*βt2a* et *βt2b*) (Glass & Donaldson, 1995) ont été utilisées. La PCR a été réalisée dans un thermocycleur (C1000 Touch, Biorad) selon le programme suivant : une étape de dénaturation initiale à 95°C pendant 2 minutes, puis 35 cycles chacun comprenant 4 étapes : une dénaturation de 15 secondes à 94°C, une hybridation à 61°C durant 15 secondes puis une étape d'élongation à 72°C durant 10 secondes, suivie d'une dernière étape à 72°C pendant 5 minutes.

Les produits de PCR ont ensuite été révélés sur un gel d'agarose à 2% puis purifiés avec le kit NucleoSpin[™] Gel and PCR clean up (Macherey-Nagel[®], Düren, Germany). Le séquençage a été sous-traité à la plateforme Microsynth AG, Balgash, Switzerland. Les séquences obtenues ont été comparées avec des séquences de référence de la base de données GenBank grâce au logiciel Blastn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

*Analyse du gène *cyp51A* et de son promoteur*

Comme les mutations sur le gène *cyp51A* seraient impliquées à 90% dans le développement de résistances aux antifongiques chez *A. fumigatus*, une amplification et un séquençage du gène et de son promoteur ont été réalisés. Pour permettre de couvrir la quasi-totalité du gène d'intérêt, un total de 5 couples d'amorces a été utilisé (Mellado *et al.*, 2001 ; Alanio *et al.*, 2011). Le programme de PCR est similaire à celui de la PCR *β-tubuline* précédemment décrit à

l'exception de la température d'hybridation des amorces qui est de 55°C. Les séquences ainsi obtenues ont été comparées à deux séquences de référence du gène *cyp51A* au moyen du logiciel Benchling (<https://benchling.com/>). Les souches de référence choisies sont des *A. fumigatus* sensibles aux antifongiques (souche n° AF 222068.1, GeneBank, pour la mutation TR₃₄/L98H et la souche n° AF 3386659, GeneBank, pour la mutation TR46/Y21F/T289A).

Détection des communautés fongiques par qPCR

La détection par qPCR a été faite sur les sols des grandes cultures et des pots de plantes ornementales, après extraction d'ADN des sols avec le kit Nucleospin soil (Macherey-Nagel®, Düren, Germany).

Les systèmes qPCR (amorces et sondes d'hydrolyse TaqMan) utilisés sont ceux décrits par Haugland *et al.* (2004) pour quantifier *Aspergillus fumigatus* et Liu *et al.* (2012) pour la quantité globale d'espèce fongiques.

La qPCR a été réalisée sur les échantillons et sur une gamme de plasmides pour la cible qPCR *A. fumigatus* ou d'ADN pour la cible qPCR panfongique. Ces gammes, allant de 100 à 0,01 fg d'ADN ont servi de témoins positifs et à la réalisation d'une courbe standard nécessaire à la quantification de l'ADN.

Chaque plaque a été validée grâce à un contrôle négatif de l'extraction et un contrôle négatif de la réaction de qPCR constitué d'eau pour biologie moléculaire (EBM). La réaction de qPCR s'est faite en plaque 96 puits, dans un volume final de 20 µL comprenant 10 µL de Master Mix taqman Gene Expression, 4 µL du mélange des amorces concentrées à 5 pM chacune, 0,8 µL de sondes pour chaque échantillon, 0,2 µL d'eau, et 5 µL de chaque échantillon. L'analyse a été faite sur un automate Applied Biosystem 7500 Fast, avec le programme suivant : 10 minutes à 95°C, 50 cycles composés de 15 secondes à 95°C et une minute à 60°C.

Une séquence nucléotidique, nommée aléa, longue de 167 pb (Knapp *et al.*, 2016) a été testée comme contrôle interne en parallèle des autres cibles pour vérifier l'absence d'inhibiteur de PCR dans chaque échantillon.

Analyse des communautés par génotypage

L'analyse a été réalisée grâce à un génotypage par microsatellite STRAf (short tandem repeat for *A. fumigatus*) (de Valk *et al.*, 2005) dans le but d'évaluer la parenté génétique entre 65 isolats d'*Aspergillus fumigatus* résistants ou sensibles isolés dans les cultures maraîchères de l'Est de la France.

