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Décryptage des mécanismes impliqués dans le biocontrôle des champignons mycotoxinogènes des céréales

Lucile Pellan

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biotechnologie et Microbiologie

École doctorale GAIA

APAB : Agro-ressources, Procédés, Aliments et Bioproduits

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Décryptage des mécanismes impliqués dans le biocontrôle des champignons mycotoxinogènes des céréales

Présentée par Lucile PELLAN

Le 6 Novembre 2020

Sous la direction de Sabine SCHORR- GALINDO

Devant le jury composé de

Guilhem DESBROSSES, Professeur, Université de Montpellier

Aziz AZIZ, Professeur, Université de Reims Champagne-Ardenne

Jérôme MOUNIER, Professeur, Université de Bretagne Occidentale

Adeline PICOT, Maître de conférences Université de Bretagne Occidentale

Caroline STRUB, Maître de conférences, Université de Montpellier

Sabine SCHORR-GALINDO, Professeur, Université de Montpellier

Président du jury

Rapporteur

Rapporteur

Examinatrice

Co-encadrante de thèse

Directrice de thèse



UNIVERSITÉ
DE MONTPELLIER

« Lorsque les sciences dévoilent les secrets de la nature,
ce que celle-ci perd de mystérieux, elle le gagne en
merveilleux. »

Paul Carvel

RESUME

Décryptage des mécanismes impliqués dans le biocontrôle des champignons mycotoxinogènes des céréales. Les céréales sont le premier facteur d'exposition des consommateurs aux mycotoxines, des métabolites secondaires toxiques synthétisés par certaines moisissures et présentant un risque pour la santé humaine et animale. Les espèces *Fusarium graminearum* et *Fusarium verticillioides* sont ainsi responsables de la production de mycotoxines de type trichothécènes et fumonisines, respectivement, d'un impact élevé surtout en pré-récolte. Avec la diminution de l'utilisation des pesticides en raison de leur toxicité, une stratégie alternative pour lutter contre ces maladies fongiques au champ, peut-être l'utilisation de micro-organismes antagonistes. Non-pathogènes, ces agents de biocontrôle (BCAs) sont capables de limiter la progression des *Fusaria* et leur synthèse de mycotoxines à l'aide de différents mécanismes biologiques cependant encore mal connus. Dans ce contexte, les objectifs de cette étude sont d'évaluer par quels mécanismes trois BCAs commerciaux sélectionnés (*Streptomyces griseoviridis*, *Trichoderma asperellum*, *Pythium oligandrum*) peuvent moduler la croissance et la production de mycotoxines des pathogènes du genre *Fusarium*, *in vitro* et *in situ*. Des activités antagonistes et des niveaux variables d'inhibition de la croissance (jusqu'à 80%) et de la mycotoxinogenèse (jusqu'à 90%) ont été observés *in vitro* selon les BCAs, les pathogènes ou les conditions de culture. Les hypothèses sont ensuite vérifiées et complétées grâce à la mise au point d'une toolbox de tests spécifiques de certains modes d'action. Leur chronologie est évaluée tout au long de l'interaction, de la germination des spores pathogènes jusqu'à la formation de périthèces de conservation, en testant une grande diversité de mécanismes des BCAs : synthèse de composés anti-germinatifs, mycophagie, synthèse d'enzymes chitinolytiques, émission de composés volatils antifongiques, inhibition des voies de biosynthèse des mycotoxines, bio-transformation de mycotoxines, compétition spatiale et nutritionnelle ou impact sur la conservation du pathogène. *S. griseoviridis* présente des capacités de compétition nutritionnelle, mais sa stratégie principale est basée sur l'antibiose, à travers la synthèse de nombreux composés antifongiques qui compense un déficit important en capacité de colonisation spatiale. Cependant, il peut être à l'origine de stimulation de production de mycotoxines. *T. asperellum* est capable d'activer un très large éventail de défenses et d'attaques combinant la synthèse de divers composés antifongiques (métabolite, enzymes, COVs), avec différentes cibles (spores, mycélium, mycotoxines), et une action directe par mycoparasitisme. L'efficacité de *P. oligandrum* est principalement due à sa forte capacité à coloniser l'environnement, avec une action directe *via* la prédation microbienne et la réduction de la formation de périthèces. Ces comportements *in vitro* ont pu également être analysés chez *F. graminearum in situ* tout au long de son cycle de vie (de la colonisation des épis à la survie dans les résidus de culture), et mis en perspectives avec les différents modes d'action identifiés chez chacun des BCAs. L'ensemble du projet a permis d'importantes avancées dans le décryptage des mécanismes d'actions des BCAs, et propose des voies d'amélioration pour optimiser l'utilisation et l'efficacité de ces antagonistes et donc limiter l'utilisation des produits phytopharmaceutiques, tout en maintenant une production saine avec de faibles niveaux de mycotoxines.

Mots clés : Agents de biocontrôle, *Fusarium*, mycotoxines, interactions microbiennes.

ABSTRACT

Deciphering the mechanisms involved in the biocontrol of mycotoxinogenic fungi of cereals. Cereals are the first factor of consumer exposure to mycotoxins, toxic secondary metabolites synthesized by particular moulds and which present a risk for human and animal health. The species *Fusarium graminearum* and *Fusarium verticillioides* are thus responsible for the production of trichothecenes and fumonisin-like mycotoxins, respectively, with a high impact especially in pre-harvest. With the decrease of pesticide use due to their toxicity, an alternative strategy to control these fungal diseases in the field may be the use of antagonistic microorganisms. Non-pathogenic, these biocontrol agents (BCAs) are able to limit the progression of *Fusaria* and their synthesis of mycotoxins through various biological mechanisms, however still poorly understood. In this context, the objectives of this study are to evaluate by which mechanisms three selected BCAs (*Streptomyces griseoviridis*, *Trichoderma asperellum*, *Pythium oligandrum*) can modulate the growth and mycotoxin production of *Fusarium* pathogens, *in vitro* and *in situ*. Antagonistic activities and varying levels of inhibition of growth (up to 80%) and mycotoxinogenesis (up to 90%) have been observed *in vitro* depending on BCAs, pathogens or culture conditions. The hypotheses are then verified and completed thanks to the development of a toolbox of tests specific to certain modes of action. Their chronology is evaluated throughout the interaction, from pathogenic spore germination to the formation of perithecia, by testing a wide variety of BCA mechanisms: synthesis of anti-germinative compounds, mycophagy, chitinolytic enzyme synthesis, emission of volatile antifungal compounds, inhibition of mycotoxin biosynthesis pathways, mycotoxin bio-transformation, spatial and nutritional competition or impact on pathogen conservation. *S. griseoviridis* presents nutritional competition capacities, but its main strategy is based on antibiosis, through the synthesis of numerous antifungal compounds that offsets a significant deficit in spatial colonization capacity. However, it can stimulate the mycotoxin production. *T. asperellum* is capable of activating a very wide range of defenses and attacks combining the synthesis of various antifungal compounds (metabolite, enzymes, VOCs), with different targets (spores, mycelium, mycotoxins), and a direct action by mycoparasitism. The efficacy of *P. oligandrum* is mainly due to its strong capacity to colonize the environment, with a direct action *via* microbial predation and reduction of perithecia formation. These *in vitro* performances could also be analyzed in *F. graminearum in situ* throughout its life cycle (from colonization of ears to survival in culture residues), and put into perspective with the different modes of action identified in each of the BCAs. The whole project has led to important advances in deciphering the mechanisms of action of BCAs and proposes ways of improvement to optimize the use and efficacy of these antagonists. Thus limit the use of chemical plant protection products, while maintaining a healthy production with low levels of mycotoxins.

Key-word : Biocontrol agent, *Fusarium*, mycotoxins, microbial interaction.

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PUBLICATIONS ET COMMUNICATIONS

Articles de recherches publiés ou en cours de publication :

1. Commercial Biocontrol Agents reveal contrasted comportment in the face-to-face against mycotoxinogenic *Fusarium* species.

Lucile Pellan^{1,*}, Noël Durand^{1,2}, Véronique Martinez¹, Angélique Fontana¹, Sabine Schorr-Galindo¹ and Caroline Strub¹

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2. Biocontrol Agents : Weapons against Mycotoxigenic *Fusarium* (Part 1: Early stage)

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Angélique Fontana¹, Caroline Strub^{1,*} and Sabine Schorr-Galindo¹.

Soumis au journal *Environmental Microbiology*, en révision.

3. Biocontrol Agents : Weapons against Mycotoxigenic *Fusarium* (Part 2 : Late stage)

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Noël Durand^{1,2}, Caroline Strub^{1,*} and Sabine Schorr-Galindo¹.

Soumis au journal *Environmental Microbiology*, en révision.

4. *Trichoderma asperellum* and *Pythium oligandrum* can modulate the mycotoxin biosynthesis genes expression in *Fusarium* cereal pathogens during their biocontrol interaction.

Lucile Pellan¹, Myriam Mamouni¹, Caroline Strub^{1,*} and Sabine Schorr-Galindo¹.

Soumis au journal *Biological Control*, en révision.

5. How Biocontrol Agents disturb life cycle interconnections of mycotoxinogenic *Fusarium graminearum* and wheat?

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Noël Durand^{1,2}, Sabine Schorr-Galindo¹ and Caroline Strub^{1,*}

Soumis au journal *Microbiological research*, en révision.

Participation à des congrès nationaux et internationaux (Posters et Communications orales) :

- Journées Mycotoxines à Bordeaux en janvier 2018 (congrès national, co-auteur d'une communication orale ;

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Pellan Lucile¹, Caroline Strub¹, Noël Durand², Angélique Fontana¹, Sabine Schorr- Galindo¹

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Decoding Biological Mechanisms Involved In Biocontrol Of Cereals Mycotoxinogenic Fungi.

Pellan Lucile¹, Caroline Strub¹, Noël Durand^{1,2}, Angélique Fontana¹, Sabine Schorr- Galindo¹

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Decoding Biological Mechanisms Involved In Biocontrol Of Cereals Mycotoxinogenic Fungi.

Pellan Lucile¹, Caroline Strub¹, Noël Durand^{1,2}, Angélique Fontana¹, Sabine Schorr- Galindo¹

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Biocontrol Agents of Mycotoxigenic Fungi: Toolbox for Elucidating Mechanisms of Action.

Pellan Lucile¹, Caroline Strub¹, Noël Durand^{1,2}, Angélique Fontana¹, Sabine Schorr-Galindo¹, Ixchel Campos-Avelar¹

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15-ADON : 15-acétyldéoxynivalénol

3-ADON : 3- acétyldéoxynivalénol

ADN : Acide Désoxyriboucléique

ARN : Acide ribonucléique

AUxC : Aire sous la Courbe

BCA(s) : Agents de Biocontrôle

CYA, CYB : Czapeck Yeast Agar, Czapeck Yeast Broth

D3G : DON-3-O-glucoside

ddPCR : Digital Droplet Polymerase Chain Reaction

DPI : Jours Post-Inoculation

DOM1 : de-époxy-DON

DON : déoxynivalénol

FB₁, FB₂, FB₃ : Fumonisine B1, Fumonisine B2, Fumonisine B3

GC-MS : Chromatographie en phase gazeuse - Spectrométrie de masse

HFB1 : fumonisine B1 hydrolysée

HPI : Heures Post-Inoculation

HPLC-MS-MS : Chromatographie en phase liquide haute performance-spectrométrie de masse en tandem

Myco : *S. griseoviridis* isolé à partir de Mycostop[®]

NIV : nivalénol

PDA, PDB : Potatoes Dextrose Agar, Potatoes Dextrose Broth

Poly : *P. oligandrum* isolé à partir de Polyversum[®]

qPCR : Quantitative Polymerase Chain Reaction

RPM : Rotation par minute

RT : Reverse Transcription

Xeda : *T. asperellum* isolé à partir de Xedavir[®]

Introduction Générale

INTRODUCTION GENERALE

De nos jours, les enjeux concernant la productivité alimentaire sont une préoccupation majeure. Les évolutions démographiques et les modes de consommations exponentielles, associés à des conditions climatiques variables, ont favorisé le développement et la dissémination de nombreux agents pathogènes de plantes, à l'origine de maladies dévastatrices des cultures. Les céréales, un des groupes de plantes d'intérêt alimentaires les plus cultivées au monde, sont fréquemment touchées par ces champignons qui mettent en danger les productions avant et après la récolte. En plus de proliférer et de diminuer la quantité de grains produits, certains de ces pathogènes synthétisent des métabolites secondaires toxiques, les mycotoxines, qui vont affecter la qualité sanitaire des récoltes. Leurs teneurs maximales sont réglementées car elles peuvent être nocives. En effet, après leur entrée dans les chaînes de transformation des produits *via* l'infection au champ, elles persistent dans les produits alimentaires jusqu'à la consommation. Elles peuvent provoquer des symptômes allant de l'intoxication alimentaire avec diarrhée ou vomissement, jusqu'à la perturbation de la reproduction et des systèmes endocriniens, des désordres neurologiques ou la mort. Ces mycotoxines font parties des contaminants naturels de l'alimentation, mais sont difficilement dégradables et très stables pendant les processus de transformation des aliments. Parmi les pathogènes producteurs de mycotoxines contaminant les céréales, *Fusarium graminearum* et *Fusarium verticillioides* vont être capables de produire des trichothécènes (NIV, DON, 15- et 3-ADON) et de la zéaralénone, ou des fumonisines (FB₁, FB₂ et FB₃) et de la beauvericine respectivement. Ils attaquent les cultures céréalières directement lors de la floraison des épis et mettent en péril les productions.

La gestion efficace des phytopathogènes a constitué un grand défi pour les scientifiques et les agriculteurs, et jusqu'à récemment, l'utilisation massive de pesticides chimiques était la solution majeure pour tenter d'éradiquer les pathogènes. Cependant, de plus en plus de ces molécules sont remises en cause pour leurs effets néfastes sur les écosystèmes et la santé des utilisateurs et des consommateurs. De plus, les agents pathogènes à l'origine des maladies des cultures ont réussi peu à peu à contourner l'effet de ces produits et à s'adapter pour maintenir ou augmenter leur niveau de virulence. Les productions agricoles sont donc soumises à un effet ciseaux avec d'une part des conditions climatiques très évolutives qui stimulent le développement microbien et la production de toxines, et d'autre part, une détermination politique de réduire l'utilisation des produits phytosanitaires. Dans ce contexte, de nouvelles voies de lutte ont été explorées et le biocontrôle semble être une alternative à considérer. Le biocontrôle regroupe les moyens de gestion des pathogènes à l'aide de substances naturelles et d'organismes antagonistes. En particulier, les micro-organismes comme les champignons et les bactéries, naturellement présents dans les agrosystèmes, sont capables d'interagir avec les plantes et les pathogènes de culture pour améliorer la protection des plantes.

Non-pathogènes, ces Agents de Contrôle Biologique ou de Biocontrôle (BCAs) vont empêcher ou réduire la progression des pathogènes par différents mécanismes directs ou indirects. Ces mécanismes sont très diversifiés et peuvent être utilisés ensembles ou seuls selon les agents de biocontrôle considérés. Ils peuvent être liés à la compétition pour l'espace ou les nutriments, à la production de composés antimicrobiens, au parasitisme ou à la dégradation de composés toxiques tels que les mycotoxines. De plus, ces mécanismes peuvent être mis en œuvre à différents stades d'évolution du pathogène, amplifiant la complexité de l'interaction microbienne. Malgré l'activité intense de recherche et de mise en application générée autour de ce domaine, des interrogations persistent sur les modes d'actions qui permettent le biocontrôle des *Fusaria* et de leur mycotoxine, mais aussi sur la spécificité, la synergie ou la chronologie de ces mécanismes.

C'est dans ce cadre que s'inscrit mon projet de thèse entrepris ces trois dernières années dans l'UMR QualiSud, à l'Université de Montpellier. L'objectif de ce travail était de décrypter quels sont les mécanismes mis en place par des agents de biocontrôle et leurs conséquences sur des pathogènes du genre *Fusarium*, producteurs de mycotoxines. Avec cet objectif en tête, trois agents de biocontrôle commerciaux contrastés ont été sélectionnés, isolés à partir de leur formulation commerciale, et mis en confrontation avec deux pathogènes mycotoxinogènes d'importance majeure *F. graminearum* et *F. verticillioides*.

- La première étude du projet s'est concentrée sur l'analyse *in vitro* des traits de vie des pathogènes et BCAs seuls ou en confrontation avec l'étude de la cinétique d'interaction des BCAs et des pathogènes dans différentes conditions expérimentales en mesurant l'impact des micro-organismes antagonistes sur la croissance des pathogènes et la production de leur mycotoxines.
- La seconde étude s'est intéressée aux mécanismes à l'origine de ces inhibitions. Une toolbox d'expériences a été mise au point afin de suivre les modes d'actions potentiels des BCAs tout au long du cycle de vie des pathogènes de la germination à la production de forme de conservation, en alliant différentes méthodes relevant de la microbiologie, la biochimie, ou la biologie moléculaire à différentes échelles d'observation. Des profils d'amplitude, de combinaisons, de spécificité et de chronologie des modes d'action dans les différentes interactions ont été établis pour permettre d'identifier les stratégies préférentielles de chacun des BCAs.
- Pour conclure ce projet, dans une troisième étude les capacités de colonisation et d'inhibition de *F. graminearum* par les BCAs ont été analysées *in situ* sur épis de blé, afin de se rapprocher au mieux des conditions naturelles d'interactions, et mis en perspective vis-à-vis des analyses réalisées *in vitro* dans une troisième étude.

Synthèse Bibliographique

1. SYNTHÈSE BIBLIOGRAPHIQUE

Cette première partie est consacrée à la description des concepts et éléments permettant de comprendre pleinement le contexte et les enjeux de cette étude.

1.1. Que sont les mycotoxines ? Comment sont-elles produites ?

1.1.1. Que sont les mycotoxines ?

Certains micro-organismes fongiques, qui attaquent les produits alimentaires au champ ou au cours du stockage, sont capables de produire des métabolites secondaires potentiellement toxiques pour notre santé, les mycotoxines (du grec ancien *mukês* : champignon et *toxikos* : poison).

L'interaction entre l'Homme et les mycotoxines a une longue histoire. Les mycotoxines sont apparues dans les réserves alimentaires humaines à peu près à l'époque où l'humanité a commencé à cultiver, récolter et stocker ses productions d'une saison à l'autre, il y a probablement 10 000 ans. Une des premières apparitions de ces molécules est répertoriée comme étant la cause d'une maladie appelée « le mal des ardents » causée par la consommation de seigle contaminé par des alcaloïdes produits par *Claviceps purpurea*, et qui a provoqué la mort de plusieurs milliers de personnes en Europe au cours du dernier millénaire (Bové, 1970; van der Linde *et al.*, 2016). D'autres importantes crises sanitaires ont pu être reliées à la présence de mycotoxines au cours des derniers siècles, comme le bérubéri cardiaque aigu associé à la consommation de riz contaminé par la citréoviridine, produite par *Penicillium citreonigrum*, au Japon en 1880 ; ou encore l'aleucie toxique alimentaire causée par des trichothécènes produits par *Fusarium sporotrichioides* en Russie dans les années 1940 (Reboux, 2006; Pitt and David Miller, 2016).

Aujourd'hui ces molécules sont définies comme des composés chimiques non-protéiques de faible poids moléculaire (0,3-0,7 kDa), qui sont sécrétés par le métabolisme secondaire de certains champignons pathogènes avant et après le stockage des aliments, et qui présentent des propriétés toxicologiques potentiellement nocives pour les organismes, parfois même à faibles doses (Organisation des Nations Unies pour l'Alimentation et l'Agriculture (JEFCA); Les mycotoxines | Anses - Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail; Mousavi Khaneghah *et al.*, 2019).

1.1.2. Quels sont leurs effets sur la santé ?

Concernant leurs effets néfastes sur la santé des organismes vivants, les mycotoxines présentent quatre modes de toxicité : aiguë, chronique, mutagénique et tératogénique. Effectivement, elles peuvent avoir des effets toxiques immédiats (mycotoxicose aiguë) mais les effets d'une exposition répétée à de faibles doses (exposition chronique) sont les plus redoutés en raison des habitudes alimentaires du consommateur et du pouvoir de rémanence de ces toxines. Étant donné la difficulté

à recueillir des données toxicologiques dans le cadre d'intoxication chronique, le rôle des mycotoxines comme cause de mortalité humaine est probablement sous-estimé (Reboux, 2006). A long terme, elles peuvent entraîner la réduction de prise alimentaire et donc de croissance chez les animaux, des infections gastro-intestinales, des maladies d'obstructions chronique des voies respiratoires, l'apparition de déficience immunitaire, ou de cancer comme le cancer hépatocellulaire (Barac, 2019).

1.1.3. Comment et où sont-elles produites ?

Il existe plusieurs milliers de mycotoxines identifiées, mais celles qui sont les plus souvent observées et qui présentent un danger sanitaire sont les trichothécènes (type A : T2/HT2 et type B : DON/NIV et dérivés acétylés), la zéaralénone, les fumonisines, les aflatoxines, l'ochratoxine A et la patuline (Gruber-Dorninger *et al.*, 2019). De par leur extrême diversité, elles peuvent être classées en fonction de leur structure chimique, des dommages qu'elles causent pour la santé de l'homme ou l'animal ou encore du type de micro-organisme qui les produit.

Les principaux genres de champignons producteurs sont : *Aspergillus*, *Fusarium* et *Penicillium* (Pitt, 2000) auxquels s'ajoutent des genres mineurs (Figure 1). Parmi eux, plusieurs ont montré des capacités à produire plusieurs mycotoxines, voire plusieurs types de mycotoxines, qui peuvent être partagées entre espèces comme l'ochratoxine A (Woloshuk and Shim, 2013).

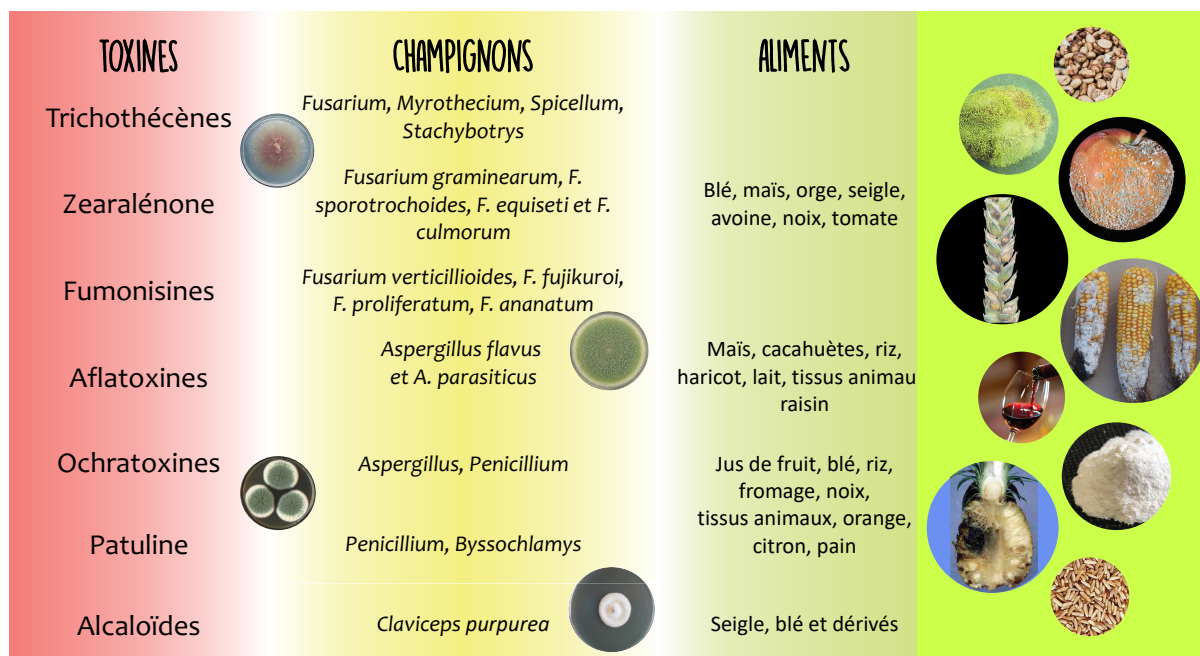


Figure 1 : Principales mycotoxines produites par des pathogènes mycotoxinogènes et aliments contaminés

Les moisissures mycotoxinogènes se développent sur différentes cultures et denrées alimentaires : céréales, oléagineux, épices, fruits, fruits séchés, noix, pommes, grains de café, et ce souvent dans un environnement chaud et humide (Marin *et al.*, 2013). Cette colonisation peut se produire avant ou après la récolte, pendant la conservation, et sur, ou, dans l'aliment lui-même. De plus, la plupart des mycotoxines sont chimiquement stables et résistent au traitement thermique (Bullerman and Bianchini, 2007). A partir des cultures de légumes ou de céréales, elles peuvent être directement absorbées par les organismes lors de l'ingestion des produits bruts ou transformés. Les plantes peuvent également servir à nourrir les animaux qui, à leur tour, pourront contaminer l'homme, par la production de viande ou de lait, que l'aliment soit transformé ou non, ce qui complexifie encore les liens entre les mycotoxines et la chaîne alimentaire.

Grâce à cette impressionnante diversité de producteurs et d'hôtes et leur stabilité, les mycotoxines ont pu se disséminer à travers le monde et n'épargnent aucune zone de la planète. On estime qu'environ 25% des cultures vivrières dans le monde sont contaminées par les mycotoxines (Eskola *et al.*, 2019). Les réseaux dynamiques de dissémination couplées aux évolutions climatiques ont contribué à de nombreuses émergences et réémergences des toxines dans plusieurs zones géographiques. Depuis plusieurs années, des sociétés internationales comme Biomin (qui commercialise des produits à destination de l'élevage animal) Cargill (spécialisé dans la fourniture d'ingrédients alimentaires et dans le négoce de matières premières), ou encore plusieurs comités comme l'Autorité européenne de sécurité des aliments (EFSA) ont entrepris de monter des programmes de surveillance globale pour suivre les contaminations par les mycotoxines sur divers échantillons agro-alimentaires à travers le globe (Figure 2). Parmi ces programmes, le « Survey Biomin Program » semble être le plus représentatif avec une grande quantité d'échantillons, prélevés à travers différents continents et analysés avec la même méthode multi-toxines (LC-MS).