Le protocole proposé par de Valk *et al.* 2005 a été suivi à l'exception de l'utilisation du fluorochrome TET (tetrachlorofluorescein) remplacé par un fluorochrome NED (2.7', 8'-benzo-5'-fluoro-2',4,7-tricloro-5-carboxyfluorescein). Un étalon de taille GeneScan™ 400 HD ROX™ (ThermoFisher, Waltham, MA, USA) a été combiné avec les produits de PCR dilués obtenus afin de détecter les fragments par électrophorèse capillaire (Applied Biosystems 3130 Genetic Analyzer, ThermoFisher, Waltham, MA, USA).

Les pics obtenus au cours de l'analyse ont été visualisés avec l'application d'analyse des microsatellites de ThermoFisher (<https://apps.thermofisher.com/apps/spa/#/dashboard>).

Pour chacun des marqueurs utilisés, le pic correct a été sélectionné et séparé des artefacts afin d'être traduit en nombre de répétitions sur la base de l'analyse STRAf d'Af293 et du nombre de répétitions déduit du génome disponible pour cette souche par un script automatisé utilisant le logiciel R Studio.

Illustrations et analyses statistiques

Les figures présentes dans ce manuscrit ont été en grande partie réalisées via le site biorender : <https://biorender.com/>.

Le logiciel RStudio (version 3.3.0, Boston, MA) a été utilisé pour construire le triangle de texture grâce aux fonctions du package *soiltexture* (Moeys *et al.*, 2018), ainsi que les diagrammes en violon grâce au package *vioplot* (Adler *et al.*, 2021). Les analyses statistiques et leurs représentations ont été réalisées grâce au même logiciel, les packages *FactoMineR*, *vegan* et *ade4* ont été utilisés pour l'analyse multivariée (Dray *et al.*, 2022 ; Husson *et al.*, 2022 ; Oksanen *et al.*, 2022).

Annexe : données complémentaires

Les valeurs obtenues pour le dosage des 25 éléments par ICP-AES pour les sols des grandes cultures prélevées en Lorraine et en Bourgogne sont présentées respectivement dans les Tableaux 16 et 17.

		Lorraine												
		Al	As	B	Ca	Cd	Co	Cr	Cu	Fe	Hg	K	Mg	
VSP		22190,1	18,9	14,5	3052,0	<LDM	19,0	38,9	14,3	24139,9	< LDM	3863,9	3472,0	
EP		20643,2	15,9	17,0	2982,4	<LQM	16,0	36,8	15,1	22881,3	< LDM	4000,9	3297,3	
TP		38374,2	18,9	26,7	7216,2	<LDM	18,6	60,2	21,0	34533,2	< LDM	5474,6	5412,3	
DJP		21998,9	25,1	15,2	3089,3	<LDM	20,7	42,8	17,2	27077,9	< LDM	3787,6	3600,2	
PCP		12555,0	6,8	12,1	1649,8	<LDM	9,3	23,9	11,1	16249,3	< LDM	3615,7	4873,1	
PP		33777,7	11,4	55,9	24285,0	<LDM	16,7	42,4	28,7	27112,0	< LDM	11519,9	21668,7	
GCP		30301,5	<LQM	66,5	85325,1	<LDM	12,1	33,6	23,3	18907,4	1,4	13083,7	58627,6	
RP		25586,7	23,0	15,7	4064,56	<LDM	26,2	39,9	16,8	32962,2	< LDM	4310,4	3592,1	
LIP		9228,8	26,8	3,6	1187,0	<LDM	5,3	18,1	20,0	8492,3	< LDM	1272,7	1139,7	
BP		32152,3	3,9	47,5	38347,0	<LDM	16,8	41,6	40,2	29676,2	0,7	11505,6	27563,8	
CBP		19979,3	8,5	20,9	1827,5	<LDM	12,0	30,0	10,4	18824,7	< LDM	4556,9	3477,9	

Lorraine													
	Mn	Na	Ni	P	Pb	S	Sb	Se	Si	Sn	Sr	Ti	Zn
VSP	1015,6	66,6	35,4	747,5	28,1	337,4	<LQM	<LQM	180,4	1,0	15,7	218,9	51,6
EP	824,6	70,2	30,5	768,4	28,2	368,6	<LQM	<LDM	228,9	1,2	17,3	260,1	54,3
TP	736,4	141,2	46,5	1010,7	35,3	579,9	7,6	<LDM	158,2	<LDM	33,0	238,7	85,2
DJP	1094,9	72,7	33,3	803,5	31,3	344,7	<LQM	<LDM	199,1	1,2	17,1	257,3	57,3
PCP	373,4	95,6	20,5	749,9	17,3	295,6	<LQM	<LQM	224,5	<LQM	9,9	196,1	63,6
PP	920,9	237,6	34,8	1064,8	31,1	1419,3	<LQM	<LDM	201,8	1,8	38,8	274,4	74,9
GCP	753,6	518,1	28,0	1369,5	24,1	1763,7	5,5	<LQM	114,5	<LDM	125,8	136,6	47,8
RP	1614,7	108,6	35,1	1234,2	38,4	415,6	5,4	<LDM	167,4	<LDM	19,8	211,2	55,3
LIP	505,5	27,2	8,2	719,1	15,3	212,6	3,5	<LDM	253,1	<LQM	6,2	106,7	26,2
BP	1067,3	272,1	34,2	805,0	30,1	842,7	6,9	<LQM	137,4	<LDM	28,4	179,8	45,4
CBP	590,0	80,4	20,5	828,6	23,7	308,6	<LQM	<LDM	189,5	1,0	10,1	116,0	31,8