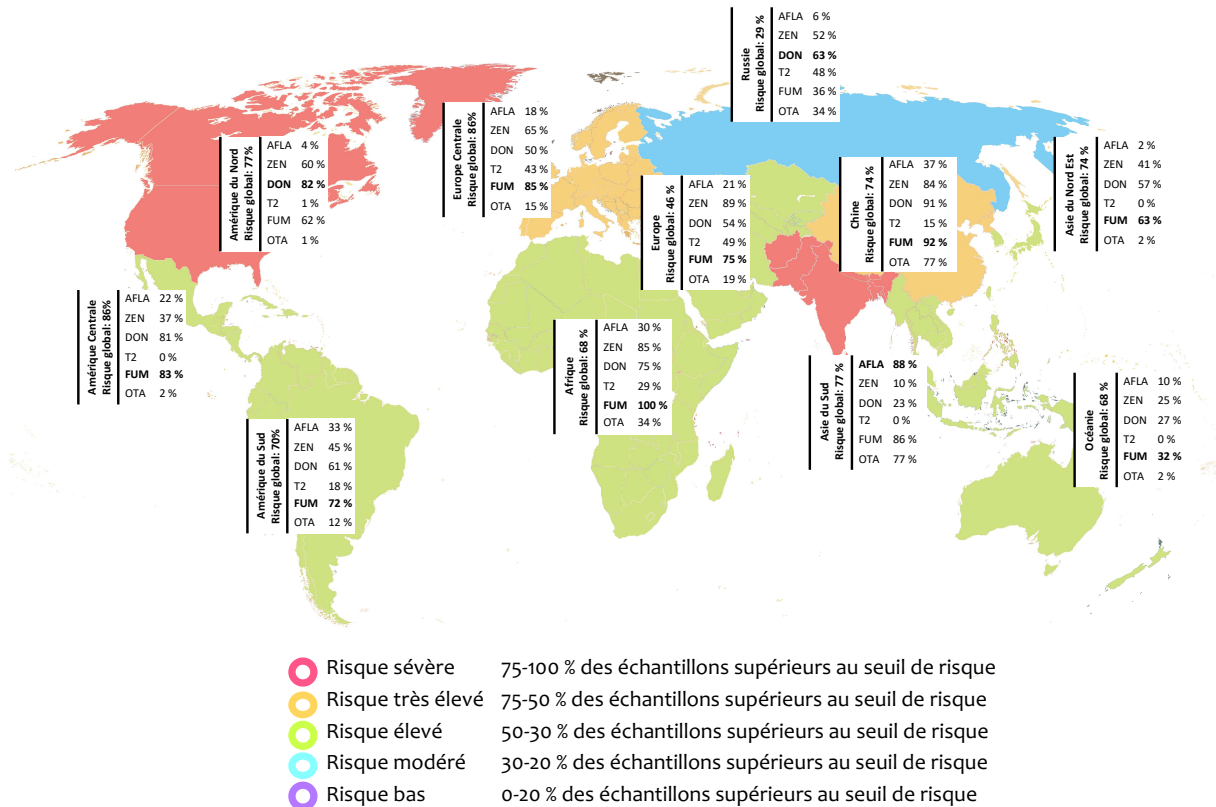


Figure 2: Carte de distribution et cooccurrence des mycotoxines à travers le monde (modifiée à partir des cartes des programmes de surveillance des mycotoxines de Biomin et Cargill 2019, (Mycotoxin Survey report by Cargill | Benison Media; Mycotoxin Survey | biomin.net))

Étant donné leur toxicité, les teneurs en mycotoxines dans les aliments sont règlementées au niveau mondial (Comité mixte FAO-OMS d'experts des additifs alimentaires, Codex Alimentarius) et européen (Règlement n°1881/2006 de la Commission européenne) (Table 1). L'objectif de ces normes est de protéger la santé publique en garantissant la sûreté sanitaire des produits et en maintenant les contaminants à des niveaux toxicologiques acceptables dans divers produits alimentaires plus ou moins exposés à ces contaminants. En plus de fixer les seuils caractéristiques des effets toxiques liés à ces mycotoxines, cette commission européenne de régulation permet d'empêcher la mise sur le marché de produits dépassant les seuils fixés, en tant que tels, après avoir été mélangés à d'autres denrées alimentaires, ou encore utilisés comme ingrédients dans d'autres denrées alimentaires.

Table 1: Niveau maximum autorisés des principales mycotoxines dans les aliments (Eskola *et al.*, 2019)

Mycotoxin	Food crop	Established levels (µg/kg)	
Codex Alimentarius standard			
Aflatoxins	Almonds, Brazil nuts, hazelnuts, peanuts, pistachios for processing	AFB1	Total
	Almonds, Brazil nuts, hazelnuts, pistachios for direct human consumption	—	15
Fumonisin B1 + B2	Unprocessed maize	4000	10
Deoxynivalenol	Cereal grains (wheat, maize and barley) for processing	2000	
Ochratoxin A	Unprocessed wheat, barley, rye	5	
European Union maximum and guidance levels			
Aflatoxins	Groundnuts (peanuts), hazelnut, Brazil nuts, other oilseeds for processing	AFB1	Total
	Almonds, pistachios, apricot kernels for processing	8	15
	Tree nuts, other than the tree nuts above for processing	12	15
	Groundnuts (peanuts), other oilseeds, other three nuts below for direct human consumption	5	10
	Almonds, pistachios, apricot kernels for direct human consumption	2	4
	Hazelnuts and Brazil nuts for direct human consumption	8	10
	All cereals except maize and rice	5	10
	Maize and rice for processing	2	4
	Unprocessed maize	5	10
Fumonisin B1 + B2	Unprocessed maize	4000	
	Maize intended for direct human consumption	1000	
Deoxynivalenol	Unprocessed cereals other than durum wheat, oats, maize	1250	
	Unprocessed durum wheat, oats, maize	1750	
Ochratoxin A	Unprocessed cereals	5	
	Cereals intended for direct human consumption	3	
Zearalenone	Unprocessed cereals other than maize	100	
	Unprocessed maize	350	
	Cereals intended for direct human consumption	75	
	Maize intended for direct human consumption	100	
T-2 + HT-2	<i>Unprocessed barley and maize</i>		200
	<i>Unprocessed oats</i>		1000
	<i>Unprocessed wheat, rye and other cereals</i>		100
	<i>Maize intended for direct human consumption</i>		100
	<i>Oats intended for direct human consumption</i>		200
	<i>Other cereals intended for direct human consumption</i>		50
USA: Action and guidance levels			
Aflatoxin B1	All food crops	20	
Fumonisin FB1 + FB2 + FB3	<i>Maize</i>		4000
Canada: Maximum and guidance levels			
Aflatoxins	Nuts	15	
Deoxynivalenol	<i>Unprocessed soft wheat</i>		2000
Australia: Maximum levels			
Aflatoxins	Peanuts, tree nuts	15	
Japan: Maximum and provisional maximum levels			
Aflatoxin B1	All food crops	10	
Deoxynivalenol	Wheat	1100 ^a	
China: Maximum and guidance levels			
Aflatoxin B1	Maize	20	
	Rice (brown rice)	10	
	Wheat, barley, other cereals	5	
	Peanuts	20	
Deoxynivalenol	<i>Maize, barley, wheat, other cereals</i>		1000
Ochratoxin A	Cereals	5	
	Beans	5	
Zearalenone	<i>Wheat and maize</i>		60

The guidance levels (the term may vary in legislation from country to country) are indicated in italics.

AFB1, aflatoxin B1; Total, total aflatoxins (sum of AFB1, AFB2, AFG1 and AFG2).

^aProvisional maximum level.

1.2. Focus sur un genre particulièrement dommageable : *Fusarium*

Parmi les pathogènes producteurs de mycotoxines, le genre *Fusarium* représente un risque majeur (Andrea He, 2018). Dans le monde en 2019, une étude réalisée sur 22 318 échantillons provenant de cultures de maïs, de blé, de soja, de leurs produits dérivés et de la nourriture pour bétail, la grande majorité des échantillons testés contiennent des mycotoxines de *F. graminearum* et *F. verticillioides* : DON, fumonisines, et zéaralénone sont les mycotoxines les plus répandues et ont été détectées au-dessus du seuil autorisé dans 64%, 60%, and 45% des échantillons. En Europe, le risque est

globalement accru avec 86% des échantillons contaminés avec des fumonisines et 67% avec de la DON (Gruber-Dorninger *et al.*, 2019).

1.2.1. *Fusarium graminearum*

1.2.1.1. Caractéristiques générales

Fusarium graminearum est un champignon ascomycète de la famille des Nectriaceae, qui commence à être isolé et caractérisé à partir des années 1920-1930 (Tu, 1929; Dickson *et al.*, 1930) (Figure 3). *In vitro* et sur milieu nutritif optimal, les colonies se développent rapidement et produisent des quantités importantes de mycélium relativement cotonneux. Le mycélium est d'abord blanc puis présente des nuances jaunes, roses ou orangées. Des sporodochies orange à rouge-brun sont produites assez lentement. Les cultures peuvent produire des pigments jaunes, oranges ou rouges particulièrement sensibles au pH (Cambaza, 2018). Les conidies portées par les sporodochies présentent une forme très caractéristique de « banane » ou fuseau mince et de longueur moyenne (50-100 µm), la surface ventrale est droite et la face dorsale est légèrement arquée (Leslie and Summerell, 2008). *Fusarium graminearum* est l'une des espèces les plus difficiles à conserver à long terme. Les collections de souches doivent être contrôlées avec une certaine régularité pour s'assurer de leur viabilité et aussi de leur toxigenèse.

Ce champignon est homothallic et peut donc former des périthèces sans avoir besoin de croiser différents isolats (Cavinder *et al.*, 2012). Ces structures de conservation se forment lors de conditions défavorables au pathogène (peu de ressources nutritionnelles, stress). Plus complexes, ils contiennent différents types de tissus produits à des stades spécifiques du développement du périthèce. Le périthèce est donc constitué de mycélium initial condensé qui donne naissance aux hyphes ascogènes, d'une paroi externe dense et résistante, de paraphyses (mycélium stérile qui occupe le centre du périthèce jusqu'au développement des asques), et des asques contenant les ascospores de forme plus ovoïdes (Trail and Common, 2000).

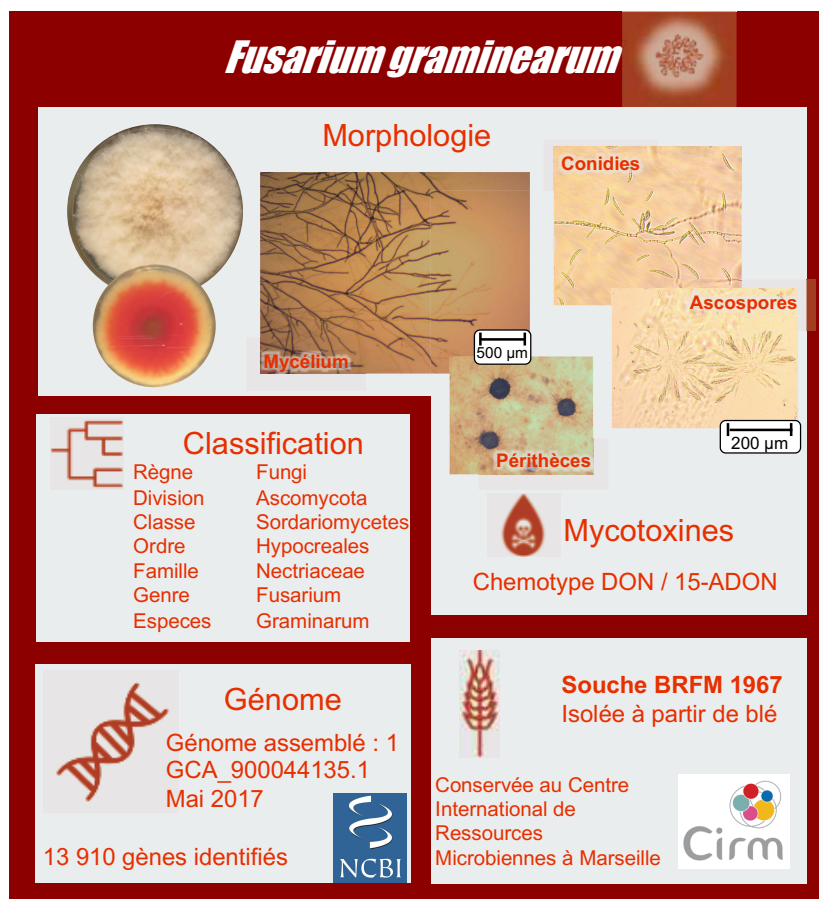


Figure 3 : Présentation de *Fusarium graminearum* et de ses caractéristiques.

Un travail important a été fourni autour du génome de *F. graminearum* ses dernières années. Sur NCBI ((National Center for Biotechnology Information), il existe 13 assemblages et annotations de diverses souches de ce pathogène, plus ou moins complet. La souche pH-1 est la plus décrite avec un génome d'une taille totale de 38Mb distribué en quatre chromosomes (dernier numéro d'accèsion GCA_9000441351, déposé en Mai 2017, base de données consultée le 15/08/2020). La base de données référence également 13 910 gènes séquencés et identifiés chez cette espèce. Plusieurs souches mutées sur des caractères morphologiques, des facteurs de régulation ou de pathogénicité sont également disponibles (Dickman and Leslie, 1992; Hou *et al.*, 2002; Han *et al.*, 2004; Dufresne *et al.*, 2008).

La souche de *F. graminearum* que nous avons sélectionnée pour réaliser cette étude est la souche BRFM1967, connue aussi sous le nom de INRA 149, et conservée au Centre de Ressources Microbiennes de l'INRAE, dans la collection des champignons filamenteux à Marseille. Cette souche est la propriété de l'INRAE, et son usage en recherche n'est pas restreint. Elle a été isolée en 2002 à partir de grain de blé tendre en France (Oise- 60). Fortement agressive et productrice de trichothécènes B sur épis de blé, elle a été testée en serre ou parcelle en plein champ et présente un chemotype DON/ 15ADON.

1.2.1.2. Le blé, l'hôte privilégié de *F. graminearum*

F. graminearum est particulièrement répandu dans le monde et il est retrouvé principalement associé avec le blé, le maïs et l'orge. Les grandes épidémies de fusarioses de l'épi survenues au cours des deux dernières décennies ont fait de lui un pathogène mondial majeur des céréales (Harris *et al.*, 2016), mais il est aussi capable d'interagir avec d'autres plantes annuelles ou vivaces, comme la pomme de terre (Estrada Jr *et al.*, 2010).

Le cycle de vie de *F. graminearum* en interaction avec les céréales et en particulier le blé est complexe, avec une phase de croissance et une phase de conservation (Figure 4). Les différents modes de trophie du pathogène durant la phase végétative ont longtemps été débattues mais la plupart des études s'accordent à dire que ce champignon est hémibiotrophe (Kazan *et al.*, 2012), caractérisé par une longue période de colonisation sans symptômes, suivie d'une nécrose tissulaire massive et d'une augmentation rapide de la biomasse (Stephens *et al.*, 2008).

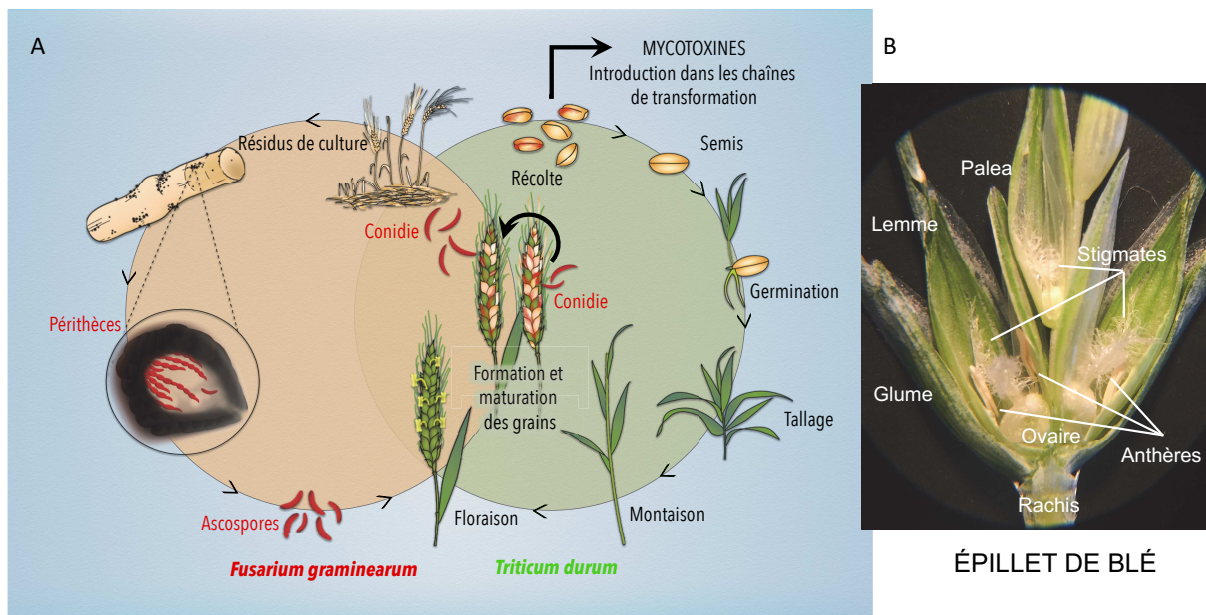


Figure 4 : Le blé, l'hôte privilégié de *F. graminearum*. A. Les cycles de vie du champignon pathogène, *Fusarium graminearum* et du blé dur, *Triticum durum* et leurs connections. B. Photographie d'un épillet en coupe longitudinale permettant de visualiser les différents tissus de l'épillet au stade de l'anthèse.

En effet, le cycle d'infection de *F. graminearum* commence lors de conditions favorables au développement du pathogène (augmentation de la température et de l'humidité) qui vont favoriser la libération d'ascospores et coïncide généralement avec le début de la floraison et l'anthèse (BBCH stades 50 à 60, fin de montaison et floraison, stades principaux et secondaires, pour les céréales (Witzenberger A. *et al.*, 1989; Lancashire *et al.*, 1991). Les spores déposées sur les tissus germent

et pénètrent les épillets par des blessures, des ouvertures naturelles ou l'espace vide entre le paléa et le lemme de l'épillet. Les hyphes commencent à se développer de manière non-ramifiée sur la surface des glumes et dans la cavité florale dans les 12 heures suivant l'inoculation (hpi), et entrent fréquemment en contact avec les stomates (Pritsch *et al.*, 2000). Cette colonisation se poursuit de manière asymptomatique et intercellulaire pendant 20 à 70 heures après l'inoculation et constitue la phase biotrophique du pathogène. Ensuite, des hyphes épais, non ramifiés, inter et intracellulaires se déploient dans les anthères et l'ensemble de l'ovaire, et le rachis est colonisé via l'ovaire 5 jours après l'inoculation (dpi). Le champignon déclenche une nécrose tissulaire par accumulation de mycotoxines et croissance intracellulaire, lui permettant de progresser rapidement à travers le système vasculaire et notamment le phloème pour envahir d'autres épillets adjacents, principalement vers le bas de l'épis (Hallen-Adams *et al.*, 2011). La production de mycotoxines est essentielle à la bonne colonisation des épis de blé par *F. graminearum*, et leur synthèse est stimulée par certains composés de défense de la plante, tels que l' H_2O_2 ou les polyamines connues pour être accumulées dans les plantes attaquées par les pathogènes (Boedi *et al.*, 2016). Elles agissent comme facteur de virulence et contribuent à l'agressivité de *F. graminearum* sur blé (Pestka, 2010). Leur synthèse durant cette phase nécrotrophe est activée de manière spécifique selon les tissus de blé inoculés. En effet, une étude a montré que l'expression de *Tri5*, un indicateur de la biosynthèse de la toxine DON était stimulé dans le rachis en comparaison avec d'autres tissus comme les anthères, qui sont pourtant les cibles initiales de l'agent pathogène (Ilgen *et al.*, 2009). Enfin, les symptômes classiques de fusariose de l'épi se manifestent par une chlorose complète de l'épi, l'apparition de nécroses et de pigments synthétisés par le champignon, caractéristiques de l'activité nécrotrophe du pathogène. Bien que la manière dont *F. graminearum* colonise un seul épillet soit bien documentée, comme décrit ci-dessus, la manière dont ce champignon passe d'un mode de vie biotrophe à nécrotrophe et se propage d'épillet en épillet reste controversée (Leonard and Bushnell, 2003; Brown *et al.*, 2010). Le cycle de vie du pathogène se poursuit ensuite de manière saprophytique jusqu'à et après la récolte du blé dans les débris de culture, qui agissent comme un réservoir d'inoculum lors de conditions défavorables. En effet, la principale source d'inoculum provient des résidus de culture infectés dans lesquels se forment les structures de conservation caractéristiques de cette espèce, les périthèces (Bujold *et al.*, 2001). Le mycélium se ramifie fortement et a tendance à s'effondrer, formant ainsi une structure dense et protectrice autour des ascospores en formation. Le pathogène peut survivre sous cette forme dans des conditions défavorables pendant une longue période : jusqu'à 16 mois sur les grains de maïs ou 23 mois sur les résidus de paille de blé (Reis, 1990; Pereyra *et al.*, 2004). Chez *F. graminearum*, cette phase de reproduction sexuée et de formation de spores sexuelles (ascospores) est un stade critique qui fait partie intégrante de son cycle de vie, responsable de l'initiation de la maladie (Sikhakolli *et al.*, 2012). En effet, lors du retour de conditions favorables, ces périthèces vont être responsables de la libération d'ascospores capables de réinfecter une nouvelle culture de céréales.

Pendant ce temps, les grains fusariés récoltés peuvent, eux, représenter un point d'entrée majeur des mycotoxines et du pathogène dans les filières de stockage et de transformation agro-alimentaire, et mettre en danger la santé des consommateurs.

1.2.1.3. Le Déoxynivalénol et ses dérivés

F. graminearum produit principalement du déoxynivalénol (DON) et ses dérivés acétylés : 3-acétyldéoxynivalénol (3-ADON) et 15-acétyldéoxynivalénol (15-ADON) (Wang *et al.*, 2012), ce qui lui confère généralement un chemotype DON/15-ADON. La DON est une molécule de formule brute $C_{15}H_{20}O_6$ et de poids moléculaire 296,31 g/mol (Figure 5).

D'une manière générale, l'extraction de la DON est réalisée à partir d'un échantillon de masse connue, homogénéisé dans un mélange de solvants organiques polaire (comme le méthanol, l'éthanol, le chloroforme, l'acétonitrile) ou l'eau avec parfois une acidification par de l'acide acétique (National Toxicology Program, 2009; Huybrechts *et al.*, 2013). Des étapes intermédiaires de purification ou de pré-concentration peuvent être appliquées avant analyses (Frenich *et al.*, 2009; Almeida *et al.*, 2012; Liao *et al.*, 2013). Les méthodes d'analyse des mycotoxines comprennent des techniques immunochimiques qui s'appliquent principalement aux contrôles de routine et à la détection rapide sur le terrain, et des techniques chromatographiques qui permettent une détermination sensible, précise et sélective des mycotoxines connues, outre l'identification multi-toxines ou de composés nouveaux ou modifiés grâce à des détecteurs de spectrométrie de masse en tandem. En effet, plusieurs études ont été réalisées pour évaluer les risques sanitaires individuels de la DON, la 3-ADON et la 15-ADON, en tant que composés d'une même famille (Soubra *et al.*, 2009; Sirot *et al.*, 2013). Cependant, ces composés dans un mélange peuvent certainement provoquer des effets synergiques plus importants que le composant individuel. Par conséquent, l'évaluation des mycotoxines prises isolément sous-estime les risques globaux des mycotoxines de la famille DON. C'est pourquoi les méthodes comme l'HPLC MS-MS sont privilégiées (Spanjer *et al.*, 2008; Hickert *et al.*, 2015), malgré le fait que ces techniques soient plus coûteuses et sophistiquées (nécessite du personnel qualifié).

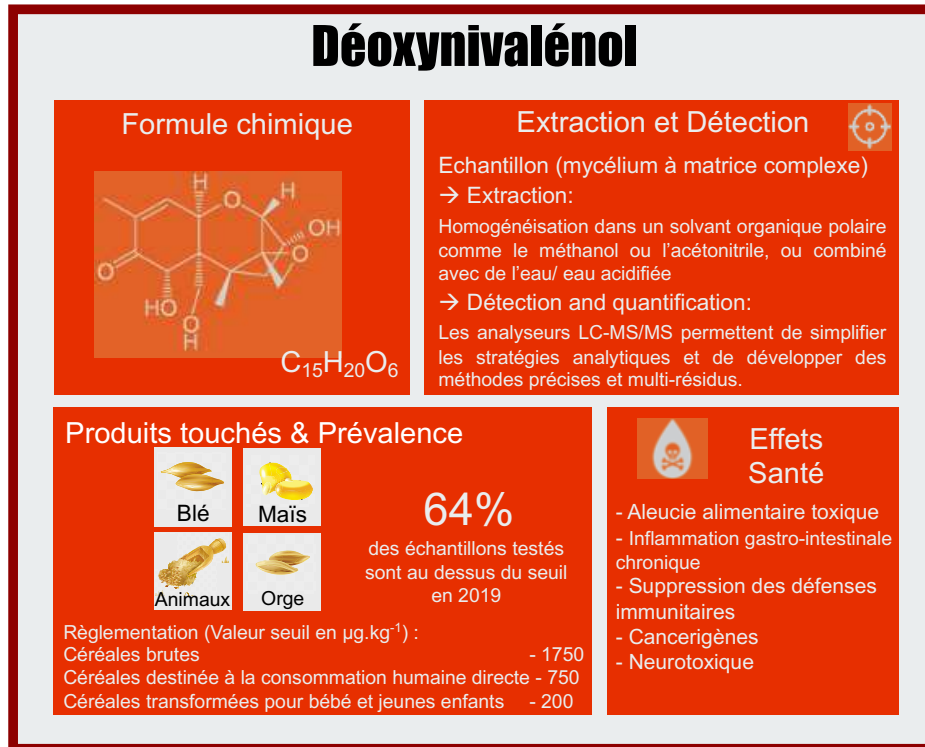


Figure 5 : Présentation du Déoxynivalénol et de ses caractéristiques.

Formule chimique : (PubChem, <https://pubchem.ncbi.nlm.nih.gov/>); Extraction et détection : (Anfossi *et al.*, 2016); Produits touchés et prévalence : (Gruber-Dorninger *et al.*, 2019) (Règlement n°1881/2006 de la Commission européenne); Effet santé : (Barac, 2019).

Les produits alimentaires sont les premières sources d'exposition humaine aux contaminations par les mycotoxines, en particulier les céréales. Le déoxynivalénol a majoritairement été détecté dans des échantillons de blé, de maïs ou d'orge, bruts ou transformés, ainsi que dans la nourriture animale formulée à base de céréales. La consommation d'aliments contaminés avec du déoxynivalénol peut entraîner des maladies humaines chroniques et aiguës telles que nausées, vomissements, troubles gastro-intestinaux et diarrhées (Marin *et al.*, 2013). Les DON acétylées peuvent engendrer une toxicité plus forte car ces toxines sont plus rapidement absorbées dans l'intestin (Gerez *et al.*, 2015). Des doses plus élevées peuvent affecter le fonctionnement de la rate, du cœur et du foie et provoquer des symptômes tels qu'une suppression des défenses immunitaires, l'apparition de cancers et de neurotoxicité (Barac, 2019)

1.2.2. Fusarium verticillioides

1.2.2.1. Caractéristiques générales

Fusarium verticillioides est aussi un champignon ascomycète de la famille des Nectriaceae, qui a été isolé et caractérisé pour la première fois à partir des années 1940-1950 (Townsend, 1943; McKeen, 1953).

In vitro et sur milieu nutritif optimal, les colonies se développent progressivement et forment des amas mycéliens très denses et aux bordures relativement lobées (Figure 6). La couleur du mycélium est d'abord blanche puis présente des nuances de roses et de violet clair. Après un développement plus important, des pigments sont produits de manière radiale avec des couleurs sombres du violet au bleu/noir. Les micro-conidies apparaissent portées par le mycélium aérien sous forme de longues chaînes et ont une forme ovoïde avec une base légèrement aplatie (4-10 µm). Elles sont produites en très grande quantité et avec des tailles assez variables même dans les mêmes conditions. La plupart des souches ne produisent que très peu de macro-conidies (Leslie and Summerell, 2008). Contrairement à *F. graminearum*, *F. verticillioides* est hétérothallique, et ne produit qu'un nombre modeste de fructifications, qui sont rarement produites en conditions naturelles (Shaner G., 2003). De plus le rôle des périthèces dans la survie et le développement du pathogène est relativement mineur (Sikhakolli *et al.*, 2012).

Fusarium verticillioides

Morphologie

- Mycélium**: Image showing dense, lobed mycelium with a 500 µm scale bar.
- Conidies**: Image showing chains of oval microconidia with a 100 µm scale bar.

Classification

- Règne: Fungi
- Division: Ascomycota
- Classe: Sordariomycetes
- Ordre: Hypocreales
- Famille: Nectriaceae
- Genre: *Fusarium*
- Especies: *Verticillioides*

Mycotoxines

Chemotype: FumB1, FumB2, FumB3

Génome

Génome assemblé : 1
GCA_003316995.2
Novembre 2018
16 297 gènes identifiés

Souche BRFM 2251
isolée à partir de maïs

Conservée au Centre International de Ressources Microbiennes à Marseille

Logos: NCBI, Cirm

Figure 6 : Présentation de *Fusarium verticillioides* et de ses caractéristiques.

D'importantes recherches ont été conduites concernant le génome de *F. verticillioides*. Sur NCBI, il existe 4 assemblages et annotations de diverses souches de ce champignon, plus ou moins complet. La souche BRIP53590 est la plus récemment décrite avec un génome d'une taille totale de 42 Mb distribué en 11 chromosomes (dernier numéro d'accession GCA_003316995.2, déposé en Novembre 2018, base de données consultée le 15/08/2020). Un douzième chromosome est surnuméraire et facultatif chez cette espèce. La base de données référence également 16 297 gènes séquencés et identifiés (Kistler *et al.*, 2013). Plusieurs mutants naturels ou obtenus en laboratoire ont été identifiés, notamment sur des caractères morphologiques (comme la production de spores ou de pigments), de métabolisation ou de pathogénicité (Butchko *et al.*, 2003; Proctor *et al.*, 2003; Glenn *et al.*, 2004; Ramamoorthy *et al.*, 2015).

La souche de *F. verticillioides* sélectionnée pour réaliser cette étude est la souche BRFM2251, connue aussi sous le nom de INRA 63, et conservée au Centre de Ressources Microbiennes de l'INRAE, dans la collection des champignons filamenteux à Marseille. Cette souche est la propriété de l'INRAE, et son usage en recherche n'est pas restreint. Elle a été isolée à partir de grain de maïs en France. Fortement productrices de fumonisines B sur épis de maïs, elle a déjà été inoculée au champ sur maïs, et l'infection des épis et la production de FB1/FB2/FB3 avait pu être mises en évidence (Picot *et al.*, 2012).

1.2.2.2. Le maïs, l'hôte privilégié de *F. verticillioides*

F. verticillioides est un agent pathogène très majoritairement associé au maïs et que l'on trouve partout dans le monde où cette céréale est cultivée. Malgré cette association dominante, il a aussi été identifié lors d'infections asymptomatiques dans d'autres mono-cotylédons et même dans la betterave sucrière (Dastjerdi and Karlovsky, 2015); ainsi qu'en association avec des maladies du blé, comme pathosystème plus émergent (Shephard *et al.*, 2005; Chehri *et al.*, 2010).

Le cycle de vie de *F. verticillioides* en interaction avec les céréales, et en particulier le maïs est très complexe. Ce champignon peut se présenter comme endophyte intercellulaire qui ne provoque pas de symptômes. Il est également capable de provoquer de multiples formes de maladies importantes du maïs comme la fonte des semis, la pourriture des racines, la pourriture de la tige et la pourriture de l'épi (Thiel *et al.*, 1991) à partir d'inoculum conservé dans les résidus de cultures (Figure 7). Au cours de ces épidémies, des symptômes apparaissent, de grandes quantités de mycotoxines sont accumulées et des dégâts importants sont observés sur la culture. Ce double comportement de *F. verticillioides* témoigne de la relation complexe entre le champignon et la plante de maïs (Bacon *et al.*, 2008).

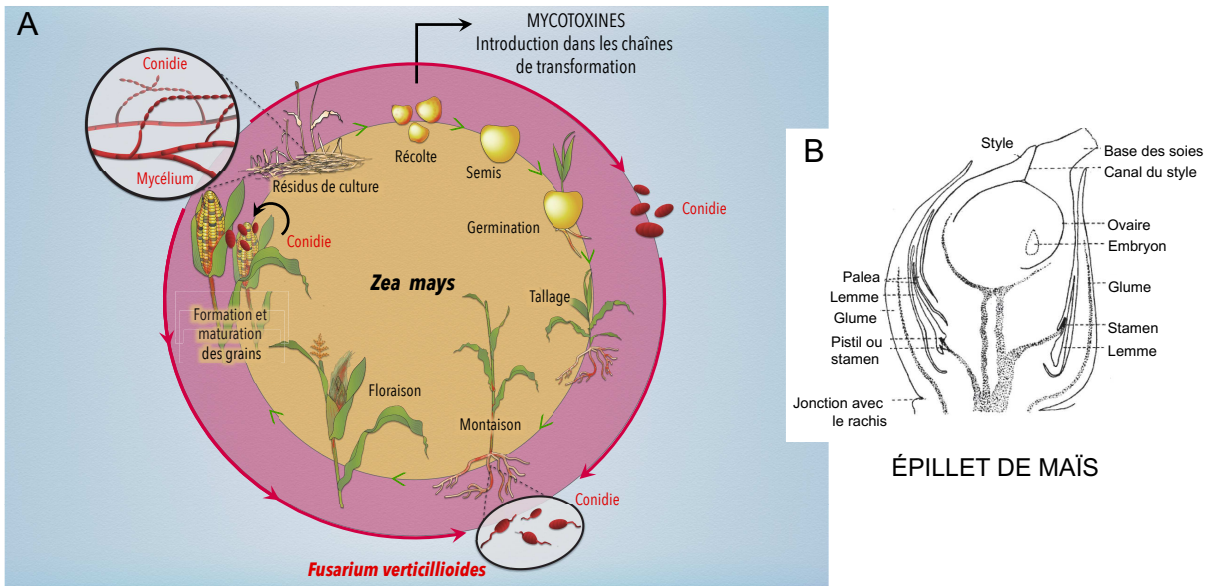


Figure 7 : Le maïs, l'hôte privilégié de *F. verticillioides*. A. Les cycles de vie du champignon pathogène, *Fusarium verticillioides* et du maïs, *Zea mays* et leur potentielles connections. B. Schéma d'un épis de maïs en coupe longitudinale permettant de visualiser les principales voies d'entrée du pathogène au stade de la floraison

L'inoculum constitué de micro-conidies est clairement prépondérant dans le cycle de vie et d'épidémie de *F. verticillioides*. La dissémination de cet inoculum primaire, conservé dans les résidus de cultures principalement, est stimulée lors des périodes de perturbation mécanique, en particulier les semis, la pollinisation, la récolte ou le débroussaillage (Donát *et al.*, 2012). Le pathogène peut ensuite pénétrer les plants de maïs de manière systémique soit à partir de la graine, par des blessures ou ouvertures naturelles sur la plante soit par infection des soies au niveau des épis (qui restent le principal point d'entrée du pathogène retrouvé dans les grains de maïs) (Munkvold *et al.*, 1997).

Lors de la présence de conditions optimales pour les semis, les spores de *F. verticillioides* vont germer et initier la croissance des hyphes qui vont peu à peu progresser à la surface des racines, puis pénétrer les racines latérales et le mésocotyle dès 72 heures après le semis dans des sols infestés, avec une croissance invasive mais intercellulaire. En 7 jours, la croissance fongique progresse mais reste limitée au tissu du mésocotyle. Quatorze jours après le début de l'infection asymptomatique, la croissance dans les tissus aériens est faible mais suffisante pour permettre aux insectes de propager l'infection. Cette croissance peut ensuite se limiter à une colonisation endophyte et asymptomatique ou entraîner l'apparition de symptômes et la pourriture des racines ou la fonte des semis (Baldwin *et al.*, 2014). Après 15 à 30 jours de culture, et dans des conditions de faible luminosité favorables à la maladie, la biomasse fongique peut augmenter fortement, provoquant la nécrose du mésocotyle de la racine principale et de la tige (Oren *et al.*, 2003).

Au stade de la floraison des épis et du développement des soies sur les fleurs femelles, le champignon colonise les grains en développement par une ouverture étroite appelée canal du style (Duncan and Howard, 2010). Le diamètre d'ouverture de ce canal dépendant du stade de développement mais aussi de la variété est un déterminant important du succès des infections par *F. verticillioides*. Les conditions environnementales influencent fortement le développement de la maladie à ce stade de développement du maïs : par exemple, les conditions humides qui suivent la levée sont propices à la pourriture de l'épi, donnant lieu au motif de "starburst" communément observé. Les stress de sécheresse provoqué par les conditions chaudes et sèches pendant le remplissage des grains sont fortement corrélées avec l'intensité de la maladie et la contamination par les fumonisines (Munkvold, 2003). Ce pathogène a été identifié à l'extrémité des épis de maïs symptomatiques et asymptomatiques, mais dans les grains symptomatiques, l'embryon et l'endosperme ont été largement colonisés et, dans certains cas, des micro-conidies ont été produites à l'intérieur du grain infecté.

Outre les mécanismes d'infection décrits ci-dessus, des insectes tels que les lépidoptères, les thrips, la pyrale ou la chrysomèle du maïs sont connus pour contribuer à l'infection des épis ou des tiges par *F. verticillioides* (Gilbertson *et al.*, 1986; Sobek and Munkvold, 1999). Ces données, associées aux méthodes connues de dissémination de l'inoculum de *F. verticillioides*, suggèrent une série d'interactions symbiotiques entre le champignon et d'autres organismes associés au maïs. De plus, la relation *F. verticillioides* - *Zea mays* ne se produit pas de manière isolée en laboratoire, et les insectes associés au maïs jouent un rôle important dans le cycle de la maladie.

Le rôle des fumonisines comme facteur de pathogénicité et de virulence de *F. verticillioides* en conditions naturelles a été examiné lors des fontes des semis, de pourriture de la tige et de pourriture de l'épi. La mycotoxine la plus produite est largement la fumonisine B1 qui est phytotoxique pour les génotypes sensibles de maïs et peut jouer un rôle dans le développement de la maladie. Elle semble favoriser la mort des cellules de maïs et la colonisation fongique nécrotrophe en activant les voies de défense de la plante, à ce titre la FB1 peut être considérée comme un effecteur nécrotique (Arias *et al.*, 2012), mais contrairement aux mycotoxines de *F. graminearum*, elles ne sont pas indispensables à l'infection. De plus, lors de la phase endophyte et sans symptômes, il peut aussi y avoir une légère production de fumonisines (Bacon *et al.*, 2008).

Enfin, lors de la récolte, les épis contaminés sont récoltés et constituent une importante source de mycotoxines qui entrent dans les processus de transformation des aliments. D'autre part, lorsque les conditions ne sont plus favorables au développement de ce champignon, il peut se conserver dans les résidus de culture, et ce pour une durée importante qui peut aller jusqu'à 630 jours à la surface et enfoui dans le sol (Cotten and Munkvold, 1998), principalement sous forme de conidies et de mycélium (peu de périthèces contrairement à *F. graminearum*). Ce réservoir d'inoculum permet ensuite le développement des maladies décrites précédemment.

1.2.2.3. Les Fumonisines

Les fumonisines sont clairement la famille de toxines la plus importante produite par *F. verticillioides*, et certaines souches peuvent produire ces mycotoxines à des niveaux très élevés (Nelson *et al.*, 1994). La fumonisine B1 est la plus connue et la mieux étudiée des fumonisines, mais on sait que d'autres dérivés sont également présents ce qui confère généralement au pathogène un chemotype FB1, FB2, FB3 (Plattner *et al.*, 1996; MacKenzie *et al.*, 1998). La fumonisine B1 est une molécule diester de formule brute $C_{34}H_{59}NO_{15}$ et de poids moléculaire 721.8 g/mol (Wang *et al.*, 2006) (Figure 8).

Les méthodes citées précédemment qui permettent d'analyser la DON et ses dérivés peuvent également s'appliquées pour l'extraction, la détection et la quantification des fumonisines. Grâce à une forte hydrosolubilité de cette molécule, l'utilisation de solvant à base d'eau permet de limiter le coût et l'exposition des manipulateurs à des produits toxiques comme les solvants organiques (de Girolamo *et al.*, 2001). De même, les méthodes d'analyses multi-toxines sont privilégiées dans le cadre d'une détection précise et fiable, comme l'utilisation d'HPLC-MS/MS, afin de ne pas sous-estimer le risque lié à la famille globale des fumonisines (de Oliveira *et al.*, 2017).

Fumonisines

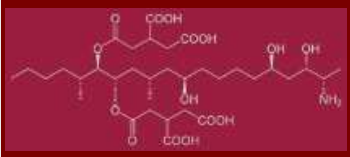



<p style="text-align: center;">Formule chimique</p> <div style="text-align: center;">  <p style="margin: 0;">$C_{34}H_{59}NO_{15}$</p> </div>	<p style="text-align: center;">Extraction et Détection</p> <p>Echantillon (mycélium à matrice complexe)</p> <p>→ Extraction: Homogénéisation dans un solvant organique polaire comme le méthanol ou l'acétonitrile, ou combiné avec de l'eau/eau acidifiée</p> <p>→ Détection and quantification: Les analyseurs LC-MS/MS permettent de simplifier les stratégies analytiques et de développer des méthodes précises et multi-résidus.</p>						
<p style="text-align: center;">Produits touchés & Prévalence</p> <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">  Maïs </div> <div style="text-align: center;">  Corn Flakes </div> <div style="text-align: center;">  Farine </div> </div> <p style="text-align: center; font-size: 1.2em; font-weight: bold;">60%</p> <p style="text-align: center; font-size: 0.8em;">des échantillons testés sont au dessus du seuil en 2019</p> <p>Règlementation (Valeur seuil en $\mu\text{g.kg}^{-1}$)</p> <table style="width: 100%; font-size: 0.8em;"> <tr> <td>Maïs brut</td> <td style="text-align: right;">- 2000</td> </tr> <tr> <td>Céréales destinée à la consommation humaine directe</td> <td style="text-align: right;">- 400</td> </tr> <tr> <td>Céréales transformées pour bébé et jeunes enfants</td> <td style="text-align: right;">- 200</td> </tr> </table>	Maïs brut	- 2000	Céréales destinée à la consommation humaine directe	- 400	Céréales transformées pour bébé et jeunes enfants	- 200	<p style="text-align: center;">Effets Santé</p> <ul style="list-style-type: none"> - Fièvre, - Œdème pulmonaire - Hémorragie - Cancérigènes - Neurotoxique
Maïs brut	- 2000						
Céréales destinée à la consommation humaine directe	- 400						
Céréales transformées pour bébé et jeunes enfants	- 200						

Figure 8 : Présentation de la Fumonisine B1 et de ses caractéristiques.

Formule chimique : (PubChem, <https://pubchem.ncbi.nlm.nih.gov/>); Extraction et détection : (Anfossi *et al.*, 2016); Produits touchés et prévalence : (Gruber-Dorninger *et al.*, 2019) (Règlement n°1881/2006 de la Commission européenne); Effet santé : (Barac, 2019)

Avec près de 60% des analyses au-dessus du seuil réglementaire, les fumonisines sont très majoritairement détectées à partir d'échantillons de maïs ou de produits à base de maïs mais sont parfois présentes dans d'autres produits comme la farine de blé (J Bend *et al.*, 2011). La consommation de maïs contaminé peut entraîner divers effets cliniques. Les fumonisines peuvent être responsables de l'apparition de fièvre d'œdème pulmonaire ou d'hémorragies (Reboux, 2006; Barac, 2019). Plusieurs études ont mis en cause les fumonisines dans le développement de cancer de l'œsophage, d'anomalies congénitales et de retard de croissance chez les enfants (Jaskiewicz *et al.*, 1987; Marasas, 2001; Gelineau-van Waes *et al.*, 2009; Shirima *et al.*, 2015).

1.3. Les moyens de luttés actuel contre les phytopathogènes et notamment les *Fusaria* des céréales

Afin de réduire les importants et divers dégâts dus aux pathogènes du genre *Fusarium* et notamment ceux décrits précédemment pour les deux pathogènes *F. graminearum* et *F. verticillioides*, plusieurs pistes peuvent être envisagées à l'échelle du bassin de production, de la parcelle, ou de la plante. (Figure 9).

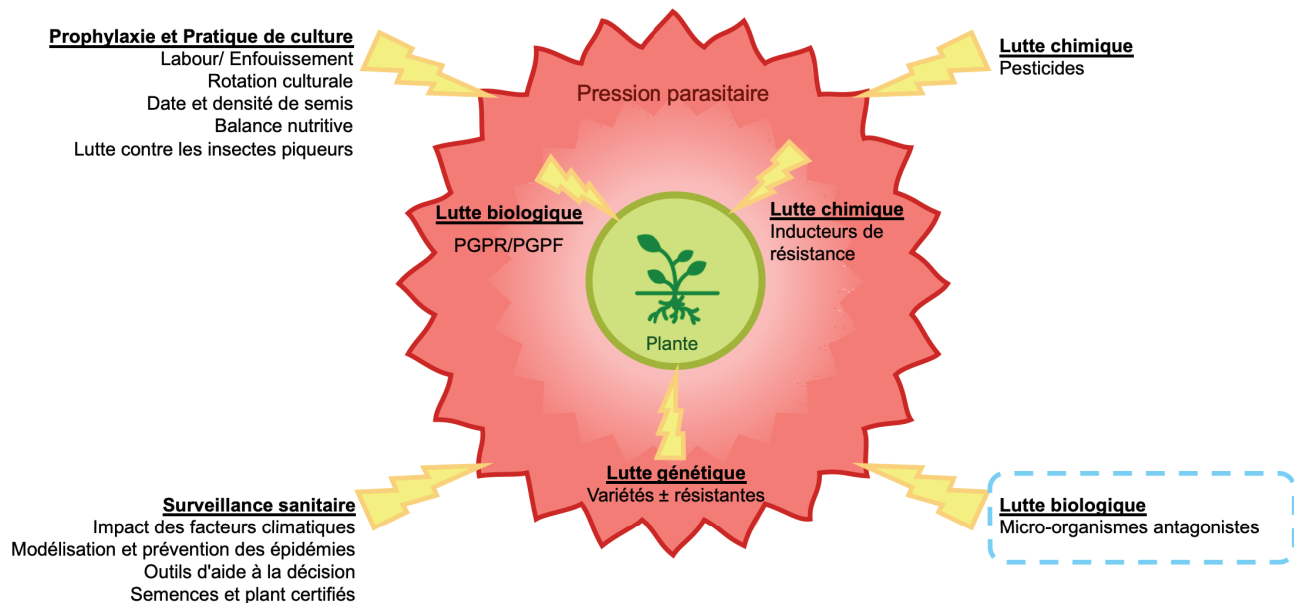


Figure 9 : Récapitulatif des moyens de lutte contre les pathogènes de plante permettant d'agir sur la plante ou sur la pression parasitaire. Le piste que nous avons choisie d'explorer est encadrée en bleu.

1.3.1. Stimulation de la résistance des plantes

Parmi les méthodes qui permettent d'agir de manière indirecte sur la plante afin de la rendre plus à même de combattre les *Fusarium* spp., plusieurs pistes sont à considérer : la lutte génétique ou variétale, la lutte chimique *via* des inducteurs synthétiques de défenses et la lutte biologique *via* des stimulateurs naturels de défense ou de croissance des plantes.

Dans le cadre des fusarioses, il n'existe pas de génotype de blé ou de maïs présentant une résistance totale à ces maladies, néanmoins des cultivars peu sensibles ont pu être identifiés. Principalement étudiés dans le cadre d'attaques de *F. graminearum*, des progrès significatifs ont été faits en blé tendre, mais encore peu de variétés sont considérées comme peu sensibles. Une dizaine de variétés du catalogue se positionne comme alternative à la référence historique sur ce critère, Apache. La résistance est multigénique et l'utilisation de l'analyse des loci liés à des caractères quantitatifs (QTL), qui identifie les régions du génome responsables de l'apparition de la résistance, a révélé que la plupart des QTLs n'expliquent qu'une proportion relativement faible de la variation du développement des maladies (Buerstmayr *et al.*, 2009; Miedaner *et al.*, 2009). Concernant le blé dur, il existe également une légère variabilité des résistances permettant de sélectionner des cultivars moins sensibles, mais qui ne permet pas de s'affranchir de l'utilisation d'autres méthodes de gestion. D'autre part, il existe des traits agronomiques/morphologiques liés à certaines variétés qui sont associés à une résistance passive aux fusarioses des épis. Chez le maïs, les deux pathogènes colonisent plus difficilement des génotypes ayant un canal styloïde plus étroit (Duncan and Howard, 2010), cette zone étant considérée comme une porte d'entrée majeure des pathogènes dans les épis. Chez le blé, Prat *et al.*, (2014) ont montré une plus forte intensité des maladies pour des cultivars présentant des plants plus petits très probablement en raison d'une différence de microclimat autour des épis de blé. De plus, comme la majorité des infections se produisent lors de la floraison (Siou *et al.*, 2014), les cultivars dont la période de floraison est plus courte peuvent être moins gravement touchés que ceux dont la période de floraison est plus longue.

Les stimulateurs de défense chimique peuvent également être utilisés pour agir de manière indirecte dans la protection des cultures contre les *Fusaria* (Small *et al.*, 2012). Ces produits sont capables d'activer les défenses des plantes et donc de limiter l'infection par les agents pathogènes (Zehra *et al.*, 2017; Alhoraibi, 2019). Ces approches chimiques sont liées à la génétique de la plante, et sont basées sur des petites molécules bioactives qui vont être perçues par la plante et déclencher une cascade de signalisation complexe impliquant l'activation de voies de défenses de la plante, et entraînant ainsi une meilleure défense au point d'infection ou de manière systémique (Jones and Dangl, 2006; Wirthmueller *et al.*, 2013). Cette signalisation passe par une accumulation de phytohormones conduisant à l'activation transcriptionnelle des gènes codant la synthèse de composés antimicrobiens tels que les phytoalexines ou les protéines liées à la pathogenèse (PR) (Pieterse *et al.*, 2014; Piasecka *et al.*, 2015; Klessig *et al.*, 2018). Cependant, l'efficacité des stimulateurs de défense des plantes en plein champ reste incertaine et peut dépendre de différents

facteurs internes et/ou externes tels que le stade de développement de la plante, la température, la sécheresse et/ou la nutrition minérale (Walters and Fountaine, 2009; Steimetz *et al.*, 2012). Néanmoins des molécules comme le sulfaméthoxazole (Smex) ou l'indole alcaloïde ont été identifiées comme inducteurs de défense de la plante et toutes deux ont réduit la gravité de l'infection par *F. graminearum* dans le blé (Schreiber, 2008).

En opposition aux stimulateurs de défense chimique, certains micro-organismes vivants ont été identifiés comme capables de promouvoir la croissance des plantes, entraînant une meilleure résistance aux potentielles épidémies. Cette amélioration de la vigueur globale de la plante passe par différents phénomènes comme l'augmentation de l'acquisition des nutriments, la production de phytohormones, la solubilisation des phosphates ou la fixation de l'azote (Singh and Jha, 2016). Par exemple, l'application de *Bacillus subtilis* et de *Pseudomonas fluorescens* a eu un effet significatif sur les facteurs de croissance du cultivar de blé Tabuki : la longueur des racines a été augmentée de manière significative lors de traitements d'enrobage et de trempage des semences, et le nombre de feuilles a augmenté de manière significative (Moussa *et al.*, 2013). En effet, ces micro-organismes présentent des caractéristiques favorisant la croissance des plantes comme la fixation de l'azote, la solubilisation du phosphate et la production d'acides organiques, de tri-calcium phosphate et d'AIA (Pal *et al.*, 2001). En dehors d'améliorer la physiologie générale de la plante, ces micro-organismes peuvent également être à l'origine de la production de stimulateurs de défense « biotiques » des plantes et agir sur l'initiation de cascades de défense chez la plante, comme développé précédemment avec les inducteurs synthétiques. L'action plus directe de certains de ces micro-organismes contre les pathogènes est détaillée dans la section biocontrôle.

1.3.2. Facteurs capables d'influencer la pression parasitaire

Hormis les méthodes présentées précédemment, la majeure partie des moyens de lutte contre les *Fusarium* spp. phytopathogènes est orientée pour limiter directement la pression parasitaire appliquée par les pathogènes. Différents leviers d'action peuvent être considérés, comme l'adaptation des pratiques culturales, la surveillance sanitaire, l'application de pesticides chimiques ou l'utilisation de micro-organismes antagonistes.

1.3.2.1. Itinéraire de culture et Prophylaxie

Comme indiqué précédemment, le sol est un important réservoir d'inoculum pour les pathogènes. Le travail du sol peut donc être un facteur important dans le développement de la fusariose du blé (Skoglund and Brown, 1988; Steinkellner *et al.*, 2002). Le labour avec retournement du sol permet d'enfouir l'inoculum primaire qui infecte les épis de blé et empêche la dispersion par éclaboussures,

il améliore le processus de décomposition des résidus et donc limite la disponibilité des nutriments et la survie de *F. graminearum* (Pereyra *et al.*, 2004), et il permet de contrôler la croissance d'adventices qui pourraient également être une source d'inoculum. Néanmoins, des études ont montré également des cas où le labour conventionnel ou l'absence de travail du sol n'avaient pas d'effet significatifs sur le développement de *F. verticillioides* et la production de fumonisines dans le maïs (Marocco *et al.*, 2008), tout comme sur *F. graminearum* lorsqu'il attaque cette même plante (Pfordt *et al.*, 2020).