Tableau 16 : Résultats du dosage par ICP-AES (en $\mu\text{g}\cdot\text{g}^{-1}$) de 25 éléments recherchés dans 11 sols de cultures céréalières de Lorraine.

Bourgogne												
	Al	As	B	Ca	Cd	Co	Cr	Cu	Fe	Hg	K	Mg
POP	35198,4	15,6	8,8	7457,8	3,0	14,3	49,5	18,2	29455,4	< LDM	5678,4	3501,2
BGP	27434,4	16,1	3,0	150924,5	1,3	9,1	34,5	10,3	25024,9	< LQM	2587,6	2379,7
CHP	26284,1	8,5	5,4	3979,4	2,8	12,0	38,1	9,7	22147,1	< LDM	3457,0	2454,4
PPF	36868,4	15,6	9,1	14419,5	3,3	13,9	52,2	15,8	28598,2	< LDM	5093,9	3398,4
SFP	36839,9	23,7	6,1	40559,3	1,6	17,6	54,2	15,0	36454,5	< LDM	5815,6	3209,2
MJP	23213,9	9,5	4,7	114120,3	1,2	9,3	31,5	17,2	16292,3	< LDM	3213,8	2465,3
LHP	30355,3	15,2	5,1	29658,5	3,4	17,3	46,2	17,3	30080,7	< LDM	4975,7	2646,5
MOP	22360,3	8,2	3,0	114452,0	0,9	8,6	29,1	13,4	17553,2	< LDM	2553,8	2256,3
MAP	33212,6	13,9	5,2	4592,3	2,8	13,5	47,6	13,7	28930,1	< LDM	5015,0	3117,8
QUP	40959,4	11,8	7,9	39109,9	1,1	13,6	56,7	16,6	31027,7	< LDM	5167,6	3724,1
PIV	24937,0	9,5	1,3	4063,8	1,0	12,4	34,7	9,8	21014,8	< LDM	3317,3	2481,7

Bourgogne													
	Mn	Na	Ni	P	Pb	S	Sb	Se	Si	Sn	Sr	Ti	Zn
POP	896,9	125,3	33,8	1094,4	34,3	429,6	< LQM	< LDM	83,9	1,7	22,8	377,4	79,7
BGP	467,4	178,3	21,5	921,9	25,2	5596,9	< LDM	< LDM	22,2	< LQM	57,1	62,7	49,6
CHP	757,2	84,8	21,9	493,5	28,2	231,6	< LQM	< LDM	36,1	< LQM	15,2	138,2	46,1
PPF	643,5	117,4	33,5	1053,1	34,7	782,9	< LQM	< LDM	52,1	1,2	21,3	250,9	67,6
SFP	1326,2	116,2	40,1	1042,2	35,7	1516,8	< LQM	< LDM	22,7	1,2	41,3	178,9	74,0
MJP	568,3	140,2	19,4	1100,9	22,1	4021,0	< LDM	< LDM	41,5	LQM	40,2	153,7	53,2
LHP	1679,6	96,6	31,8	1048,9	33,4	1098,8	< LQM	< LDM	24,6	1,2	26,4	161,5	63,7
MOP	466,0	128,6	19,2	713,2	28,2	4339,1	< LDM	< LDM	20,5	< LQM	45,3	65,1	49,5
MAP	828,2	99,4	31,5	680,8	32,4	318,7	< LQM	< LDM	34,7	1,5	17,9	193,6	62,9
QUP	708,6	86,6	32,8	690,5	34,7	1495,8	< LQM	< LDM	36,9	< LQM	31,0	102,8	71,2
PIV	779,8	67,8	21,4	574,8	28,2	276,4	< LQM	< LDM	20,1	4,4	15,3	132,2	47,7

Tableau 17 : Résultats du dosage par ICP-AES (en $\mu\text{g}\cdot\text{g}^{-1}$) de 25 éléments recherchés dans 11 sols de cultures céréalières de Bourgogne.