Le précédent cultural et le choix des rotations de culture peut être un facteur important déterminant le risque des maladies dues aux espèces de *Fusarium* sur le blé (M. Váňová *et al.*, 2008). Les *Fusaria* considérés ne sont pas spécifiques à l'hôte et peuvent être isolés à partir de nombreuses espèces de plante. Une rotation impliquant le maïs, également un hôte de ces espèces, doit être évitée. Des auteurs (Dill-Macky and Jones, 2000) ont démontré une diminution légère mais significative de la gravité du FHB et du DON après la plantation de blé dans des résidus de soja par rapport aux résidus de blé ou de maïs. D'autres (Schaafsma *et al.*, 2005) ont constaté que le nombre de propagules viables piégées à l'anthèse dans les champs de blé plantés après du maïs ou du blé était plus élevé que le nombre de propagules viables dans les champs de blé suivant des cultures non-hôtes. Réciproquement, les mêmes observations peuvent être recueillies dans le cas de culture de maïs, en rotation avec du blé (Khonga and Sutton, 1988; Munkvold, 2003).

L'étape du semis peut aussi être modulée pour diminuer la pression parasitaire. En général, une densité modérée de semis, une profondeur de semis peu importante ainsi qu'un semis précoce sont positivement corrélée avec une diminution des niveaux d'infection (Blandino *et al.*, 2008, 2009; Parsons and Munkvold, 2012).

Une fois la culture implantée, la balance nutritionnelle équilibrée par l'apport de fertilisant peut impacter la propagation des pathogènes. Différentes formes d'azote ont des effets différents sur la survie des *Fusarium* spp. D'une part, dans certains cas, le nombre de propagules de *F. graminearum* dans les résidus augmente avec la fertilisation azotée inorganique et avec l'application de nitrate d'ammonium et de calcium (Bateman and Coşkun, 1995; Yi *et al.*, 2006). D'autre part, l'urée peut entraver la reproduction des champignons, inhiber la formation de spores et empêcher la maturation des ascospores (Teich, 1989).

Enfin, les insectes ont été identifiés comme un facteur en étroite corrélation avec l'apparition de fusarioses, notamment dues à *F. verticillioides* sur les épis de maïs et en particulier dans les zones tempérées où et cela semble être la voie d'infection la plus importante (Avantaggiato *et al.*, 2002). Il a également été démontré que le maïs transgénique toxique pour les lépidoptères réduit l'incidence de la maladie et l'accumulation de fumonisines dans des conditions de forte pression parasitaire. Les cultivars dont l'enveloppe est plus serrée et plus complète constituent également une barrière physique contre les thrips et les insectes qui s'en nourrissent, ce qui réduit le niveau d'infection par *F. verticillioides* (Munkvold, 2003).

1.3.2.2. Surveillance sanitaire

Comme les épidémies de fusarioses sont très dépendantes du climat, de nombreux facteurs environnementaux peuvent être liés à des variations d'incidence des maladies. Ces facteurs peuvent être liés au pH, à la lumière, à la disponibilité en oxygène ou encore à la disponibilité de l'eau et la température qui sont deux facteurs très importants pour le développement des pathogènes (Leplat *et al.*, 2013). L'humidité permet en effet le développement des fusarioses et par conséquent l'accumulation de DON ou de fumonisines dans les grains. En l'absence de précipitations naturelles excessives, une gestion de l'irrigation visant à prévenir une humidité excessive dans les champs de blé ou de maïs irrigués peut réduire considérablement le risque de développement des pathogènes (Gilbert and Tekauz, 2000), en particulier pendant les périodes d'anthèse et de remplissage précoce des grains.

La température et en particulier les effets potentiels sur les cultures et les agents pathogènes du changement climatique (Chakraborty and Newton, 2011) sont aussi à considérer dans le cas des fusarioses. La réorganisation à grande échelle des zones de production pourrait aussi être une piste intéressante dans la gestion globale du risque épidémique.

Étant donné cette forte sensibilité aux conditions environnementales, différents Outils d'Aide à la Décision (OAD) ont été mis au point pour optimiser les traitements et les variétés afin de prévenir les maladies du blé (de Wolf *et al.*, 2003; Shah *et al.*, 2013). La plupart des outils de prévision contre les fusarioses mis à disposition des agriculteurs en France ont été élaborés à partir des données météorologiques, de piégeages de spores dans des parcelles, ainsi que sur des facteurs de risque associés à des pratiques culturales (Prandini *et al.*, 2009), comme Taméo® (conçu par ARVALIS & Météo-France), Septo-LIS carto® (ARVALIS), Opti-Protech® (option de l'outil Mesparcelles diffusé par les Chambres d'agriculture), ATLAS-xarvio FIELD MANAGER (BASF), application Météus® (ISAGRI), etc. Ces outils en constante amélioration sont également utilisés pour établir le niveau de risque dans une région donnée et pour différents cas-types (Gourdain and Grignon, 2019). Par exemple, le Baromètre Maladies d'Arvalis permet de calculer instantanément un niveau de risque associé aux cultures de blé sur 7 jours, centré sur le jour de la simulation, pour 5 maladies dont la fusariose des épis (Baromètre Maladies blé tendre, ARVALIS - Institut du végétal).

L'utilisation de plants ou graines certifiés peut être un moyen de limiter l'apport de pathogènes dans les cultures, mais étant donné le type de rotation et la conservation des deux principaux pathogènes du genre *Fusarium* dans les résidus de culture et le sol comme inoculum majeur, ce levier reste très minoritaire. L'activité de certification des semences et plants réalisée par le Service Officiel de Contrôle et de Certification qui s'applique aux espèces agricoles, prend en compte la garantie de l'identité et de la pureté variétale, la garantie des qualités technologiques des semences mais pas la qualité sanitaire dans le cas des céréales. Cependant un traitement fongicide des semences est

quasi-systématique, afin de protéger la graine des pathogènes qu'elle peut porter, mais aussi de ceux présents dans le sol sur les débris de culture (Schaafsma *et al.*, 2005). De même qu'utiliser des semences saines bien triées, non porteuses de maladie permet de limiter la propagation.

1.3.2.3. Produits Phytopharmaceutiques

L'utilisation de fongicides chimiques a été et reste considérée comme le moyen de lutte le plus répandu dans le monde pour lutter contre les épidémies de fusarioses des céréales (D'angelo *et al.*, 2014). De très nombreux produits ont été formulés au cours du temps : 2091 formulations fongicides existent pour traiter les parties aériennes des céréales, et 203 sont autorisées en France (ephy | Catalogue des produits phytopharmaceutiques). Différentes études sur la gestion des fusarioses du blé ont montré que, selon le niveau de maladie et de sensibilité du cultivar, un traitement à base de propiconazole, prothioconazole, tebuconazole, metconazole, ou tebuconazole + prothioconazole permettait une réduction de 40 à 70% de la propagation de *F. graminearum* (Paul *et al.*, 2008). L'application de fongicides au stade de la floraison semble être le meilleur moment pour réduire l'infection par *Fusarium* spp. (Müllenborn *et al.*, 2008). De plus, les effets bénéfiques de l'utilisation de cultivars résistants et de l'application de fongicides efficaces sont cumulatifs (Willyerd *et al.*, 2012).

Cependant, de nombreuses études ont aussi rapporté l'existence d'effets secondaires fortement indésirables des traitements fongicides contre les fusarioses. L'application de fongicides chimiques sur des semis de maïs avant la plantation n'a pas été efficace dans la gestion de la maladie, et entraîne plutôt une augmentation significative des concentrations de mycotoxines dans les plantes (Ramirez *et al.*, 2004). De même certains herbicides à base de glyphosate peuvent stimuler la croissance de *F. graminearum* sur les résidus de culture dans le champ, ce qui entraîne une augmentation des infections de la base de la tige du blé et des épis (Fernandez *et al.*, 2009). Dans le cas de *F. verticillioides*, la lutte chimique a été généralement décrite comme "spectaculièrement infructueuse" en raison de son caractère principalement cryptogamique et du maintien important des populations dans le sol (Leslie and Summerell, 2013). A cela s'ajoute le fait que de nombreux pathogènes développent une résistance aux fongicides. Enfin, des risques importants pour la santé, allant d'irritations et maux de tête jusqu'à l'asthme, le diabète ou le cancer, mais aussi des risques pour l'environnement (perturbation des écosystèmes, résidus dans les aliments et l'eau potable) ont été identifiés en lien avec l'utilisation de pesticides (Kim *et al.*, 2017). En réponse à cette inquiétude grandissante du public, les chercheurs concentrent aujourd'hui leurs efforts sur l'identification et le développement de méthodes alternatives plus respectueuses.

1.3.2.4. Vers le biocontrôle ...

Afin de limiter l'utilisation de ces produits, deux pistes sont principalement considérées. D'une part la meilleure façon de lutter contre la fusariose dans le blé et le maïs consiste à appliquer plusieurs stratégies ; une seule stratégie échoue généralement en cas d'épidémie (Mcmullen *et al.*, 2008). A ce titre, les méthodes développées précédemment doivent être considérées dans un plan global et intégratif de lutte contre les pathogènes, en combinant par exemple : une bonne gestion des résidus de récolte, un travail du sol qui accélèrent leur décomposition (broyage et enfouissement), des dates de semis précoces, un choix variétal favorisant les cultivars précoces, ainsi que l'utilisation d'OAD pour raisonner les traitements.

D'autre part, FUSATOX, un réseau européen de chercheurs travaillant sur les fusariotoxines, soutient également la mise en place de système de gestion privilégiant l'association de pratiques intégrant l'ensemble des maillons de la chaîne de contamination, de la caractérisation et disponibilité de l'inoculum fongique à la parcelle jusqu'à l'identification des paramètres biotiques et abiotiques contrôlant son développement et l'expression de sa toxinogénèse dans le grain. Il a été établi qu'une bonne connaissance des facteurs climatiques et agronomiques favorisant la contamination des plantes puis des grains n'est pas suffisante pour limiter la progression des *Fusaria*. Une des orientations particulièrement prometteuses ciblée par les chercheurs pour limiter les contaminations, tout en limitant l'utilisation de produits phytopharmaceutiques nocifs, est une meilleure compréhension de l'écologie microbienne. Cette démarche a d'ores et déjà permis des avancées scientifiques significatives et des propositions d'outils de gestion du risque tels que des antagonistes microbiens, des structures chimiques à propriétés antifongiques et anti-mycotoxines et de nouveaux traits génétiques associés à la résistance des plantes. Pour être capable d'anticiper et prévoir toute évolution potentielle du risque mycotoxine à travers l'utilisation d'organismes antagonistes, il est aussi indispensable d'approfondir les connaissances concernant les modes d'interactions de ces agents (Florence Forget, 2018).

1.4. Le biocontrôle

1.4.1. Définition et utilisation

Basé sur le concept de service écosystémique, c'est-à-dire les services rendus à l'Homme par la nature, le biocontrôle (ou lutte biologique, contrôle biologique) *via* l'utilisation de micro-organismes antagonistes est un concept largement décrit et étudié au cours des deux derniers siècles qui de plus permet une réciprocité de service de l'homme vers nature. William Roberts a démontré en 1874 l'action antagoniste des micro-organismes entre *Penicillium glaucum* et d'autres bactéries et a introduit le terme d'antagonisme (Foster and Raoult, 1974). Le contrôle biologique a été utilisé pour la première fois par C. F. Von en 1914 (Paulitz *et al.*, 1987; Cook, 1988) dans le cadre de la gestion des maladies des plantes. Les premières stratégies de biocontrôle portaient sur l'exploitation de caractère particulier aux agents bénéfiques, comme le parasitisme de *Trichoderma lignorum* contre

plusieurs agents pathogènes de plantes ou la production d'antibiotiques dans le sol par les espèces appartenant aux genres *Aspergillus*, *Penicillium*, *Trichoderma* et *Streptomyces*.

Aujourd'hui le biocontrôle est définie comme « l'utilisation d'organismes bénéfiques, de leurs gènes et/ou de produits, tels que des métabolites, pour réduire les effets négatifs des agents pathogènes des plantes et favoriser les réponses positives de la plante » (Vinale *et al.*, 2008; Tranier *et al.*, 2014). L'International Biocontrol Manufacturers Association (IBMA) définit le biocontrôle comme l'utilisation d'agents ou de produits qui affectent naturellement les ravageurs et les agents pathogènes des cultures en limitant leur propagation. Les micro-organismes considérés peuvent être appelés agents de biocontrôle (BCAs).

En comparaison avec l'utilisation de pesticides chimiques, il semblerait que le biocontrôle peut présenter de nombreux avantages notamment liés aux coûts ou temps de développement, au risque de résistance ou aux effets secondaires (Table 2) d'après une étude de Ram *et al.*, 2018, mais ces conclusions restent assez marginales dans la littérature.

Table 2 : Paramètres de développement et évaluation des risques des pesticides chimiques et des agents de biocontrôle (Ram *et al.*, 2018).

	Chemical Control	Biological Control
Developmental costs	US\$ 150 million	US\$ 2 million
Success ratio	1:200,000	1:10
Developmental time	10 years	<10 years
Benefit/cost ratio	2:1	20:1
Risks of resistance	Large	Very small
Specificity	Less	High
Harmful side-effects	Many	Nil/few

Modified from http://www.iobc-global.org/download/IOBC_InternetBookBiCoVersion6Spring2012.pdf.

De plus, plusieurs études ont montré que les céréales produites biologiquement présentaient des taux de contamination plus faible par les espèces de *Fusarium* et également des niveaux de mycotoxines plus bas que celles produites en agriculture conventionnelle (Bernhoft *et al.*, 2010). Dans des cultures de maïs, les *Fusaria* ont été détectés à hauteur de 34,93 % dans les cultures conventionnelles contre seulement 18,15 % dans les cultures biologiques (Ariño *et al.*, 2007), mais il n'y avait pas de différences concernant la contamination en mycotoxines.

1.4.2. Les agents de biocontrôle identifiés contre les deux *Fusaria* étudiés

Étant donné les avantages apportés par l'utilisation de BCAs, de nombreux travaux ont permis d'identifier des antagonistes des *Fusaria* des céréales ces dernières années. Ces organismes sont

de divers types (bactéries, champignons, oomycètes, levures) et ont été testés dans des conditions expérimentales très variables (stade de développement des plantes ou pathogènes, matrices, conditions) (Table 3).

Table 3 : Souches antagonistes présentant des effets de biocontrôle sur les Fusarioses de l'épi (FHB) ou les agents pathogènes qui les provoquent : *F. graminearum* et *F. verticillioides*. (Adapté de (Legrand *et al.*, 2017; Sarrocco and Vannacci, 2018), et enrichi)

BCA	Culture	Maladie / pathogène	Reference
<i>Trichoderma gamsii</i>	Blé	FHB / <i>F. graminearum</i>	(Matarese <i>et al.</i> , 2012) (Sarrocco <i>et al.</i> , 2013)
<i>Trichoderma harzianum</i>	Blé Maïs	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Mahmoud, 2016), (Saravanakumar <i>et al.</i> , 2017), (Saravanakumar <i>et al.</i> , 2018) (Nayaka <i>et al.</i> , 2010)
<i>Clonostachys rosea</i>	Blé Maïs	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Kosawang <i>et al.</i> , 2014) (Karlsson <i>et al.</i> , 2015) (Palazzini <i>et al.</i> , 2013) (Xue <i>et al.</i> , 2014) (Gilbert and Haber, 2013) (Xue <i>et al.</i> , 2014), (Samsudin <i>et al.</i> , 2017)
<i>Aureobasidium pullulans</i>	Blé Maïs	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Wachowska and Głowacka, 2014) (Chulze <i>et al.</i> , 2015)
<i>Pythium oligandrum</i>	Blé	FHB / <i>F. graminearum</i>	(Faure <i>et al.</i> , 2018)
<i>Streptomyces</i> spp.	Blé	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Palazzini <i>et al.</i> , 2017) (Strub <i>et al.</i> , 2019a, 2019b) (Cavaglieri <i>et al.</i> , 2004; L. R. Cavaglieri <i>et al.</i> , 2005), (Baharlouei <i>et al.</i> , 2010)
<i>Streptomyces plicatus</i>	X	FHB / <i>F. graminearum</i>	(Zhao <i>et al.</i> , 2014),
<i>Bacillus subtilis</i>	Blé Maïs	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Mahmoud, 2016) (Danial <i>et al.</i> , 2020), (Snook <i>et al.</i> , 2009)
<i>Bacillus cereus</i>	Blé Maïs	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Gilbert and Fernando, 2004), (Figueroa-López <i>et al.</i> , 2016)
<i>Bacillus mycoides</i>	Blé	FHB / <i>F. graminearum</i>	(Gilbert and Fernando, 2004)
<i>Bacillus amyloliquefaciens</i>	Blé	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Schisler <i>et al.</i> , 2002); (Khan <i>et al.</i> , 2004) (Comby <i>et al.</i> , 2016), (L. Cavaglieri <i>et al.</i> , 2005; L. R. Cavaglieri <i>et al.</i> , 2005) (Pereira <i>et al.</i> , 2011)
<i>Microbacterium oleovorans</i>	Maïs	<i>F. verticillioides</i>	(Cavaglieri <i>et al.</i> , 2004; L. R. Cavaglieri <i>et al.</i> , 2005)
<i>Lysobacter enzymogene</i>	Blé Maïs	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Jochum <i>et al.</i> , 2006), (Palumbo <i>et al.</i> , 2007)

<i>Pseudomonas fluorescens</i>	Blé Maïs	FHB / <i>F. graminearum</i> <i>F. verticillioides</i>	(Nourozian <i>et al.</i> , 2006) (Khan and Doohan, 2009), (Nayaka <i>et al.</i> , 2009)
<i>Lactobacillus plantarum</i>	X	<i>F. graminearum</i> <i>F. verticillioides</i>	(Baffoni <i>et al.</i> , 2015), (Lavermicocca <i>et al.</i> , 2000) (de Melo Nazareth <i>et al.</i> , 2019)
<i>Pediococcus pentosaceus</i>	Maïs	<i>F. verticillioides</i>	(Dalié <i>et al.</i> , 2012)
<i>Azotobacter armeniacus</i>	Maïs	<i>F. verticillioides</i>	(L. R. Cavaglieri <i>et al.</i> , 2005)
<i>Cryptococcus flavescens</i>	Blé	FHB / <i>F. graminearum</i>	(Schisler <i>et al.</i> , 2002); (Khan <i>et al.</i> , 2004), (Dunlap <i>et al.</i> , 2015)
<i>Cryptococcus aureus</i>	Blé	FHB / <i>F. graminearum</i>	(Schisler <i>et al.</i> , 2011)

Champignons
 Oomycètes
 Bactéries
 Levures

Parmi les nombreuses souches qui montraient une efficacité contre les *Fusaria* toxigènes, très peu avaient fait l'objet de tests de toxicité sur l'homme ou l'environnement, ou de formulation, ce qui peut être un frein très important pour nombre d'entre elles afin de passer de l'étape préliminaire de screening à celle de la commercialisation (Yang *et al.*, 2011). En effet, la commercialisation des produits de biocontrôle doit être soumise à des processus en plusieurs étapes, notamment l'isolement des micro-organismes de l'écosystème naturel, l'évaluation du bio-agent à la fois *in vitro* et dans des conditions de serre, l'essai du meilleur isolat dans des conditions de terrain, la production de masse, la formulation, la livraison, la compatibilité, l'enregistrement et la commercialisation (Junaid *et al.*, 2013).

Dans le contexte de notre étude, le choix de travailler avec des souches commercialisées offrait plusieurs avantages : les micro-organismes avaient montré une efficacité contre des pathogènes de plantes dont des *Fusarium* spp., ils ne présentaient pas d'effets nocifs pour l'Homme ou l'environnement, ils étaient formulables et applicables au champ en condition naturelle, et leurs effets et mécanismes d'action sur les pathogènes mycotoxinogènes et leur production de mycotoxine n'était que partiellement caractérisés.

En Europe, il n'existe que deux agents de biocontrôle commercialisés contre *F. graminearum* sur les épis de blé : Polyversum® à base de *Pythium oligandrum* (DeSangosse) et Cerall® à base de *Pseudomonas chlororaphis* (Belchim Crop Protection). Cette faible offre disponible en termes d'agents commercialisés spécifiquement contre les fusarioses des épis nous a conduit à ouvrir la sélection à des BCAs dont les *Fusaria* des céréales ne sont pas la cible stipulée. De plus, dans la plupart des criblages de nouveaux BCAs, peu de différences peuvent être observées entre les candidats potentiels d'une même espèce, d'où l'intérêt d'étudier des BCAs de différents types pour observer un maximum de phénotypes (Luongo *et al.*, 2005; Schisler *et al.*, 2006). De plus, même parmi les agents commercialisés, certains mécanismes d'action restent incompris et constituent des zones d'ombre qui ne permettent pas une utilisation optimale contre les pathogènes mycotoxinogènes. Parmi les BCAs commercialisés, une attention particulière a été portée sur la

sélection de trois agents antagonistes qui représentaient une grande variété de types de micro-organismes, de cibles et d'usages recommandés. L'ensemble de ces critères nous a conduit à sélectionner les trois BCAs suivants : Mycostop/*Streptomyces griseoviridis* (Lallemand Plant Care®), Xedavir/*Trichoderma asperellum* (Xeda International®), et Polyversum/*Pythium oligandrum* (DeSangosse®).

1.4.2.1. *Streptomyces griseoviridis*

Streptomyces griseoviridis est une bactérie actinomycète de la famille des *Streptomycetaceae*, qui commence à être isolée à partir de tourbe de sphaigne de couleur claire, en Finlande, et caractérisée à partir des années 1980 (Tahvonen, 1982) (Figure 10). *In vitro* et sur milieu nutritif optimal, les colonies se développent lentement et produisent des filaments très fins qui s'apparentent à des hyphes mycéliens, ce qui fait partie des caractéristiques des actinomycètes. Ce mycélium est principalement blanc puis présente des nuances brunes ou orangées, et s'organise de manière très dense. Les conidies portées directement par le mycélium présentent une forme sphérique, de petites tailles (0.5-2 µm), elles sont souvent disposées en chaînette ou isolées et sont produites en grande quantité.

Streptomyces griseoviridis

Morphologie

500 µm

Mycélium

Conidies

200 µm

Classification

- Règne: Bacteria
- Division: Actinobacteria
- Classe: Actinobacteria
- Ordre: Actinomycetales
- Famille: Streptomycetaceae
- Genre: Streptomyces
- Espèce: Griseoviridis

Souche K61
Isolée à partir de:
Tourbe de sphaigne,
en Finlande

Nom commercial: **LALLEMAND**
Mycostop® LALLEMAND PLANT CARE

[5.10⁵ CFU.g⁻¹]

Année de commercialisation:
2014

Usage:
Traitement de sol généraux

Génome assemblé : 1
GCA_005222485.1
Mai 2019

0 gènes identifiés

NCBI

Figure 10 : le BCA *Streptomyces griseoviridis* et ses caractéristiques


Plusieurs études ont exploré le génome de *S. griseoviridis* ces dernières années. Sur NCBI, il existe 2 assemblages d'une unique souche, avec un génome d'une taille totale de 8 Mb (dernier numéro d'accèsion GCA_005222485.1, déposé en Mai 2019, base de données consultée le 15/08/2020, (National Center for Biotechnology Information)). La base de données ne référence pas de gènes séquencés et identifiés chez cette espèce.

La souche de *S. griseoviridis* que nous avons sélectionnée pour réaliser cette étude est la souche K61, commercialisée en France sous le nom de Mycostop (abrégé Myco) par la marque Lallemand Plant Care® à partir de 2014. Elle est recommandée pour des traitements généraux de sol à conditions que les légumes et fruits traités ne soient pas en contact avec le sol et pour les plantes non alimentaires et, est efficace contre *Fusarium* spp. (ephy | Catalogue des produits phytopharmaceutiques). Elle est formulée avec une concentration de 5.10^5 CFU.g⁻¹.


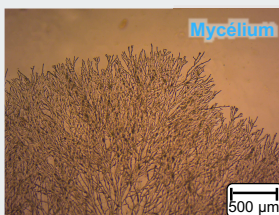

1.4.2.2. Trichoderma asperellum

Trichoderma asperellum est un champignon ascomycètes de la famille des *Hypocreaceae*, qui commence à être isolé autour de 1882 mais réellement caractérisé et classifié en 1999 (Samuels *et al.*, 1999) (Figure 11). *In vitro* et sur milieu nutritif optimal, les colonies se développent rapidement et produisent des filaments très ramifiés qui recouvrent la surface du milieu de culture. Son mycélium est d'abord blanc puis présente rapidement des nuances bleues à vertes principalement provoquée

Trichoderma asperellum



Morphologie

Classification

Règne	Champignon
Division	Ascomycota
Classe	Sordariomycetes
Ordre	Hypocreales
Famille	Hypocreaceae
Genre	Trichoderma
Espèce	Asperellum


Souche TV1

Isolée à partir de:
Racine de plants de tomates, en Italie


Génome

Génome assemblé : 1
GCA_013423425.1
Juillet 2020

12855 gènes identifiés



Nom commercial:

Xedavir® 

[1.10⁷ CFU.g⁻¹]

Année de commercialisation:
2013

Usage:
Traitement de sol généraux

par la synthèse de spores colorées mais peu de pigments sont diffusés dans le milieu de culture. Les conidies sont portées par des conidiophores ramifiés, généralement associés par paire à des phialides droits. Les conidies ont une forme globulaire à sous-globulaire et sont de petite taille (2-3 µm). Produites en très grandes quantité, elles sont donc pigmentées et disposées de manière relativement isolées (Samuels *et al.*, 1999).

Figure 11 : le BCA *Trichoderma asperellum* et ses caractéristiques

Plusieurs études ont exploré le génome de *T. asperellum* ses dernières années. Sur NCBI, il existe 4 assemblages provenant de l'étude de plusieurs souches, avec un génome d'une taille totale de 36Mb (dernier numéro d'accèsion GCA_013423425.1, déposé en Juillet 2020, base de données consultée le 15/08/2020, (National Center for Biotechnology Information)). La base de données référence 12 849 gènes séquencés et identifiés chez cette espèce. La souche de *T. asperellum* que nous avons sélectionnée pour réaliser cette étude est la souche TV1, isolée à partir de racines de plant de tomates dans une ferme près de Crémone en Italie. Cette souche est commercialisée en France sous le nom de Xedavir (abrégé Xeda) par la marque XedaInternational® depuis 2013. Elle est recommandée pour des traitements généraux de sol (ephy | Catalogue des produits phytopharmaceutiques), et est formulée avec une concentration de 1.10^7 CFU.g⁻¹.

1.4.2.3. *Pythium oligandrum*

Pythium oligandrum est un oomycète de la famille des *Pythiaceae*, qui commence à être isolé et caractérisé à partir des années 1940 (Drechsler, 1943). *In vitro* et sur milieu nutritif optimal, les colonies se développent très rapidement et recouvrent le milieu d'une fine couche de mycélium très ramifiés (Figure 12). Le mycélium est blanc, se propage de manière très régulière et ne produit pas de pigments. La reproduction de ce micro-organisme passe notamment par une phase asexuée, et une phase sexuée impliquant la production de structures très caractéristiques appelées oogones, sphériques et dentelées (20 µm), et qui contiennent des oospores (You *et al.*, 2019).

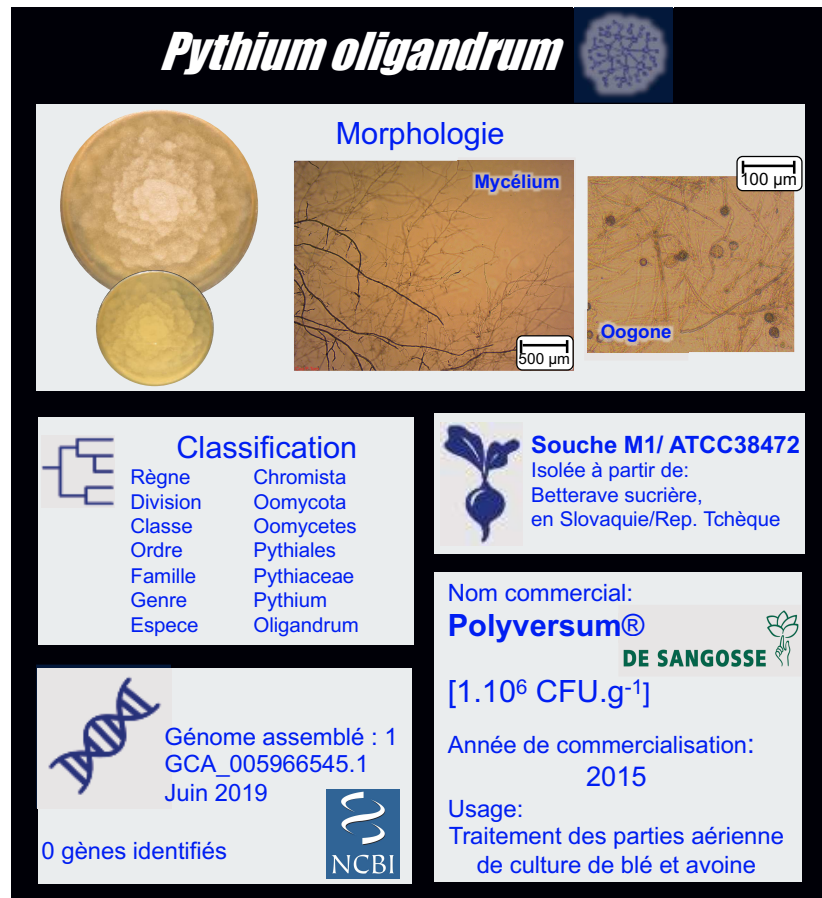


Figure 12 : le BCA *Pythium oligandrum* et ses caractéristiques.

D'importantes avancées ont pu être mises en évidence concernant le génome de *P. oligandrum* ces dernières années. Sur NCBI, il existe 3 assemblages et annotations de diverses souches, plus ou moins complets. La souche sélectionnée pour cette étude (n°ATCC38472) est la plus récemment décrite avec un génome d'une taille totale de près de 42 Mb (dernier numéro d'accèsion GCA_005966545.1, déposé en Juin 2019, base de données consultée le 15/08/2020, (National Center for Biotechnology Information)). En contrepartie, peu d'études ont cherché à séquencer et annoter des gènes spécifiques et la base de données ne référence pas de gènes chez cette espèce. Cette même souche de *P. oligandrum* est utilisée pour produire du Polyversum (abrégé Poly), une formulation commercialisée par DeSangosse®. Elle est recommandée pour des traitements des parties aériennes des céréales notamment le blé et l'orge (ephy | Catalogue des produits phytopharmaceutiques), et est formulée avec une concentration de 1.10⁶ CFU.g⁻¹.

1.4.3. Mécanismes d'action des BCAs :

Malgré leur commercialisation, des incertitudes persistent sur leurs efficacités contre les champignons des céréales du genre *Fusarium* et leur impact sur la production de mycotoxines mais également quant à certains de leurs mécanismes d'action connus (Nicot, 2012). Le développement de stratégies efficaces pour l'utilisation de ces BCAs dépend essentiellement de la compréhension des processus clés du biocontrôle et de leurs interactions. Sans une estimation fiable de ces processus, il est donc impossible d'améliorer les stratégies d'application des BCAs (Pal and Gardener, 2006).

Il est donc essentiel de comprendre les mécanismes à la base de l'interaction entre BCAs et pathogènes mycotoxinogènes dans le but d'optimiser leurs usages et de repérer des mécanismes particulièrement actifs qui par la suite pourraient être recherchés chez d'autres micro-organismes candidats afin de faciliter le screening de nouveaux BCAs efficaces.

1.4.3.1. Interactions trophiques

Comme le biocontrôle des maladies implique à la fois des plantes et des micro-organismes, les interactions qui conduisent à la lutte biologique ont lieu à plusieurs niveaux d'échelle. Le biocontrôle peut être considéré comme le résultat positif résultant d'une série d'interactions spécifiques et non spécifiques. En ce sens et afin de comprendre les mécanismes de contrôle biologique, il est utile d'apprécier les différentes stratégies d'interaction des organismes. La description de ces différents types d'interactions est extraite de (Pal and Gardener, 2006). Les types d'interactions ont été désignés comme suit depuis l'interaction la plus positive jusqu'à la plus néfaste pour les deux organismes considérés : mutualisme, commensalisme, neutralisme, compétition ou concurrence, amensalisme, parasitisme et prédation (Odum and Odum, 1953). Il est important de noter que ces types d'interactions ne peuvent avoir lieu sans un certain contact ou proximité entre les deux organismes considérés. Le mutualisme est une association entre deux ou plusieurs espèces dont les deux espèces tirent un bénéfice. Parfois, il s'agit d'une interaction obligatoire tout au long de la vie, impliquant un contact physique et biochimique étroit, comme ceux entre les plantes et les champignons mycorhiziens. Cependant, elles sont généralement facultatives et opportunistes. Le commensalisme est une interaction symbiotique entre deux organismes vivants, où un organisme profite et l'autre n'est ni lésé ni avantagé. La plupart des microbes associés aux plantes sont supposés être commensaux par rapport à la plante hôte, car leur présence, individuellement ou en totalité, entraîne rarement des conséquences ouvertement positives ou négatives pour la plante. Le neutralisme décrit les interactions biologiques lorsque la densité de population d'une espèce n'a absolument aucun effet sur l'autre. A partir de ce niveau, les autres modes d'interactions entre organismes peuvent présenter de réels atouts dans le biocontrôle. La compétition au sein des espèces et entre elles entraîne une diminution de la croissance, de l'activité et/ou de la fécondité des

organismes en interaction. Des BCAs peuvent entrer en compétition avec des agents pathogènes pour acquérir des nutriments dans et autour de la plante hôte. L'amensalisme est une interaction entre deux organismes vivants, où un organisme ne tire aucun bénéfice et l'autre est lésé. Le parasitisme est une symbiose dans laquelle deux organismes sans lien phylogénétique coexistent sur une période prolongée. Dans ce type d'association, un organisme appelé parasite, bénéficie de la symbiose et l'autre appelé hôte subit des dommages mesurables. Les activités de divers hyperparasites capable d'attaquer les pathogènes de plantes, peuvent donc donner lieu à du biocontrôle. Enfin, la prédation fait référence à la chasse et à la mise à mort d'un organisme par un autre pour la consommation et la subsistance. Si le terme de prédateur désigne généralement les organismes qui se nourrissent aux niveaux trophiques supérieurs dans le monde macroscopique, il a également été appliqué aux actions des microbes, par exemple les protistes, et de la mésofaune, par exemple les nématodes fongiques et les microarthropodes, qui consomment la biomasse des agents pathogènes (Pal and Gardener, 2006). Les modes de trophie de pathogènes ou non peuvent également influencer les interactions entre les êtres vivants : biotrophie (se développent que sur des hôtes vivants), nécrotrophie (tuent les cellules puis se développent sur des tissus morts), saprophytisme (dépendent d'une base nutritive constituée d'éléments morts) sont à considérer. De ces différents modes de vie globaux découlent différents mécanismes d'action à différents degrés et constitue la base du biocontrôle. En effet, la lutte biologique viable et à grande échelle résulte le plus souvent de l'optimisation du mutualisme entre plantes et organismes bénéfiques ou de l'antagonisme entre organismes bénéfiques et agents pathogènes (compétition, parasitisme, prédation).

1.4.3.2. Mécanismes d'action identifiés

Les principaux mécanismes des BCAs contre les *Fusarium* spp., mais aussi d'autres pathogènes de plantes peuvent être liés à l'antibiose, au parasitisme, à la compétition ou à l'inhibition de la synthèse des mycotoxines (Torres *et al.*, 2019) (Figure 13)

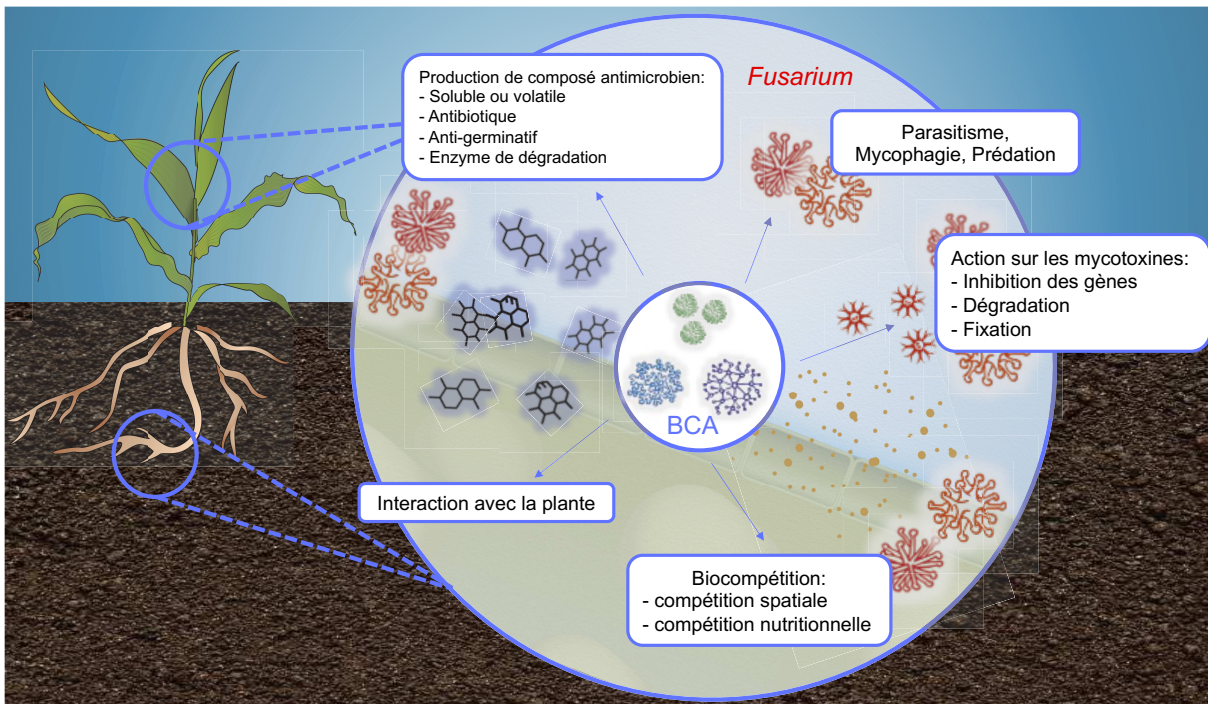


Figure 13 : Principaux mécanismes de biocontrôle potentiels pour lutter contre les *Fusaria* mycotoxinogènes (d'après certaines informations de (Torres *et al.*, 2019)).

1.4.3.2.1. Production de composés antimicrobiens

La production de composés antimicrobiens, associée au terme d'antibiose, peut regrouper les BCAs capables de synthétiser différents composés ayant une action sur la physiologie des pathogènes comme les antibiotiques, les enzymes lytiques, ou d'autres composés antifongiques (Reino *et al.*, 2008; Vallet *et al.*, 2017). Les BCAs peuvent posséder un arsenal de métabolites ciblant différentes structures de l'agent pathogène qui freinent sa croissance, réduisent la production de facteurs de virulence ou le tuent (effets anti-germination, dégradation des parois, des membranes, anti-mycotoxinogènes, même à faible dose). Par exemple, certaines enzymes lytiques peuvent hydrolyser une grande variété de composés polymères, notamment la chitine, les protéines, la cellulose, l'hémicellulose et l'ADN des pathogènes (Pal and Gardener, 2006). Les métabolites secondaires antifongiques peuvent avoir diverses formes (soluble, volatile, etc.). Parmi ces composés, ont été identifiés : les peptaïbols, le 6-pentyl- α -pyrone, la viridine, la gliovirine, les glisoprénines, la gliotoxine et d'autres (Daguerre *et al.*, 2014). Ils sont toxiques pour les champignons pathogènes à des concentrations qui dépendent du composé et de la cible (Raaijmakers *et al.*, 2009), et peuvent agir directement ou à distance.

Ces composés ont pu être identifiés pour de nombreux genres et espèces de BCAs incluant : *Trichoderma* spp., *Clonostachys* spp., *Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp., *Lactobacillus* spp., *Cryptococcus* spp., *Kluyveromyces* spp. et *Saccharomyces* spp. (Abdallah *et al.*, 2018).

Bien qu'il ait été prouvé que l'antibiose soit une arme majeure des BCAs contre les pathogènes des plantes, une résistance des maladies pourrait apparaître. Un exemple est connu pour *F. verticillioides* dans lequel un gène codant pour la lactamase a été identifié, et permet à l'agent pathogène de résister à certaines phytoanticipines produites dans les plantes, mais aussi éventuellement aux lactames microbiens (Kettle *et al.*, 2015), des produits de défense contre les phytopathogènes.

1.4.3.2.2. Parasitisme, mycophagie et prédation

Le mycoparasitisme est le parasitisme d'un micro-organisme fongique par un autre micro-organisme (Lecomte *et al.*, 2016). Ce mécanisme implique une attaque par contact direct du pathogène par le BCA qui va dégrader ou consommer celui-ci jusqu'à sa mort (Kim and Vujanovic, 2016). Il se traduit généralement par la pénétration physique du mycoparasite dans les hyphes de l'hôte via le développement d'organes particuliers tels que les haustoria (Jeffries, 1995). Le parasitisme implique généralement la synthèse de divers métabolites ou enzymes conduisant à la dégradation des structures fongiques suivie de l'absorption de nutriments/métabolites par le champignon hôte (Daguerre *et al.*, 2014). La limite très fine entre la capacité à se nourrir du mycélium vivant (plutôt appelée mycophagie) et la capacité à détourner les constituants du mycélium après synthèse de composés antifongiques fragilisant ou tuant celui-ci reste assez peu discutée. Il existe plusieurs parasites connus de pathogènes des plantes, capables d'agir en attaquant les structures de conservation des champignons pathogènes (par exemple *Coniothyrium minitans* capable d'attaquer les sclérotés) (Ridgway *et al.*, 2001) tandis que d'autres s'attaquent aux hyphes en développement (par exemple *Trichoderma*) (Haggag and Mohamed, 2002). Les mycoparasites les plus étudiés sont les espèces de *Trichoderma*, qui sont prédominants et généralistes dans les environnements naturels, végétaux et pédologiques (Annie Rousseau *et al.*, 1996). Mais le parasitisme a aussi été identifiés chez de nombreux genres et espèces (Adams, 1990), comme : *Clonostachys* spp. (Lübeck *et al.*, 2002), *Pythium nunn*, *Pythium oligandrum* (You *et al.*, 2019), *Talaromyces flavus* (Madi *et al.*, 1997), (*Para*)*Coniothyrium minitans* (McLaren *et al.*, 1996), mais aussi chez des BCAs bactériens comme *Streptomyces* spp. (Tu, 1986; Tarkka *et al.*, 2009). Un parasite spécifique de *F. graminearum* a aussi été décrit en association avec les cultures de blé : *Sphaerodes mycoparasitica* (Goh *et al.*, 2009).

Contrairement au mycoparasitisme, la prédation microbienne est plus générale, donc non spécifique à un agent pathogène et offre généralement des niveaux moins prévisibles de contrôle de la maladie. Certains BCAs présentent un comportement prédateur dans des conditions nutritionnelles limitantes (Pal and Gardener, 2006).

1.4.3.2.3. Biocompétition

D'autre part, les BCAs peuvent modifier des facteurs abiotiques qui influencent le développement des *Fusarium* spp. et la production de mycotoxines comme le pH ou la disponibilité en O₂, en nutriments

et en eau (Gardiner *et al.*, 2009; Picot *et al.*, 2010). Ces interactions conduisent à des phénomènes de biocompétition, lorsque deux ou plusieurs organismes sont présents simultanément (Abdallah *et al.*, 2018). Ils peuvent entrer en compétition nutritionnelle lorsqu'ils ont besoin du même nutriment essentiel (capacité intrinsèque à utiliser les macro-nutriments ou micro-nutriments libérés par la plante hôte), ou en compétition spatiale lorsqu'ils occupent le même espace limité (exclusion compétitive de l'accès à certains habitats privilégiés) (de Boer *et al.*, 2003). Cette biocompétition peut donc se produire lorsque des niches écologiques particulières sont simultanément occupées par des agents pathogènes et des BCAs qui ont les mêmes exigences physiologiques (Samsudin *et al.*, 2016). Il est plus difficile d'évaluer ce type de mécanismes, c'est pourquoi seulement peu de BCAs ont directement été reliés à la bio-compétition : (Celar, 2003; Liu *et al.*, 2013). Néanmoins, plusieurs études ont montré que ce phénomène pouvait être à l'origine de protection importante contre *F. graminearum* chez les céréales (Dawson *et al.*, 2004; Lakhesar *et al.*, 2010)

1.4.3.2.4. Mécanismes en lien direct avec la production de mycotoxines.

De nombreuses études ont mesuré l'impact de BCAs sur les *Fusaria* et parfois les mycotoxines, mais comme précisé précédemment les mécanismes spécifiques à la réduction des mycotoxines sont souvent ignorés dans le cadre des études concernant l'action de BCAs sur les souches mycotoxinogènes de *Fusarium*. Et pourtant, elles jouent un rôle très important dans l'interaction entre les agents et les pathogènes. Écologiquement parlant, les mycotoxines permettent aux souches de *Fusarium* considérées de faciliter leur colonisation des tissus végétaux mais surtout de se défendre contre d'autres micro-organismes présents dans le même environnement. Elles pourraient donc fortement affecter le comportement des agents de biocontrôle, mais cet aspect est rarement évalué (Lutz *et al.*, 2003). Réciproquement, les agents de biocontrôle ont développé des armes permettant de combattre spécifiquement ce trait de virulence des pathogènes, et ces modes d'action pourraient représenter un atout majeur en termes de sûreté sanitaire des aliments. Parmi ces mécanismes, l'inhibition des gènes de synthèse des toxines chez les pathogènes ou la biotransformation de ces molécules sont des moyens de maîtrise du risque mycotoxine.

La production de mycotoxines est gouvernée par un ensemble de gènes dépendant du type de mycotoxines et du pathogène (Choi and Shim, 2008; Alexander *et al.*, 2009). Certains agents antagonistes ont déjà montré des capacités à interagir directement avec les gènes de biosynthèse des mycotoxines. L'expression de plusieurs de ces gènes a été fortement inhibée en présence des BCAs et ont ainsi permis de réduire en amont la quantité de mycotoxine produite par les pathogènes. Parmi ces agents, le champignon mycoparasite *Clonostays rosea*, a été identifié comme inhibiteur des voies de biosynthèse des mycotoxines de *F. verticillioides* (Samsudin and Magan, 2016) ; ou *Pseudomonas* spp. qui a modifié l'acétylation des histones et ainsi bloquer la synthèse de DON (Chen *et al.*, 2018). Décrypter les mécanismes conduisant à l'induction ou à la répression de la biosynthèse

de ces toxines est indispensable pour pouvoir espérer définir des stratégies de maîtrise du risque (Atanasova-Penichon *et al.*, 2018).

En plus de réduire la production des mycotoxines, les BCAs sont capables de bio-transformer/détoxifier les mycotoxines, une fois qu'elles ont été produites par les pathogènes. Ces mécanismes en aval de la synthèse des toxines peuvent être liés à des phénomènes de dégradation enzymatique, de modification/complexation des toxines ou d'adhésion/fixation, permettant une réduction de la toxicité de ces composés. Plusieurs études ont montré la capacité de bactéries et de champignons à synthétiser des enzymes capables de dégrader ou modifier les mycotoxines, comme les bactéries lactiques ou des champignons des genres *Aspergillus*, *Rhizopus* et *Penicillium* spp. ou bien l'espèce *Clonostachys rosea* (Gromadzka *et al.*, 2009). Concernant la DON, différentes activités peuvent intervenir pour réduire la toxicité de cette molécule comme : de-époxydation réductive, acétylation, oxydation, épimérisation, glycosylation ou minéralisation (Karlovsky, 2011). Ces réactions donnent place aux principaux composés de dégradation de la DON : 3epi-DON, 3keto-DON, DOM1 (de-époxy-DON) et D3G (DON-3-O-glucoside). Concernant les fumonisines, la détoxification passe par une hydrolyse via une carbométhyl-transférase ainsi qu'une déamination. Ces réactions donnent place aux principaux composés de dégradation des fumonisines : fumonisine B1 hydrolysée (HFB1) ou 2-keto FB1 hydrolysée (McCormick, 2013). Enfin, certains micro-organismes sont littéralement capables de fixer ou d'adsorber des mycotoxines, incluant la DON. Ces phénomènes d'adhésion physique ont notamment été démontrés avec les parois bactériennes de bactéries lactiques (Haskard *et al.*, 2000; Fazeli *et al.*, 2009). D'autres recherches ont également montré que des spores fongiques peuvent adsorber des mycotoxines avec une efficacité entre 29% et 60%, grâce à des interactions hydrophobes avec la paroi (Jard *et al.*, 2011).

Les agents de biocontrôle les plus efficaces utilisent simultanément une combinaison de différents types d'interactions pour contrôler les pathogènes (Pal and Gardener, 2006). Ces différents niveaux d'échelles d'action des BCAs contre les pathogènes de plantes impliquent différents niveaux de compréhension de mécanismes et de leurs effets : par exemple, l'antibiose peut être démontrée par la présence de halos d'inhibition de la croissance de l'agent pathogène lors de confrontation *in vitro*, mais aussi par une analyse précise du ou des métabolites responsables de cette inhibition. De plus, ces modes d'actions peuvent également être liés les uns aux autres, par exemple la sécrétion d'une enzyme de dégradation peut être l'une des composantes de la stratégie globale de lutte basée sur le parasitisme ; ou plusieurs modes d'action indépendants peuvent avoir un effet synergique, comme la compétition nutritionnelle et l'antibiose.

La complexité de ces mécanismes à différents stades de l'évolution des agents pathogènes et leur synergie rendent leur analyse difficile, soulignant l'importance de mettre au point des démarches expérimentales fiables pour mieux comprendre leur fonctionnement.

1.5. Méthode de recherche et d'étude des mécanismes de biocontrôle

La plupart des pipelines de sélection et caractérisation des BCAs sont basés sur des approches fonctionnelles. En réponse à des problématiques fondamentales ou appliquées, des essais sont développés pour cribler ou caractériser, dans une collection de micro-organismes, les capacités d'inhibitions de différents traits pathogènes, ou la production de composés antifongiques. Le critère écrasant de discrimination des BCAs est leur capacité à réduire la croissance des pathogènes, vient ensuite leur capacité à produire des enzymes lytiques, des composés antifongiques et la mesure de l'impact pathogène sur la plante (chlorose, nécrose, etc.) (Raymaekers *et al.*, 2020). Ces recherches impliquent différents niveaux d'étude (*in vitro*, *in situ*, *in planta*) (Lecomte *et al.*, 2016). Le potentiel antagoniste du candidat est souvent testé lors d'un premier criblage en conditions *in vitro*, suivi par des tests d'efficacité dans des essais biologiques sur matrices végétales ou en conditions naturelles au champ (Köhl *et al.*, 2011). La caractérisation des mécanismes biologiques des BCAs engage l'intégration de nombreux champs disciplinaires (microbiologie, microscopie, biochimie, biologie moléculaire, etc.) (Figure 14). Le coût et le temps impliqué dans chacune de ces méthodes en fonction des objectifs est une question centrale lors du choix de stratégie à mettre en place dans le montage de projets. De plus, bien que ces recherches soient motivées par l'apport important en termes d'applications agronomiques, sanitaires et donc économiques, il permet aussi d'explorer l'aspect fondamental du décryptage des mécanismes de communication et d'interaction plante-BCA-pathogène.

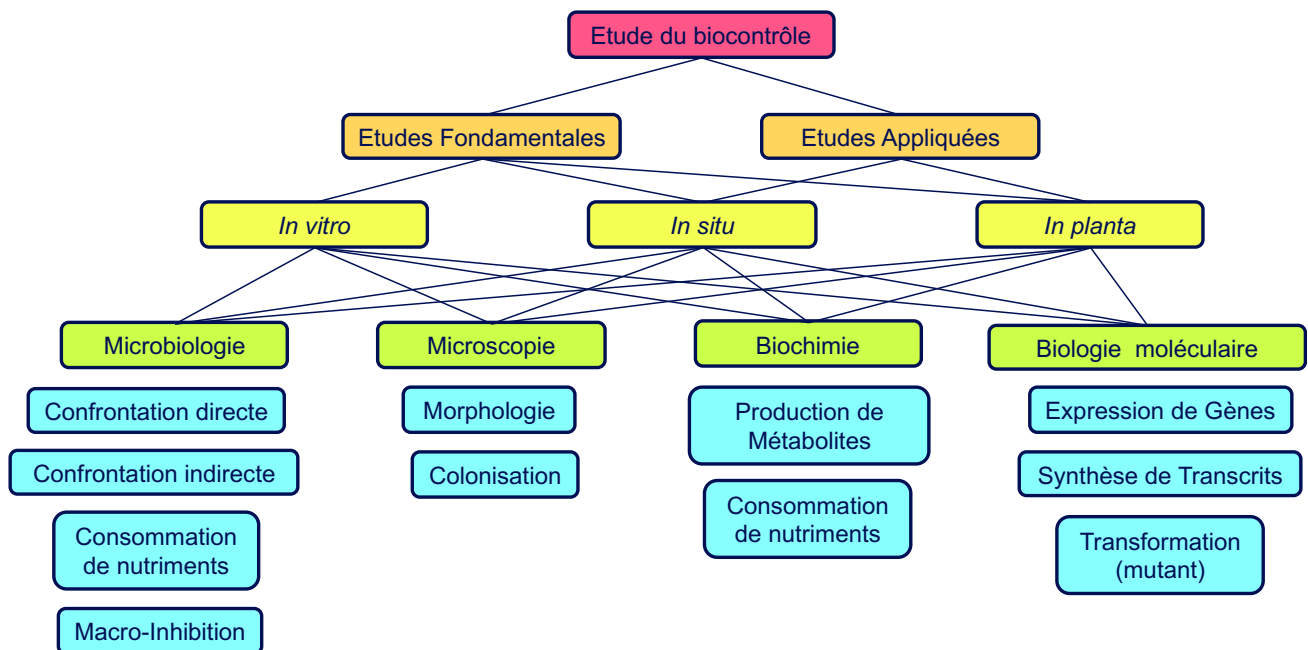


Figure 14 : Vue globale des méthodes de recherche et d'étude des mécanismes de biocontrôle.