Titre : Evaluation de l'impact de la dissémination des fongicides triazolés en agriculture sur *Aspergillus fumigatus* et proposition de solutions.

Mots clés : triazolés, *Aspergillus fumigatus*, pollution environnementale, résistance aux antifongiques, cyclodextrines, agriculture

Résumé : L'impact des activités anthropiques sur les services écosystémiques est avéré. Les études tournées vers l'environnement dans des approches pluridisciplinaires sont nécessaires pour mieux comprendre et limiter cet impact. L'une des problématiques majeures est le développement de la résistance aux antimicrobiens, dont un des exemples est la résistance d'*Aspergillus fumigatus* aux triazolés. Cette espèce de moisissure ubiquiste est un pathogène opportuniste pour l'Homme et les animaux. Les alternatives thérapeutiques pour combattre cette moisissure sont minces et sont essentiellement basées sur une famille de molécules, les triazolés. Des mécanismes de résistance à ces traitements sont décrits sur tous les continents et sont liés, en partie, à l'utilisation, dans l'environnement, de molécules triazolées pour combattre les moisissures phytopathogènes, dont les structures chimiques sont très proches des triazolés à usage médical. Cet impact, qualifié de non intentionnel, est une source d'émergence et de diffusion de souches fongiques résistantes. Ce travail de recherche vise à comparer différents domaines d'application des molécules triazolées, allant des champs de cultures céréalières aux exploitations maraîchères et aux parterres de plantes ornementales, afin de mieux comprendre les conditions d'émergence et/ou de diffusion de cette résistance. Cette approche multi-échelle a permis de mettre en évidence un gradient de résistance en fonction des domaines étudiés. Ainsi, alors que les sols des cultures céréalières ne présentent aucun isolat résistant, 71% des isolats retrouvés dans les plantes ornementales entourant le centre hospitalier de Besançon se sont avérés résistants aux triazolés, classifiant les bulbes de ces plantes traitées aux triazolés comme des « hotspots » potentiels de résistance. La transition vers l'utilisation de plantes à bulbes issues de l'agriculture biologique est nécessaire pour éviter l'exposition de populations à risque autour de l'hôpital notamment. Dans d'autres types d'agriculture où ces fongicides garantissent de bons rendements de production, des approches innovantes doivent être développées pour limiter l'émergence de ce problème de santé publique. Les cyclodextrines, molécules cages issues de la dégradation de l'amidon, semblent être une piste intéressante.

Title: Evaluation of the impact of the dissemination of triazole fungicides in agriculture on *Aspergillus fumigatus* and proposal of solutions.

Keywords: triazoles, *Aspergillus fumigatus*, environmental pollution, antifungal resistance, cyclodextrins, agriculture

Abstract: The negative impact of human activities on the ecosystem is significant. Environmentally oriented studies in multidisciplinary approaches are needed to better understand and limit this impact. One of the major issues is the development of antimicrobial resistance, of which one example is the resistance of *Aspergillus fumigatus* to triazoles. This ubiquitous mould species is an opportunistic pathogen for humans and animals. The therapeutic alternatives to combat this fungus are few and essentially based on triazoles. However, mechanisms of resistance to these treatments have been described worldwide and are partly linked to the excessive use of triazole molecules in the environment to fight against phytopathogenic moulds, whose chemical structures are very close to those of triazoles in medical use. This impact, described as unintended, is a source of emergence and spread of resistant fungal strains. The aim of this research was to compare different areas of application of triazole molecules, ranging from cereal crop fields to vegetable farms and ornamental plant beds, in order to better understand the conditions in which this resistance develops. This multiscale approach enabled us to highlight a gradient of resistance according to the areas studied. Thus, while the soils of cereal crops did not present any resistant isolates, 71% of the isolates found in the ornamental plants surrounding Besancon hospital centre proved to be resistant to triazoles, classifying the bulbs of these triazole-treated plants as potential resistance "hotspot". Therefore, the transition to the use of organically grown bulbous plants is necessary to avoid exposure of populations at risk, especially around the hospital. While in certain agricultural types where these fungicides guarantee good production yields, innovative approaches must be developed to limit the emergence of this public health problem. Cyclodextrins, cage molecules resulting from the degradation of starch, seem to be an interesting avenue.