1.5.1. Antagonismes *in vitro*

1.5.1.1. Confrontation en milieu solide

Le test de confrontation directe en boîte de Petri est l'un des tests les plus répandus pour détecter des activités antagonistes chez les BCAs en co-culture avec des pathogènes. Dans ce type d'essai, des milieux de culture (semi-)solide (à base d'agar) sont inoculés avec l'agent pathogène cible et le BCAs à tester. Les essais peuvent comprendre la confrontation d'un à six antagonistes et d'un agent pathogène. L'inoculation de l'antagoniste se fait à partir d'un point marqué dans la boîte et peut également varier en inoculant soit une goutte contenant une certaine quantité de CFU/ml, soit par dépôt à l'aide d'oeuse microbiologique ou soit à l'aide d'un plug de culture (cellules bactériennes, spores fongiques ou fragments mycéliens). Malgré une volonté d'utiliser des milieux de culture qui reflètent l'habitat nutritionnel des agents pathogènes, ces confrontations sont majoritairement réalisées sur des milieux de culture très riches et génériques comme le PDA, PCA, etc., (Adesina *et al.*, 2009; Zhao *et al.*, 2014; Schöneberg *et al.*, 2015; Berg *et al.*, 2017; Comby *et al.*, 2017) pour permettre le développement de deux micro-organismes qui peuvent être très différents. Peu d'importance est accordée au choix de ces milieux, pourtant il peut avoir une influence non-négligeable sur les activités de contrôle biologique observées, que ce soit en termes qualitatifs ou quantitatifs (Bosmans *et al.*, 2016). De plus, une bonne sélection des composants de ces milieux de culture peut permettre de favoriser la production des mycotoxines par les pathogènes afin d'identifier les BCAs qui auront un impact considérable même en cas de forte production (Gardiner *et al.*, 2009). Après incubation, différentes observations macroscopiques peuvent être réalisées. Des outils d'analyses d'images ont été développés pour mesurer l'inhibition du pathogène à partir des photos de boîte de Petri de confrontation (Szekeres *et al.*, 2006) mais d'autres peuvent être envisagés pour caractériser la production de pigments localisés, ou des zones de croissance superposées des deux micro-organismes. En effet, les modes de contacts des micro-organismes peuvent également donner des informations sur leur mode d'action : la présence d'inhibition de croissance du pathogène avant contact avec le BCA traduit la sensibilité du pathogènes aux composés antifongiques produits par le BCA (McGarvey *et al.*, 2012; Zhao *et al.*, 2014).

D'autre part, ces co-cultures peuvent être détournées en fonction du type de mécanisme que l'on cherche à identifier. Une des adaptations les plus décrites est la co-culture permettant l'identification de composés volatiles d'intérêt. Pour garantir le fait que l'inhibition observée repose exclusivement sur les composés volatils excrétés par le BCA, le BCA et l'agent pathogène doivent être cultivés dans un même volume mais séparés physiquement ce qui permet de différencier les effets des composés diffusibles dans le milieu de ceux des composés volatiles (Ryu *et al.*, 2003). Ce type de système peut être obtenus en scellant deux boîtes de Petri en sens inverses et sans couvercles (Humphris *et al.*, 2001; Stinson *et al.*, 2003), en créant une zone sans milieu de culture entre les points d'inoculation

de deux micro-organismes ou en utilisant des boîtes de Petri spécifiques avec des compartiments séparés (Ezra *et al.*, 2004; Fernando *et al.*, 2005; Fialho *et al.*, 2011)

Ces essais sont très pratiques, car ils permettent de tester un grand nombre d'organismes, et d'émettre des hypothèses sur leurs modes d'action, et ce à moindre coût, c'est pourquoi ils ont été largement décrits dans des publications récentes du monde entier pour rechercher de nouveaux antagonistes. Ces méthodes permettent d'évaluer l'effet des BCAs sur la croissance, le phénotype, la pigmentation des pathogènes lors de confrontation, cependant, lors de la recherche de BCAs présentant des mécanismes de biocontrôle très spécifiques, il n'est pas toujours possible de distinguer un ou plusieurs des cinq types de mécanismes présentés précédemment. De plus, de nombreux paramètres peuvent influencer l'interaction entre les micro-organismes comme la composition des milieux (même à échelle fine comme le type d'agar ou d'extrait de levure), le pH, l'activité de l'eau (a_w), etc. (Köhl *et al.*, 2011; Tanaka *et al.*, 2014; Bosmans *et al.*, 2016) et sont à réaliser en prenant toutes les précautions pour monitorer ces facteurs lorsque cela est possible.

1.5.1.2. Caractérisation microbiologique en milieu solide et liquide

Des méthodes microbiologiques ont également été développées dans le but de caractériser des traits spécifiques des BCAs.

Parmi ces méthodes, une des plus anciennes repose sur des substrats solides permettant spécifiquement la détection de composés en lien avec la production d'enzymes lytique. Par exemple, le milieu trypticase soja agar (TSA) est utilisé pour détecter les protéases ; la formation d'un halo transparent autour de la colonie indique une activité protéolytique (Cattelan *et al.*, 1999). Le même type d'observation peut être réalisée avec une grande quantité d'enzyme. Une étude (Tokpah *et al.*, 2016) a récemment évalué le comportement enzymatique de 935 isolats bactériens en terme de sécrétion de chitinases, cellulases, glucanases et protéases, à l'aide de milieux de culture adaptés. Les sidérophores impliqués dans la compétition nutritionnelle peuvent également être détectés avec un milieu de culture CAS (chrome azurol-sulfonate) (Schwyn and Neilands, 1987). Si les sidérophores sont sécrétés, ils chélateront le fer (Fe) dans le milieu et formeront un complexe Fe-sidérophores, faisant ainsi passer la couleur du milieu du bleu au jaune-orange. Cependant, le développement sur milieu solide et la mesure de halo d'inhibition/coloré ne permet pas toujours une identification fiable des capacités des BCAs (Calvente *et al.*, 2001).

Le profilage nutritionnel des micro-organismes est aussi une piste intéressante pour mieux comprendre les phénomènes de compétition pour différents nutriments exercés par les BCAs. La croissance des BCAs et de l'agent pathogène peut être facilement observées en mesurant la densité optique d'une culture liquide, et des systèmes de phénotypage des capacités de croissance de micro-organismes sur différents nutriments en microplaques ont été mis au point pour caractériser de manière fondamentale les capacités d'assimilation des micro-organismes (Gardiner *et al.*, 2009; Atanasova and Druzhinina, 2010) et pourrait être utilisés dans l'étude du biocontrôle.

Via la culture des BCAs en milieu liquide, il est aussi possible de produire de grandes quantités de composés sécrétés et potentiellement antimicrobiens par les BCAs. Pour détecter l'action des composés diffusibles dans le milieu, les surnageants de culture sont généralement purifiés à partir de ces milieux liquides en séparant la biomasse microbienne) (Zacky and Ting, 2015; Ambrico and Trupo, 2017). Ces « Cell-Free-Extract » peuvent ensuite être utilisés directement pour évaluer l'impact des composés excrétés par les BCAs sur les pathogènes, ou réservés pour des analyses plus poussées.

1.5.2. Antagonismes *in situ* sur organes détachés

Bien que l'identification de BCAs pour des effets antagonistes directs soient les plus couramment réalisés *in vitro*, des essais sur du matériel végétal ont également été signalés (par exemple, (Abraham *et al.*, 2010; Comby *et al.*, 2017; Perez *et al.*, 2017; Zhang *et al.*, 2017). Malgré quelques inconvénients pratiques, ces essais *in situ* permettent de tester le potentiel antagoniste du BCA dans des conditions plus proches des conditions naturelles ; et permet aussi le criblage contre des agents pathogènes difficiles à cultiver *in vitro*, comme les biotrophes obligatoires. En général, ces types de criblage sont effectués sur les parties de la plante sur lesquelles les infections ont lieu et où les symptômes de maladies sont observés. Les inoculations se font à partir d'inoculum produit en milieu synthétique, déposé ou pulvérisé sur l'organe, ou le matériel végétal est directement plongé dans l'inoculum. L'évaluation de l'impact des BCAs se fait principalement à partir d'évaluation visuelle de symptômes, via des échelles de notations. Plusieurs études ont notamment mis en évidence l'intérêt du test sur des épis de blé détachés qui fournit des informations sur la pathogénicité, la capacité de croissance et l'efficacité des souches de BCAs sur la plante ciblée, avant de les tester sur des plantes entières(Comby *et al.*, 2017; Rojas *et al.*, 2020). De plus, cela peut permettre de caractériser le caractère endophyte des BCAs ou encore de s'assurer de l'absence de pathogénicité sur les plantes cibles (Ait Bahadou *et al.*, 2018).

1.5.3. Antagonismes *in planta* en serre ou au champ

Après une validation *in vitro* et sur organes détachés, la seconde étape d'évaluation a lieu lors d'essais aux champs ou en serre. Ces essais qui se déroulent de manière similaire à ceux sur organes détachés mais sur des plantes entières, présentent l'avantage majeur d'être le plus proche des conditions environnementales naturelles du pathosystème (Pereira *et al.*, 2011). En effet, la capacité antagoniste de certaines souches est également altérée par différentes espèces de plantes hôtes. Cela s'explique par le fait que certaines espèces de BCAs endophytes préfèrent coloniser des génotypes de plantes particuliers et ainsi interagir plus efficacement avec les agents pathogènes (Arnold and Lutzoni, 2007; Martín *et al.*, 2015). Toutefois, en ce qui concerne la culture des plantes

d'essai, l'application des BCAs et des agents pathogènes, et l'évaluation du développement des symptômes, ces essais sont en général laborieux, envahissants et longs. C'est pourquoi les criblages à haut débit sont moins évidents, et un nombre relativement faible de BCAs sont testés de cette manière (Akram *et al.*, 2013). De plus sur le terrain, les paramètres abiotiques et biotiques variables limitent la reproductibilité des résultats et peuvent compliquer l'analyse et la comparaison mutuelle d'un grand nombre de candidats (Pliego *et al.*, 2011), encore plus celle de l'analyse de modes d'action en particulier.

De manière objective, malgré le fait que beaucoup de ces analyses aient permis d'identifier des agents de biocontrôle efficaces, ils font l'objet d'un débat long et intense. L'une des raisons est que de nombreux organismes efficaces pour la stimulation de la croissance des plantes et le biocontrôle *in vivo* ne présentent aucun antagonisme *in vitro* (Adesina *et al.*, 2009; Schreiter *et al.*, 2014). Les avis sont très partagés sur le fait de privilégier l'une ou l'autre des approches et sur la corrélation entre études *in vitro*, *in situ*, *in planta*. Selon une étude (Köhl *et al.*, 2011), lors de confrontations sur organes détachés, les partenaires du système tripartite (plante, agent pathogène et BCA) interagissent de manière optimale dans des conditions contrôlées qui sont représentatives du stade épidémiologique et des conditions environnementales ciblés dans la culture. Une autre étude portée par (Stadler and von Tiedemann, 2014) a corrélé des activités de biocontrôle entre les conditions de laboratoire *in vitro* et *in vivo*, mais ils n'ont pu confirmer aucune inhibition lors des essais sur le terrain. Dans une autre étude de criblage de BCAs sur épis de blé, une réduction de l'expression des symptômes et une biomasse réduite de *Fusarium graminearum* a été observée sur les épis détachés infectés, et a été confirmée dans les essais d'inoculation d'épi en serre. Il est intéressant de noter qu'aucun de ces isolats n'avait montré une réduction de la croissance de *F. graminearum* dans les essais de confrontation *in vitro* (Rojas *et al.*, 2020). Ces différences peuvent s'expliquer par l'impact du métabolisme actif de la plante sur l'interaction BCA-pathogène, comme décrit par (Boedi *et al.*, 2016) lors de l'analyse de l'impact des pathogènes sur blé vivant ou mort : la plante vivante supprimait activement la croissance fongique et favorisait une production de toxines beaucoup plus élevée par rapport au tissu végétal identique sans métabolisme, ce qui suggère que les molécules signalant l'induction de métabolites secondaires ne sont pas préexistantes ou ne sont pas stables dans la plante en quantité suffisante avant l'infection. Enfin, il apparaît clairement que seul une combinaison de différentes méthodes de criblage permet de tirer les avantages des différentes approches, notamment la manipulation à haut débit *in vitro* et le criblage *in vivo* dans des conditions naturelles (Raymaekers *et al.*, 2020)

1.5.4. Analyses d'échantillons

Avec des niveaux de complexité croissants, tous ces modes d'expérimentation peuvent donner lieu au prélèvement que ce soit de l'ensemble du système (milieu de culture, organes ou plants entiers) ou d'échantillons afin d'évaluer de manière plus précise leur composition. Le choix de l'échantillonnage et le mode de prélèvement sont essentiels et doivent être choisis pour être représentatif du système analysé.

1.5.4.1. Microscopie

La microscopie notamment permet une visualisation des interactions. Sans avoir besoin de matériel très perfectionné, des observations macro- et microscopiques de l'interaction entre BCA et pathogènes à l'aide de loupe binoculaire ou de microscope optique (Vallet *et al.*, 2017) permettent de visualiser la formation de structures de contact particulières comme les haustoria, d'observer des inhibitions de la germination des spores, ou des dégradations ou déformations de mycélium soutenant les hypothèses d'activités enzymatique ou antifongique. Des outils tels que la microscopie électronique à balayage, microscopie confocale ou à fluorescence, peuvent être très instructifs, ces équipements permettent également la visualisation de colonisation ou d'interaction entre micro-organismes (pathogène ou BCA) transformé(s) à l'aide de gènes rapporteurs réactifs à certaines longueurs d'onde lumineuses (comme la GFP) (Ilgen *et al.*, 2009).

1.5.4.2. Biochimie

Même si ce domaine de compétence est abordé précédemment dans des expériences à l'échelle macroscopique et qualitative, des outils bien plus performants sont à la disposition des chercheurs pour détecter ou identifier des métabolites fongiques d'intérêt ou les niveaux d'activité des enzymes les produisant.

Différentes activités enzymatiques peuvent être évaluées dans des prélèvements de culture liquide de BCAs, notamment les activités cellulase, chitinase, et glucanase, sont mesurées par la dégradation de substrats spécifiques marqués qui peuvent ensuite être quantifiés par des méthodes spectrophotométriques (de los Santos-Villalobos *et al.*, 2012). Différentes optimisations de ces méthodes ont permis de miniaturiser les expériences en microplaques, ou sous forme de kits, et donc de faciliter l'utilisation de ce type de test (Ramada *et al.*, 2010; Sharma *et al.*, 2013). D'autre part l'ajout d'inhibiteur a permis de les rendre plus spécifiques de l'action de certaines enzymes (Martínez-Absalón *et al.*, 2014). Des techniques bio-analytiques plus performantes comme la chromatographie liquide à haute pression (HPLC), sont maintenant utilisées pour détecter et quantifier les antibiotiques produits par des micro-organismes *in vitro* ou *in situ* (Bonsall *et al.*, 1997). La polyvalence, la capacité de résolution et la précision quantitative de cet outil en fait l'une des meilleures méthodes pour détecter la synthèse d'antibiotiques ou d'autres composés antifongiques par les BCAs.

Ces analyses peuvent se faire aussi sans *a priori* en comparant l'ensemble des molécules produites et détectées dans différentes conditions afin de relier la présence de certains de ces composés à des activités particulières, comme lors d'analyses métabolomiques. Elles peuvent être un atout pour démêler les stratégies impliquées dans les interactions entre agents pathogènes et BCAs. De plus, l'interaction directe entre les métabolites secondaires dérivés des agents de biocontrôle et les phytopathogènes modifie le profil du protéome et du transcriptome des plantes. Par conséquent, le profilage des métabolites est une approche précise et bien établie pour étudier les réponses des plantes à divers stress (White *et al.*, 2012).

En ce qui concerne les composés volatils, si la détection d'antagonisme est relativement simple à travers l'utilisation de systèmes séparés (comme présenté précédemment), pour déterminer et différencier les différents profils des BCAs ou pathogènes, on procède principalement grâce à des chromatographies en phase gazeuse associées à un spectromètre de masse (GC-MS), qui est une méthode puissante pour identifier et quantifier les composés volatils. Une méthode basée sur la micro-extraction en phase solide couplée à la GC-MS a été particulièrement documentée ces dernières années (Kluger *et al.*, 2013; Giorgio *et al.*, 2015; Wu *et al.*, 2015). Cependant des dispositifs plus perfectionnés pourraient être mis au point en particulier dans le cas de confrontation des micro-organismes avec des vitesses de croissances atypiques ou un développement volumineux, ou pour des suivis cinétiques.

Spécificité liée aux pathogènes mycotoxinogènes, la détection et quantification des mycotoxines doivent être plus largement considérées lors de l'étude des mécanismes de biocontrôle. L'analyse des concentrations en mycotoxines dans des échantillons traités ou non avec les BCAs peut être un bon point de départ (Juodeikiene *et al.*, 2017). Pour aller plus loin, il n'existe pas de méthode consensus pour analyser l'impact direct des BCAs sur la production des mycotoxines, néanmoins de nombreux travaux ont porté sur les capacités de détoxification des BCAs. Ces essais sont en général réalisés en mettant en contact le BCA avec la mycotoxine purifiée, en milieu solide ou liquide, puis en monitorant sa disparition. Grâce à des méthodes d'analyses spectrales perfectionnées, type HPLC-MS-MS, il est possible de rechercher les produits de dégradation de ces mycotoxines pour mieux comprendre les activités enzymatiques ou chimiques à l'origine de ces transformations (Ikunaga *et al.*, 2011).

Enfin des méthodes plus performantes permettent d'aller plus loin dans l'analyse de la répartition, du type ou de la structure de composés identifiés comme bénéfiques contre les pathogènes de plantes. Ce type d'analyses, ayant pour but la découverte de nouveaux composés antimicrobiens ou une meilleure compréhension de leur rôle, peuvent être réalisées via des pipelines complexes impliquant des outils tel que spectrométrie de masse à transformée de Fourier par résonance cyclotronique d'ions à ultra-haute résolution (ICR-FT/MS) ou/et la spectroscopie de résonance magnétique nucléaire (RMN) pour identifier toutes les substances bioactives dans des systèmes complexes (Haesler *et al.*, 2008), ou encore l'imagerie par spectrométrie de masse (MSI) (Vallet *et al.*, 2017).

Cette dernière est maintenant communément utilisée pour la cartographie chimique des systèmes microbiens

1.5.4.3. Biologie moléculaire

Les techniques de biologie moléculaire ciblée jusqu'au séquençage dernière génération représentent un puissant outil encore sous-exploré/sous-employé dans le domaine du biocontrôle et de la sécurité alimentaire. L'éco-génomique offre la possibilité de mieux comprendre les caractéristiques écologiques des organismes bénéfiques, tant au niveau de la souche unique qu'au niveau de la communauté, et également en vue de leur dissémination volontaire dans l'environnement. Ce domaine de recherche relativement nouveau offre de nombreuses possibilités de découvrir les principaux mécanismes moléculaires qui contrôlent les interactions entre les micro-organismes et les plantes et entre les micro-organismes bénéfiques et pathogènes (Martin, 2014). Ces approches peuvent se faire de manière ciblée ou totale, que ce soit sur l'ensemble des gènes ou des transcrits et comprend l'identification, l'analyse de séquence, l'expression voire de la transformation (Massart and Jijakli, 2007).

1.5.4.3.1. Gènes, Transcrit, Mutants : approche ciblée

Une première approche impliquant la biologie moléculaire peut être ciblée, comme la détection directe et précise de ces micro-organismes. Pour chacun des micro-organismes, l'ADN est extrait à partir de matrice plus ou moins complexe et des gènes cibles sont recherchés et quantifiés. Par exemple, la qPCR est un excellent outil pour détecter et quantifier les pathogènes dans différents tissus de l'hôte, mais aussi plus spécifiquement détecter et quantifier les champignons producteurs de mycotoxines présents (Hamada *et al.*, 2012; Sanzani *et al.*, 2012). Les tests qPCR spécifiques aux gènes de production de toxines peuvent être utilisés pour étudier la structure des populations d'agents pathogènes, la concurrence entre les sous-populations productrices et non productrices de toxines et les effets des stratégies de gestion des maladies sur la réduction des contaminations par les toxines (Luo *et al.*, 2009; Sanzani *et al.*, 2009)

Du côté du BCA, ces mêmes approches peuvent être utilisées pour détecter et quantifier la colonisation d'un agent (Savazzini *et al.*, 2008), ou encore identifier ses capacités potentielles, comme la synthèse d'antibiotique (Zhang *et al.*, 2006). De la même manière, si une étape de transcription inverse est ajoutée avant analyses ou si c'est l'ARN qui est extrait, il est possible pour ces deux types de micro-organismes de suivre l'activation de certains gènes cibles (par exemple l'activation des gènes de biosynthèse des mycotoxines des pathogènes ou l'expression de gènes en lien avec des mécanismes de biocontrôle comme les gènes codant des chitinases ou d'antibiotiques des BCAs) (Samsudin and Magan, 2016).

D'autres méthodes de transformation impliquant des gènes rapporteurs, comme la GFP, peuvent permettre une meilleure compréhension de l'activité de l'un ou l'autre des micro-organismes. Le grand

avantage de cette protéine est qu'une fois exprimée par le micro-organisme étudié, la visualisation de ce dernier est immédiate sous microscopie à fluorescence. Cette technique, qui gagne rapidement en popularité, a été particulièrement exploitée pour étudier les interactions plantes-pathogènes (Chen *et al.*, 2003; Bolwerk *et al.*, 2005) cependant comme très peu de BCAs ont été manipulés génétiquement (Lübeck *et al.*, 2002), cette technologie n'a pas encore été pleinement exploitée pour l'étude des interactions complexes plantes-pathogènes-BCAs. Des transformations plus poussées ont conduit à la production de mutant. Par exemple, il a été démontré que des souches mutantes incapables de produire des phénazines (Bonsall *et al.*, 1997) ou des phloroglucinols (Keel, 1992) sont toujours aptes à coloniser la rhizosphère mais beaucoup moins capables de supprimer les maladies racinaires transmises par le sol que les souches correspondantes de type sauvage.

1.5.4.3.2. Génome et Transcriptome : approche globale

De la même manière que pour la biochimie et l'analyse des métabolites, en biologie moléculaire l'analyse des gènes et des transcrits peut se faire sans a priori afin de détecter parmi l'ensemble des gènes ceux qui sont potentiellement liés à l'interaction entre BCAs et pathogènes. Le séquençage complet du génome de plusieurs agents de biocontrôle tels que *Trichoderma atroviride*, *T. virens*, *T. reesei*, *Bacillus brevis*, *Pantoea vagans*, *Microbacterium barkeri* et *Pseudomonas fluorescens* F113 est actuellement disponible, facilitant ainsi la recherche (Ram *et al.*, 2018). L'analyse du transcriptome des BCAs afin d'identifier des gènes liés aux propriétés de biocontrôle de la souche est un travail long et fastidieux. Par exemple, une étude a séquencé 3298 ADNc de *Trichoderma harzianum* par la technique EST (Expressed Sequence Tag) (Liu and Yang, 2005). Ils ont identifié des ADNc présentant une similarité avec les protéines impliquées dans le biocontrôle et les ont classés selon leurs mécanismes d'action potentiels : action antifongique, mycoparasitisme, compétition pour l'espace et les nutriments, et inactivation de la protéase des pathogènes. Une étude plus récente conduite sur *Bacillus velezensis* grâce à des approches de « génome mining » (qui consiste à obtenir diverses informations sur l'organisme à partir de l'analyse du génome), a montré l'existence de 13 groupes de gènes biosynthétiques (BGC) codant la synthèse de métabolites secondaires ayant des fonctions de biocontrôle, ce qui a été confirmé par des analyses chimiques (Chen *et al.*, 2018). De même, l'analyse du transcriptome de l'agent pathogène peut conduire à l'identification d'effets répressifs du BCAs sur certaines fonctions biologiques, comme notamment la synthèse de mycotoxines.

C'est l'ensemble de toutes ces approches qui permet une vision plus large des interactions entre BCAs et pathogènes. L'idéal dans notre cas de figure, est d'avoir le maximum d'informations pour pouvoir relier les phénomènes entre eux, et ne pas passer à côté de mécanismes de biocontrôle efficaces. En effet, il est crucial d'envisager différentes approches expérimentales, car l'efficacité des BCAs peut varier considérablement en fonction des conditions d'incubation (Schöneberg *et al.*, 2015).

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Objectifs de la Thèse

2. OBJECTIFS DE LA THESE

Grâce à cette synthèse bibliographique, nous avons pu aborder les problématiques majeures liées aux mycotoxines, et à leurs producteurs du genre *Fusarium*, notamment les risques sanitaires importants que peuvent représenter ces molécules toxiques à travers le monde. Pour lutter contre ces champignons, plusieurs stratégies alternatives aux produits phytosanitaires ont été abordées mais une solution semble particulièrement prometteuse dans le cadre d'une gestion intégrée des cultures : le biocontrôle via l'utilisation de micro-organismes antagonistes. Ces agents de biocontrôle (BCAs) vont réduire par différents mécanismes la progression de ces champignons et limiter l'accumulation de mycotoxines dans les productions agricoles. Ces mécanismes sont très complexes, ils peuvent être : liés à la synthèse de composés antimicrobiens, au parasitisme, à la compétition ou à une action directe sur les mycotoxines ; directs ou indirects ; combinés ou seuls ; spécifiques ou non d'un pathogène ou d'un stade de vie. La mise en place de telles méthodes de protection nécessite une meilleure compréhension de ces mécanismes biologiques et de leurs particularités lors de l'interaction entre les moisissures mycotoxinogènes et BCAs, afin de favoriser les phénomènes d'antagonismes, optimiser l'utilisation des BCAs, mais aussi faciliter la sélection de nouveaux candidats de biocontrôle en fonction des processus les plus prometteurs.

Dans ce contexte les objectifs de cette étude sont les suivants :

- (i) Évaluer les effets d'Agents de Biocontrôle sélectionnés sur la croissance et la production de mycotoxines des pathogènes du genre *Fusarium* en conditions contrôlées.
- (ii) Décrypter les mécanismes d'actions potentiels des BCAs à l'origine des inhibitions observées, grâce à la mise au point et la réalisation de tests spécifiques, à l'identification et la quantification de certains modes d'action, mais aussi par leur chronologie dans la cinétique d'interaction BCA-pathogènes.
- (iii) Transposer les confrontations *in situ* et mettre en relation les effets *in vitro* et *in situ* des BCAs sur *F. graminearum*

La suite du manuscrit est donc structurée en 3 études expérimentales correspondantes, présentées au format d'articles scientifiques avec parfois des résultats complémentaires.

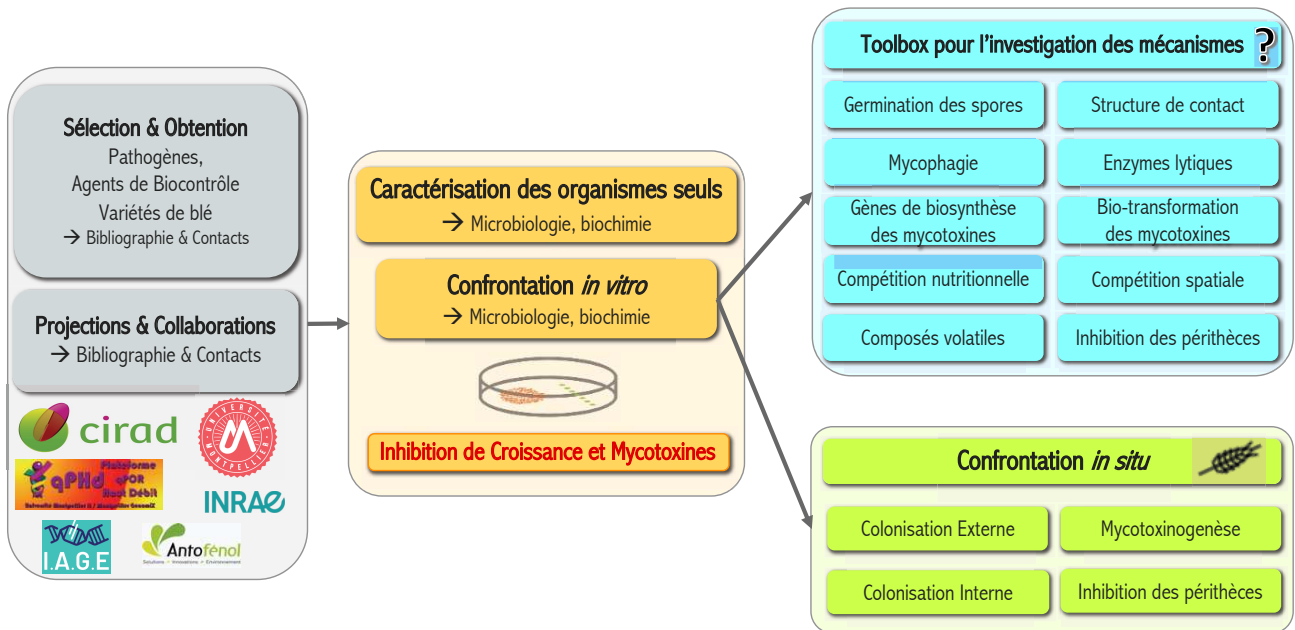


Figure : Schéma général du projet de thèse.

Partie Expérimentale

Partie Expérimentale

Étude n°1

3. PARTIE EXPERIMENTALE

3.1. Étude n°1 :

Évaluation *in vitro* des effets des Agents de Biocontrôle sur des souches mycotoxinogènes de *Fusarium*.

Cette partie fait l'objet d'une communication écrite, sous forme d'article de recherche parue dans le journal *Toxins* :

Commercial Biocontrol Agents reveal contrasted comportment in the face-to-face against mycotoxinogenic *Fusarium* species.

Lucile Pellan ^{1,*}, Noël Durand ^{1,2}, Véronique Martinez ¹, Angélique Fontana ¹, Sabine Schorr-Galindo ¹ and Caroline Strub ¹

¹Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Montpellier, France

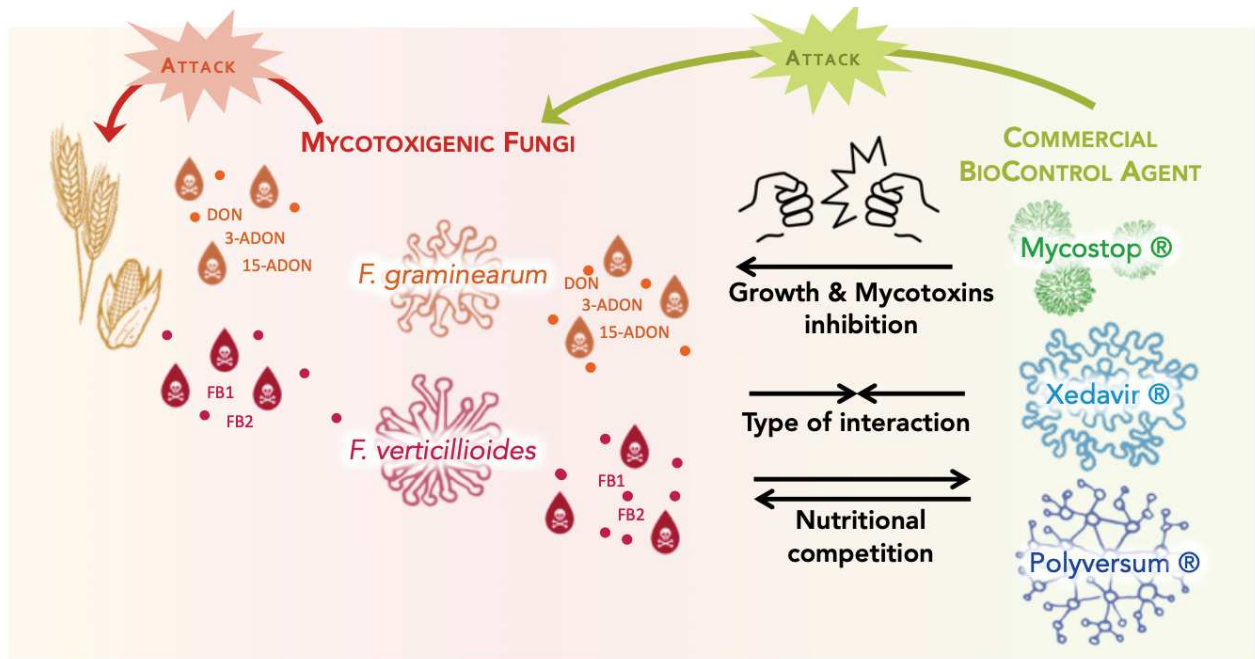
²CIRAD, UMR Qualisud, F-34398 Montpellier, France

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Dans cette première partie, l'impact *in vitro* des trois BCAs sélectionnés a été évalué contre les deux pathogènes *F. graminearum* et *F. verticillioides*. L'analyse primaire des comportements des micro-organismes sur différents milieux de culture a permis d'identifier les conditions optimales pour la croissance et la production de mycotoxines des pathogènes tout en permettant une croissance des BCAs, et ainsi optimiser les essais en confrontation. Ces essais réalisés en boîte de Petri et sur lame, sur un milieu de culture commun aux différents BCAs et un milieu de culture spécifique, ont permis d'avoir une première approche concernant les interactions entre BCA et pathogène. Les analyses des cinétiques de confrontation ont révélé des activités antagonistes variables avec différents schémas d'inhibition impliquant différentes stratégies de biocontrôle des pathogènes. Le premier BCA, *S. griseoviridis* (Myco) provoque une zone d'inhibition de la croissance du pathogène avant contact où la structure du mycélium de l'agent pathogène est altérée. Ce type d'observation suggère la diffusion de composés antimicrobiens, bien que son effet global sur la croissance et la production de mycotoxines soit limité par sa faible capacité de croissance. En revanche, les deux autres BCAs, *T. asperellum* (Xeda) et *P. oligandrum* (Poly) sont capables de coloniser les milieux plus rapidement que les agents pathogènes. Les observations microscopiques de l'interaction entre *T. asperellum* et les pathogènes ont montré une dégradation du mycélium pathogène lors du contact, suggérant cette fois des activités chitinolytiques. Lors de la co-culture avec *F. graminearum*, ce BCA a réduit la croissance et la concentration de mycotoxines de 48% et 72%, respectivement. Contre *F. verticillioides*, 78% d'inhibition de la croissance et 72% d'inhibition de la production de mycotoxines ont été mesurées. D'autre part, *P. oligandrum* était capable de progresser par-dessus les colonies d'agents pathogènes, ce qui suggère un type d'interaction étroite comme le mycoparasitisme, et qui

semble être confirmé par les observations microscopiques. Il a également pu agir sur la croissance et la production de mycotoxines chez *F. graminearum* avec 79 % et 93 % de réduction respectivement, et chez *F. verticillioides* avec 49% et 56% d'inhibition respectivement. Étant donné l'impact important des milieux de culture sur les interactions, un profilage nutritionnel des micro-organismes a également permis d'explorer les potentielles compétitions nutritionnelles entre les BCAs et pathogènes.

Mots-clé : agents antagonistes, confrontation *in vitro*, mycotoxines, compétition nutritionnelle.




Résumé graphique



Article

Commercial Biocontrol Agents Reveal Contrasting Compartments Against Two Mycotoxigenic Fungi in Cereals: *Fusarium Graminearum* and *Fusarium Verticillioides*

Lucile Pellan^{1,*}, Noël Durand^{1,2}, Véronique Martinez¹, Angélique Fontana¹, Sabine Schorr-Galindo¹ and Caroline Strub¹ 

¹ Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Montpellier, France; noel.durand@cirad.fr (N.D.); veronique.martinez@umontpellier.fr (V.M.); angelique.fontana@umontpellier.fr (A.F.); sabine.galindo@umontpellier.fr (S.S.-G.); caroline.strub@umontpellier.fr (C.S.)

² CIRAD, UMR Qualisud, F-34398 Montpellier, France

* Correspondence: lucile.pellan@umontpellier.fr

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Abstract: The aim of this study was to investigate the impact of commercialized biological control agents (BCAs) against two major mycotoxigenic fungi in cereals, *Fusarium graminearum* and *Fusarium verticillioides*, which are trichothecene and fumonisin producers, respectively. With these objectives in mind, three commercial BCAs were selected with contrasting uses and microorganism types (*T. asperellum*, *S. griseoviridis*, *P. oligandrum*) and a culture medium was identified to develop an optimized dual culture bioassay method. Their compartment was examined in dual culture bioassay in vitro with both fusaria to determine growth and mycotoxin production kinetics. Antagonist activity and variable levels or patterns of mycotoxinogenesis inhibition were observed depending on the microorganism type of BCA or on the culture conditions (e.g., different nutritional sources), suggesting that contrasting biocontrol mechanisms are involved. *S. griseoviridis* leads to a growth inhibition zone where the pathogen mycelium structure is altered, suggesting the diffusion of antimicrobial compounds. In contrast, *T. asperellum* and *P. oligandrum* are able to grow faster than the pathogen. *T. asperellum* showed the capacity to degrade pathogenic mycelia, involving chitinolytic activities. In dual culture bioassay with *F. graminearum*, this BCA reduced the growth and mycotoxin concentration by 48% and 72%, respectively, and by 78% and 72% in dual culture bioassay against *F. verticillioides*. *P. oligandrum* progressed over the pathogen colony, suggesting a close type of interaction such as mycoparasitism, as confirmed by microscopic observation. In dual culture bioassay with *F. graminearum*, *P. oligandrum* reduced the growth and mycotoxin concentration by 79% and 93%, respectively. In the dual culture bioassay with *F. verticillioides*, *P. oligandrum* reduced the growth and mycotoxin concentration by 49% and 56%, respectively. In vitro dual culture bioassay with different culture media as well as the nutritional phenotyping of different microorganisms made it possible to explore the path of nutritional competition in order to explain part of the observed inhibition by BCAs.

Keywords: antagonistic agents; in vitro dual culture bioassay; mycotoxins; nutritional competition

Key Contribution: Complementary and integrative approaches to characterize the impact of three distinct commercial biocontrol agents on the pathogenic capacities of two mycotoxigenic fungi, including the monitoring of growth, spore production, mycotoxin production or nutritional profile.

1. Introduction

Many plant pathogens endanger agriculture, particularly in cereal production. Some of these phytopathogens can produce mycotoxins and have multi-destructive effects through reducing the yield and nutritional properties of grain, and can also affect its health-related characteristics. Among mycotoxigenic fungi, the genus *Fusarium* is the most prevalent and represents a significant risk [1].

Mycotoxins are small toxic molecules resulting from the secondary metabolism of these fungi. *Fusarium graminearum* is able to produce deoxynivalenol (DON) and its derivatives 15 and 3-ADON. *Fusarium verticillioides* mainly produces fumonisins B₁ and fumonisins B₂ (FB₁ and FB₂). They are ubiquitous in agricultural production, and cereal crops are the primary source of consumer exposure to mycotoxins [2]. Mycotoxins are quite stable molecules which are very difficult to remove or degrade, and are found along the food chain while retaining their toxic properties. They can cause acute and chronic intoxication in both humans and animals depending on different factors (intake levels, duration of exposure, toxin type, mechanisms of action, metabolism, and defense mechanisms) [3]. The symptoms can be very variable depending on organisms considered, but can range from food poisoning or gastric-intestinal problems to suppression of the immune system, the induction of cancer, or death [4,5]. Sometimes the combination within trichothecenes/fumonisins or with other mycotoxins such as ochratoxins or aflatoxins can produce a synergistic toxic effect [6,7]. Given their high toxicity, DON and fumonisins have been regulated by the European Union and the maximum recommended levels of mycotoxins are 750 µg kg⁻¹ and 1000 µg kg⁻¹, respectively, for processed cereal intended for human consumption (Commission Regulation (EC) No 401/2006). Economically, these contaminants hamper international trade and significantly affect the world economy. Because their appearance can occur before or after harvesting, one of best ways to reduce the presence of mycotoxins in food and feed is to prevent their formation in the crop [8].

Agricultural production is subject to a scissor effect with, on one hand, very evolutive climatic conditions that promote microbial development and, on the other hand, the political determination to reduce the use of phyto-chemical products. The adverse effects of plant protection products on environment and on human and animal health have encouraged the European Union to promote the search for alternative and environmentally friendly solutions such as integrated pest protection and the use of biological control agents (BCAs) [9]. Non-pathogenic, they can prevent or reduce the progression of these fungi through different mechanisms. These mechanisms are very diverse, direct or indirect, and can be used together or alone depending on the biocontrol agent. The main direct mechanisms may be related to antibiosis, competition, parasitism, or toxin biotransformation [10]. Antibiosis includes the production of secondary metabolites like antibiotics, cell wall-degrading enzymes, and molecules that can suppress growth or kill pathogens [11–13]. Competition can occur when two or more fungi are present simultaneously. Microorganisms can then enter into nutritional competition when they need the same essential nutrient, or into spatial competition when they occupy the same limited space [14,15]. Parasitism is a direct attack of pathogen by the BCA that will degrade or consume the pathogen host until it dies [16]. Some mechanisms can be indirect. Colonization of the plant by beneficial microorganisms can trigger local or systemic defensive responses, enhancing resistance against phytopathogens [17,18].

Although the production of mycotoxins by toxigenic pathogens is of economic importance, many studies do not take this into account when studying biological control strategies. These studies are then limited to the fungicidal or fungistatic effects of BCAs, while the effect of BCAs on mycotoxin production is often neglected [19]. Moreover, most studies on the biocontrol of other plant pathogens do not consider the potential presence of several mycotoxins simultaneously in the crop, nor the side effects of BCAs on the mycotoxigenic agents present. Despite more than 70 years of intensive research and some promising results, only a few BCAs are currently available on the market, and for *Fusarium spp.* for instance in Europe, only *Pseudomonas chlororaphis* and *Pythium oligandrum* are available, as the commercial products Cerall® (Belchim Crop Protection) and Polyversum® (Biopreparáty/De Sangosse), respectively.

The lack of commercially available BCAs for mycotoxigenic fusaria may be due to numerous factors (unstable biocontrol efficacy under field conditions, strict storage and transport conditions for BCAs, complexity of registration) and more importantly a lack of knowledge about biocontrol mechanisms [20], especially concerning mycotoxins.

Indeed, a better understanding of the mechanisms that allow the biocontrol of pathogens, especially on marketed agents that have passed all the stages of formulation and registration, will make it possible to identify particularly effective mechanisms or biomarkers of mechanisms. This information could be used to optimize the use of existing BCAs but also to refine the selection of new BCAs.

The ultimate goal of the present study was to characterize the impact of commercial biocontrol agent on life traits of mycotoxigenic fungi (growth and mycotoxinogenesis). Therefore, the objectives were (1) to develop optimal methods of dual culture bioassay, allowing the analysis of the interaction between pathogens and BCAs; (2) to identify effect of BCAs on growth and mycotoxins of *Fusarium graminearum* and *Fusarium verticillioides*; and (3) to propose a hypothesis on the modes of action of BCAs against mycotoxigenic pathogens. With these objectives in mind, three commercial BCAs were selected with contrasting uses and microorganism types (*T. asperellum*, *S. griseoviridis*, *P. oligandrum*) and studied with in vitro dual culture bioassay against *F. graminearum* and *F. verticillioides* with an optimized method promoting mycotoxin production. Variable levels of mycotoxinogenesis and growth reduction were observed depending on the microorganism type of BCAs or on the culture conditions (e.g., different nutritional sources), suggesting contrasting biocontrol mechanisms. Macroscopic and microscopic observation of interactions support this hypothesis, finding various structural formations of BCA. Nutritional phenotyping was used to detect the possibility of nutritional competition between microorganisms, and to identify the trophic requirements of BCAs.

2. Results

To permit the interaction between BCAs and pathogens during dual culture bioassay, the first step was to select appropriate media to allow the development of each microorganism's pairing (BCA/pathogen). BCA/pathogen selection criteria and descriptions are presented in Section 5, Material and Methods. Then, to characterize this interaction on selected media, integrative approaches were used. Microscopic and macroscopic observations were undertaken to identify specific compartment of BCAs and their effects on *Fusarium* physiology. The BCA treatment's impact on growth, global mycotoxins, and specific mycotoxin production kinetics of pathogens was analysed. Finally, the impact of differential nutritional resources during dual culture bioassay was analysed and linked with the nutrient profiling of all microorganisms.

2.1. Compartments of Microorganisms and Culture Media Selection

After 8 days of incubation, all microorganisms were able to grow on all different media, with the exception of Polyversum® (Poly), which was unable to grow on Czapeck agar (CZA) synthetic medium (Figure 1). Mycostop® (Myco), which has the most limited growth (area under the growth curve, $AUGC < 1.85 \times 10^2$), was an excellent spore producer (spores $> 3.85 \times 10^3$), especially on International *Streptomyces* Project Medium 2 (ISP2). Xedavir® (Xeda) was minimally affected by the culture medium variation. Polyversum® (Poly), the oomycete BCA, was characterized solely on growth capacity because oospore obtention was a long and complex process which is difficult to achieve, and to obtain oospores under these specific in vitro conditions. *F. graminearum* produced a low quantity of spores affected by medium variation. For both pathogens, global mycotoxin production was a very discriminant variable for medium selection. The culture media on which pathogens *F. graminearum* and *F. verticillioides* produced the most mycotoxins were respectively CZA and PDA.

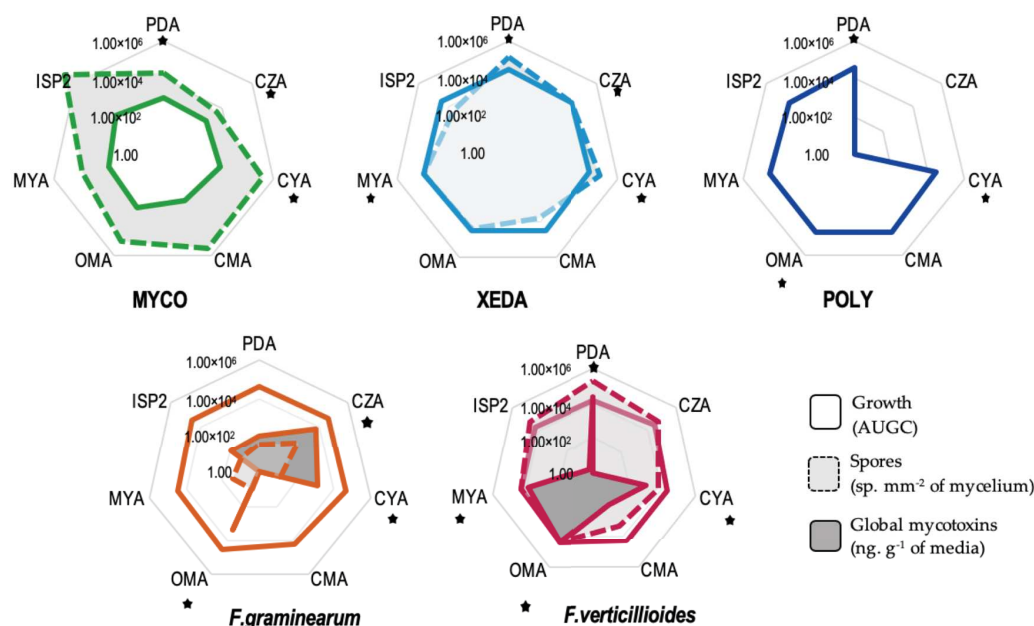


Figure 1. Comportment of all micro-organisms (commercialized biological control agents (BCAs) and pathogens) on different media. Stars indicate the final selected medium for each microorganism. Myco: Mycostop[®], Xeda: Xedavir[®], Poly: Polyversum[®]. AUGC: area under the growth curve, PDA: potato dextrose agar, CZA: Czapeck agar, CYA: Czapeck yeast agar, CMA: corn meal agar, OMA: oat meal agar, MYA: malt yeast agar, ISP2: International *Streptomyces* Project Medium 2 (8 days at 25 °C). Levels of global mycotoxins are given as a sum of the mycotoxins from a pathogen chemotype (*Fusarium graminearum*: deoxynivalenol (DON) + 15ADON and *Fusarium verticillioides*: fumonisins B₁ (FB₁) + fumonisins B₂ (FB₂)).

In order to promote pathogen development and mycotoxin production during interaction, the selection of dual culture bioassay media was firstly based on the mycotoxin production capacity of pathogen (Table 1). Then, the ability of BCAs for growth on this medium was checked. To observe the differential capacity of BCAs to impact mycotoxigenic fungi, one common medium (CM) for each pathogen was selected: for *F. graminearum*–BCA dual culture bioassay CYA was chosen, and for *F. verticillioides*–BCA dual culture bioassay PDA was selected. Other interesting media which did not allow all BCAs to grow were assigned to a specific dual culture bioassay duo (SM), and were selected for their ability to promote mycotoxinogenesis. These selected culture media were used to test antagonist activity and allowed identification of the impact of BCAs on pathogenic growth and mycotoxin production. The use of different culture media enabled the characterization of the impact of nutrient resources on the dual culture bioassay of microorganisms.

Table 1. Selected media for dual culture bioassay. CM: common medium for all BCAs confrontation of concerned pathogen; SM: Specific media for each BCA–pathogen confrontation of concerned pathogen. Myco: Mycostop[®], Xeda: Xedavir[®], Poly: Polyversum[®].

Selected Media for Dual Culture Bioassay (Pathogen–BCA)				
Pathogens	<i>F. Graminearum</i>		<i>F. Verticillioides</i>	
BCA	CM	SM	CM	SM
Myco	CYA	CZA	PDA	CYA
Xeda	CYA	CZA	PDA	MYA
Poly	CYA	OMA	PDA	OMA

2.2. Macroscopic and Microscopic Interaction

During the dual culture bioassay test, the macroscopic observation of plates (7 days after inoculation) revealed differential compartments between microorganisms (Figure 2). For both pathogens, mycelial development was limited by the presence of all BCAs compared to the control. Myco formed a very small colony related to pathogens, but an inhibition zone of pathogen growth was observed (especially in dual culture bioassay with *F. verticillioides*). With respect to pathogens, accumulation of pigment was observed in the dual culture bioassay zone (on the reverse side of Petri dish, not shown). The Xeda colony grew as fast as the pathogen and filled the free space in the Petri dish. Once again, accumulation of pathogenic pigment in the confrontation zone could be observed on the reverse side of Petri dishes. The Xeda colony could exude fine black droplets on its surface solely during confrontation. Poly was able to grow faster than the pathogen (especially faster than *F. graminearum*) and fill the free space in Petri dish. During co-culture, it was able to progress on both the pathogenic colony and inhibit the homogenous and natural pink pigmentation of *F. graminearum* completely.

At the microscopic scale, *F. graminearum* alone produced rapidly long, dense, and straight hyphae, while *F. verticillioides* alone produced fine and extra fine lightly branched hyphae (Figure 2). With microscopic interactions between BCAs and pathogens photographed over time, many characteristic structures could be observed. Myco was able to cause anarchic ramifications of *F. graminearum* mycelia before contact (+72 h) and vesicle formation at the tip of pathogen hyphae when they were close (+96 h). Growth inhibition by Myco was clearly identified at the microscopic level in co-culture with *F. verticillioides*, with an early antigerminative action (+72 h) and a linear/regular growth inhibition after 96 h. Xeda induced deformation of *F. graminearum* mycelia, which could extend to the formation of loops after 96 h. The progression of this BCA face-to-face with *F. verticillioides* created a mechanical barrier that slowed the progression of the mycotoxigenic fungus. In the dual culture bioassays of both pathogens, Xeda caused a degradation of pathogenic mycelia in contact zones (+96 h). The co-culture *F. graminearum*–Polyversum showed dispersed contact structures. The BCA was able to pass through the mycelium of the pathogen, and induced formation of lysed wall hyphae. It adopted a completely different compartment in the dual culture bioassay against *F. verticillioides* and induced the transient formation of micro-vesicles at the extremity of pathogenic hyphae (between 24 and 72 h of dual culture bioassay). When it was close to the pathogen (+96 h), it deployed long mycelial filaments to colonize the environment.

2.3. BCA Impact on Growth and Mycotoxin Production

During the antagonistic in vitro test, the growth and the mycotoxin production of both pathogens in confrontation against the three BCAs were monitored over 12 days. Classic batch curves were obtained for pathogen growth and major mycotoxin production (latent phase, exponential phase, stationary phase). The area under growth/global mycotoxins curve (respectively AUGC and AURMC) was calculated (Figure 3). All BCA treatments impacted growth of *F. graminearum*, especially Xeda and Poly, causing growth reductions of respectively 48% and 79%.

In addition to reducing the pathogens mycelial development, Xeda and Poly had a direct action on global DON production, with reductions in mycotoxin concentrations of 73% and 93% respectively. *F. graminearum* 15-ADON production was affected and complete inhibition of 15-ADON peak production (8 days after inoculation) was observed with all BCA treatments (see Figure A2). Dual culture bioassays with *F. verticillioides* showed a completely different response profile to BCAs. Even if the growth reduction of pathogen was significant face-to-face with Myco (24%), the specific production was lightly stimulated. It was mainly with the reduction of pathogen growth that Xeda restricted fumonisin production, with the most important inhibition levels (78%) among all BCA tested.

In summary, Poly was more efficient in the *F. graminearum* dual culture bioassay and Xeda was more efficient in the *F. verticillioides* dual culture bioassay using common media (CM).

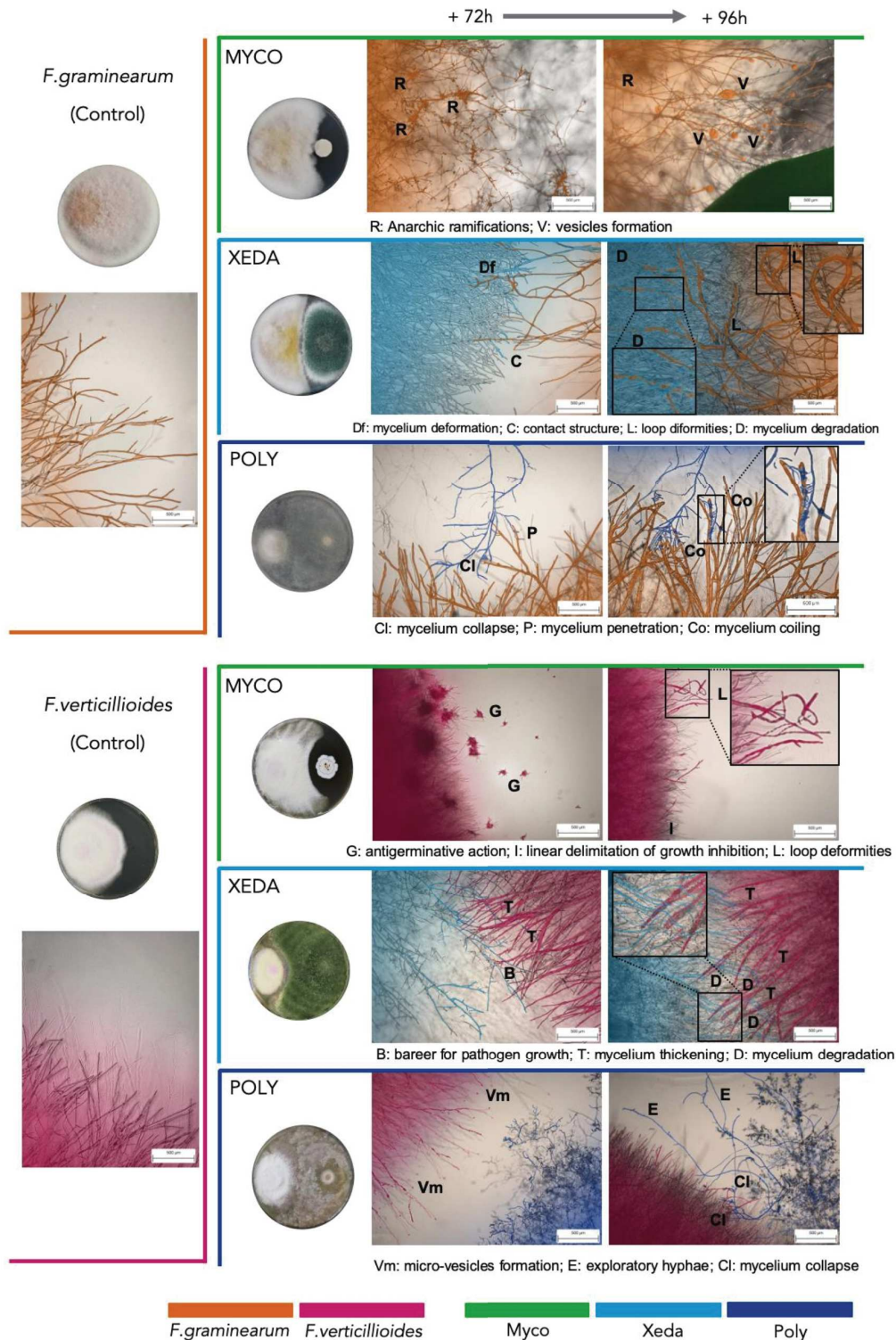
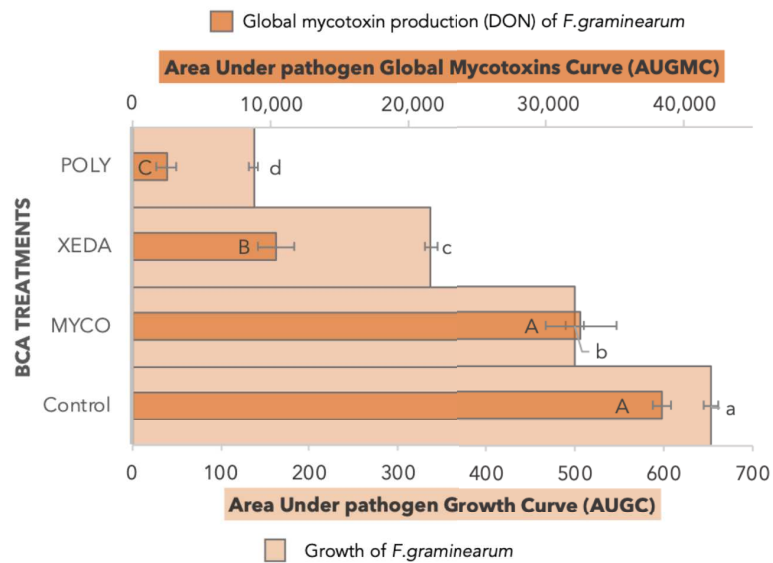
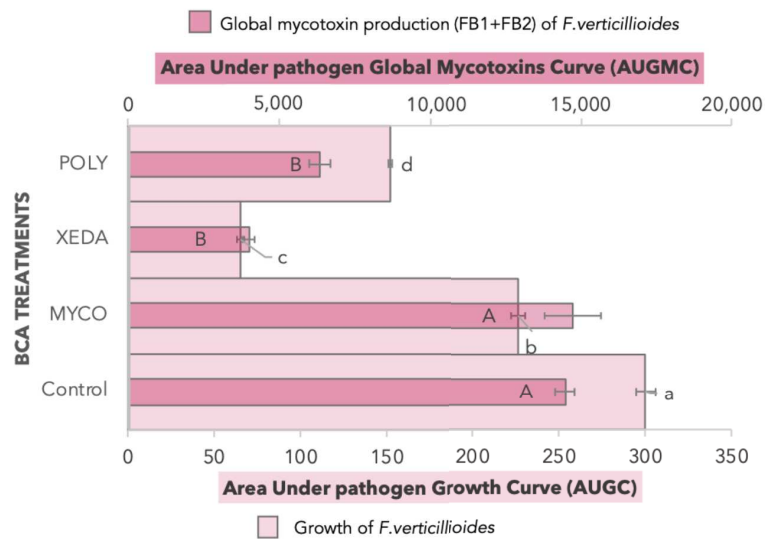


Figure 2. Macroscopic and microscopic interactions during pathogen–BCA dual culture bioassay. The *F.graminearum* and *F.verticillioides* control and confrontation plates respectively had CYA and PDA media (CM, 7 days after inoculation). The same media were used for the slide confrontation (+72/96 h after inoculation) indicated at the top of the figure. Colors indicate the type of microorganisms, and letters indicate specific structures. Myco: Mycostop[®], Xeda: Xedavir[®], Poly: Polyversum[®].



(A)



(B)

Figure 3. Comparative effects of BCA treatments on the evolution of pathogens. Dual culture bioassays for all BCAs against one pathogen were performed on common medium (CM). (A) *F.graminearum*, on CYA, in orange; (B) *F.verticillioides*, on PDA, in pink, for 12 days. Myco: Mycostop[®], Xeda: Xedavir[®], Poly: Polyversum[®]. Growth (pastel colors, bottom axis) and mycotoxin production levels (pastel colors, top axis) are expressed in the area under growth/global mycotoxin curves (respectively AUGC and AUGMC). Original kinetic curves are available in Figure A2. ANOVA test, Growth comparisons (a, b, c, d) and Mycotoxin comparisons (A, B, C). p -value < 0.05. FB₁ + FB₂ are represented together because the quantity of FB₂ was not significant.

2.4. Impact of Media Variation on Pathogenic Factors during Dual Culture Bioassay (Pathogens–BCAs)

Principal component analysis (PCA) analysis was performed in order to visualize global inhibition profiles of pathogens depending on BCA treatment, but also to assess the impact of culture medium on those inhibition profiles (Figure 4). For both biplots, the variables are well-projected and equivalently projected, and the two first components explained 90.7% and 94.8% of data distribution for *F. graminearum* (Figure 4A) and *F. verticillioides* (Figure 4B), respectively. The growth factor mainly contributed to Dim2, mycotoxin factors mainly contributed to Dim1, and all variables were in correlation. With all culture media combined, Myco and Control samples were regrouped in both graphs in the positive part of biplot. However, culture media influenced the separation of samples on CZA (*F. graminearum* control or confronted with Myco). Control samples were correlated with DON and 15-ADON factors, in contrast to Myco treatment samples, on the other side of Dim2. In the *F. graminearum* bi-plot (Figure 4A), Xeda and Poly samples are regrouped in the bottom-left quarter of graph, suggesting strong opposition to the studied pathogen factor. For the Poly-confronted samples, an increase of mycotoxin inhibition was observed in OMA, highlighting the important effect of culture media on dual culture bioassay, illustrated here for *F. graminearum*. Global antagonist activity of Xeda against *F. graminearum* in the CYA medium was higher than on CZA. On the *F. verticillioides* bi-plot (Figure 4B), these two BCAs (Xeda and Poly) caused the most antagonistic activity. However, against this pathogen, Xeda showed the strongest growth and mycotoxin inhibition. MYA-confronted samples were further from the specific mycotoxin production factor than PDA-confronted samples, indicating a higher specific mycotoxin reduction in this culture medium. Poly was able to inhibit pathogenic factors in both culture media, but with OMA the separation between control and confronted samples was greater, suggesting a stimulation of antagonistic capacities on this culture medium.

2.5. Nutrient Profiling and Nutritional Competition

Given the observed medium impact, nutrient profiling of all microorganisms was characterized using phenotype microarray, and a comparison between microorganisms was performed to identify potential nutritional competition. Along 138 h of incubation, a contrasting capacity to consume the different nutritional resources appeared. As shown in Figure 5, globally, two clusters of microorganisms can be distinguished based on the capacity to metabolize C sources (Figure 5A), with separate microorganisms in two clusters: one with Poly and *F. graminearum*, which were able to consume less C sources than others (high trophic requirements), and one including *F. verticillioides*, Xeda, and Myco, which could consume a broad range of C sources (low trophic requirements). Some C compounds strongly stimulated the growth of microorganisms, such as tyramine/glucose, N-acetylglucosamine/sucrose, threonine/tartaric acid, glycerol/uridine, and proline/sucrose, respectively, for Myco, Xeda, Poly, *F. graminearum*, and *F. verticillioides*. Sucrose was therefore the carbohydrate allowing the strongest growth of two antagonistic microorganisms (Xeda and *F. verticillioides*). On other hand, threonine is a compound that is consumed only by the Poly BCA and inhibits the growth of other microorganisms, including the two mycotoxigenic pathogens. Concerning the aptitude of microorganisms to degrade nitrogen sources (Figure 5B), two different clusters were formed: one with of Poly, Xeda, and Myco, which were able to consume less N sources than others (specialized); and one including *F. graminearum* and *F. verticillioides*, which could consume a broad range of N sources (unspecialized). This clustering was in accordance with the type of microorganism (BCAs or pathogens), and Poly has a nitrogen consumption profile which is in complete contrast to the *F. verticillioides* profile. Some N nutrients increase the growth of particular microorganisms: histidine/lysine, uric acid/serine, xanthine/L-cysteine, uric acid/allantoin, and serine /L-pyroglutamic acid, respectively, for Myco, Xeda, Poly, *F. graminearum*, and *F. verticillioides*. Preferential nitrogen sources of Xeda were also the preferential nitrogen sources of the two pathogens.

Nutrient consumption and the resulting growth capacity were difficult to relate to the synergistic effects that could be observed in the dual culture bioassay on complex synthetic media. Nevertheless, results show that Poly, because of its high trophic requirement, has more difficulty consuming nutrients than pathogens. This observation is confirmed with regard to the major elements that comprised the

culture media of the dual culture bioassay (the two CM pathogens PDA and CYA, and the SM BCAs OMA), even on OMA, where it showed very strong antagonistic capacities during dual culture bioassay. Xeda had better biocontrol activity against *F. verticillioides* on MYA (global mycotoxin inhibition), and against *F. graminearum* on CYA (growth and global mycotoxin inhibition). The MYA medium was composed of fructose and mannitol, which the pathogen/BCA duo can degrade in equivalent proportions. CYA medium contains sucrose and nitrate, two compounds for which Xeda has a stronger affinity than *F. graminearum*.

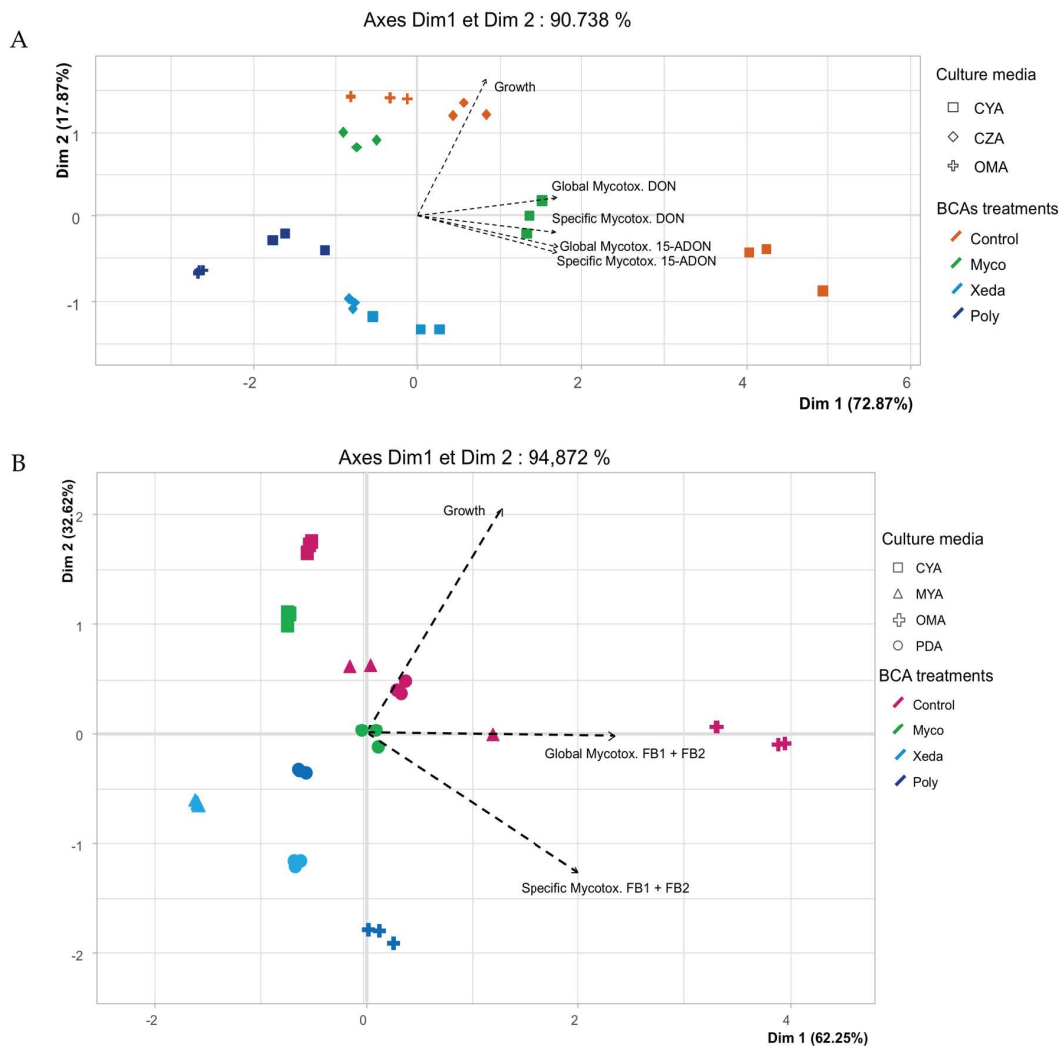


Figure 4. Principal component analysis (PCA) bi-plot with respect to the growth and mycotoxin production data set (—) for the two considered pathogens, (A) *F. graminearum* and (B) *F. verticillioides*, obtained after BCA confrontation (indicated by colors) in different selected culture media (indicated by forms). Myco: Mycostop[®], Xeda: Xedavir[®], Poly: Polyversum[®].



Figure 5. Growth induction of microorganisms during growth on 95 carbon sources (PM1) (A) or 95 nitrogen sources (PM3) (B). Both panels show optical density measurements over a 138-h time course (i.e., area under the growth curves, AUGCs). Color keys of the heat map (from purple to yellow) are expressed for one nutrient in comparison with other microorganisms (row comparison). Micro-organisms are indicated by colors at the end of each column. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. Major elements included in the composition of selected media are indicated by forms next to the concerned nutrient. Medium compositions are available in Figure A1.

3. Discussion

Based on the presented results, the development of dual culture bioassay methods and in particular the choice of culture media should be considered during in vitro studies between mycotoxigenic pathogens and BCAs. Globally, complex culture media selected for the affinity that the microorganisms have for them allow good growth of all microorganisms, with the exception of Poly which does not grow on CZA, a minimum medium. However, the mycotoxinogenesis of pathogens is strongly influenced by the type of media, as shown by our results and as widely reported in the literature [21,22]. A study conducted by Gardiner et al. [22] on the nutritional profile of *F. graminearum* identified the same type of profile, with preferential consumption of the same nutrient as in our results. It is therefore necessary to select culture media that will promote production of this mycotoxin in order to identify BCAs that will have a considerable impact even during high production. Most of the in vitro dual culture bioassays between pathogenic fungi and BCAs are performed on PDA [23,24], which does not stimulate the production of tricothecenes by *F. graminearum*. The use of two media per BCA/pathogen coupling allows the observation of whether nutritional conditions impact the inhibition caused by BCAs. The high mycotoxigenic capacity of the strains was verified. Many variations can be observed between the two pathogens. In natural conditions, they originate from different environments, as *F. graminearum* is isolated from wheat spikes while *F. verticillioides* is isolated from maize kernels. In addition, the pathogens produce distinct types of mycotoxins (tricothecene for *F. graminearum* and fumonisins for *F. verticillioides*) and their production levels are also different. According to the analyses that we performed on both pathogenic strains, the maximum production of fumonisin and tricothecene was about 5000 and 15,000 ng g⁻¹ of media, respectively. Moreover, their colonization strategies are in contrast: *F. graminearum* grew rapidly while producing fewer spores, unlike *F. verticillioides* for which expansion was slower, but it compensated with a substantial production of spores, allowing it to colonize the environment in a comparable way. For these reasons, most of the analyses are carried out independently.

In dual culture bioassay with pathogens, Myco did not have time to develop, which did not allow it to compete spatially with pathogens. The halos of inhibition and the absence of contact during the observation period as well as the deformation of the pathogenic hyphae of *F. graminearum* suggest the synthesis of diffusible antimicrobial compounds by the BCA. Actinomycetes, and particularly *Streptomyces* species, are well known for their production of a wide spectrum of antibiotics [25,26]. In dual culture bioassay with *F. verticillioides*, they were even able to remotely prevent germination of pathogenic spores and completely inhibit pathogen progression. Several compounds were emitted by *Streptomyces* spp. and identified (e.g., as methyl vinyl ketone) for their ability to inhibit the spore germination of phytopathogens [27,28]. The appearance of vesicles at the tips of the hyphae of *F. graminearum* may be related to the synthesis of mycotoxins. Some studies have found that mycotoxins, notably in *F. graminearum*, were synthesized in toxisomes, a kind of vesicle excreted out of the cells and at the tip of hyphae [29,30]. The pathogen's perception of BCA may stimulate this production. Based on the results of the antagonistic tests, Myco tends to stimulate specific mycotoxin production despite significant inhibition of the growth of both pathogens. Therefore, it is essential to test the ability of BCAs to impact mycotoxin production and not only growth. Its ability to degrade many sources of C allows it to grow under many conditions, which could make it a preventive treatment. However, to effectively control pathogens, the ratio of inoculum from Myco must be superior than the quantity of pathogens.

Xedavir adopts a completely different strategy. With good colonization capacities, can compete with the development of pathogens. It can form a mechanical barrier that physically blocks the development of pathogenic fungi and is also able to establish connections between its hyphae and those of pathogens through the formation of haustorium [31]. When in contact with pathogens, it can also synthesize compounds that degrade the fungal walls, probably chitinases, preventing the progression of pathogens. Numerous studies have shown this ability in different species of *Trichoderma* [32,33]. Not surprisingly, the genomes of the mycoparasitic *Trichoderma* spp. are rich in

gene-encoding enzymes like chitinases and glucanases [34,35]. This wall degradation will provide a preferential substrate for further development. It will rather have a hyperparasitic behavior [36]. Its goal is to kill pathogens. Faced with *F. graminearum*, it will act directly on the specific mycotoxinogenesis independently of growth reduction. It will therefore be able to act directly on the mycotoxin biosynthesis pathways, or by biotransformation of the mycotoxins produced (transformation, degradation, binding). It would be interesting to explore this potential because recent studies have identified mycotoxin degradation capacities in other BCAs of the *Trichoderma* genus [37]. During in vitro dual culture bioassays on selected media, culture media seem to influence the impact of Xeda on pathogens. The nutritional resources of microorganisms will therefore impact the inhibition of pathogens by BCAs. One culture medium could even allow Xeda to act more drastically on specific mycotoxinogenesis (MYA–*F. verticillioides*), and thus reduced the mycotoxin concentrations of samples. These results suggest that nutritional competition phenomena could be one of the weapons used by Xeda to fight pathogens. This path is supported by the comparison of nutritional profiles of Xeda and pathogen nutritional profiles. It appears that for certain compounds, the capacities of assimilation will be equivalent or to the advantage of BCAs, notably certain culture media compounds used during the dual culture bioassays (like sucrose). Indeed, culture media that allow a better inhibition of pathogens (MYA and CYA, respectively for *F. verticillioides* and *F. graminearum*) will be considered as resources for which microorganisms are in competition. Recently, Wei and collaborators showed that root-associated bacterial communities with a clear niche overlap with the pathogen reduce pathogen invasion success, constrain pathogen growth, and have lower levels of diseased plants in greenhouse experiments [38].

Concerning interactions between Poly and fusaria pathogens, Poly is one of the most competitive BCAs despite its high nutritional requirements. It is able to proliferate very rapidly under favorable conditions. Against the two pathogens, it will be able to invade the space left free more quickly, until it grows on the colonies of the pathogen. This behavior suggests an interaction requiring close contact, like parasitism. Poly is known for its capacities to colonize fungi and oomycetes [39]. The perception of the pathogen could be done via the perception of ergosterol from pathogens. In fact, sterols have been identified first as stimulating the sexual reproduction of *Pythium* species (like Poly) [40–42]. The same stimulation effects have been observed during dual culture bioassays with fungal pathogens [43]. This ability is a considerable advantage for the invasion of Poly and its mechanisms of action against pathogens. In addition to limiting the spatial spread of the pathogen, the absence of pathogenic pigment suggests a direct interaction with the metabolism of mycotoxigenic pathogens. At the same time after inoculation, compared to Xedavir, which has a slower progression, the contact took place in a very subtle manner. It seems that the BCA did not want to be perceived by the pathogens and only few wall degradation enzymes were synthesized. In dual culture bioassay against *F. graminearum*, Poly was able to pass through the mycelium of the pathogen and induce formation of lysed wall hyphae. They could be considered as privileged spaces for nutrient sampling by the BCA, suggesting again a close-contact interaction, such as mycoparasitism. These observations are entirely consistent with another study by Charlene Faure et al., who had observed the same type of behavior by Poly, capable of crossing the mycelium of *F. graminearum* without forming any particular structure [43]. During the antagonist activity test, Poly showed very good antagonistic capacities, allowing a reduction of the growth of both pathogens. However, the impact on mycotoxin production was much stronger on *F. graminearum*, against which it was particularly effective. By integrating the impact of the growing medium, Poly was the BCA that was most sensitive to change in nutritional resources. From the beginning of the study, it was the microorganism that was the most sensitive to culture media, with an inability to produce oospores in vitro in synthetic culture media that are not enriched in sterols. In contrast, it was able to deploy its mechanisms of action during dual culture bioassays because it was stimulated by the presence of the pathogen and by the complex nutrients in the culture media. In fact, it was most effective on a culture medium composed mainly of cereals (OMA). The results showed that Poly was less adapted in terms of nutrient consumption than the pathogens on all the culture media of the dual culture bioassays, even on OMA, where it showed very strong antagonistic capacities during

dual culture bioassay test. It would therefore appear that unlike Xeda, nutrient competition was not a preferential mechanism for Poly to deal with mycotoxigenic fungal pathogens. Nevertheless, it is interesting to note that threonine was a compound that was consumed only by Poly BCA and did not promote growth of other microorganisms, including the two mycotoxigenic pathogens. It was interesting to test the enrichment of commercial formulations with compounds of this type in order to boost the growth of Poly during treatment in real conditions.

The three BCAs therefore have contrasting and complementary strategies, with different capacities to inhibit the propagation of pathogens. Their behavior was identical in one aspect: all inhibited the peak production of 15-ADON produced by *F. graminearum* (see Figure A2A). 15-ADON is a precursor to the formation of DON [44]. This inhibition could therefore be a sign of an action on the biosynthesis pathway, in particular the *Tri8* gene involved in the transformation of 15-ADON into DON.

4. Conclusions

In the present study, three commercial biological control agents (BCAs) were selected and tested to evaluate their antagonistic activities against *F. graminearum* and *F. verticillioides*, two mycotoxigenic fungi. Results suggest that these three BCAs revealed contrasting effects on growth and mycotoxin production of pathogens, particularly Xedavir® against *F. verticillioides* and Polyversum® against *F. graminearum*, the two most effective combinations. In addition, several paths could be explored concerning the various observed modes of action of these BCAs, like antibiosis, parasitism, or mycotoxin degradation. It would appear that the inhibition effect of BCAs could be dependent on the nutritional condition of interaction, providing new insight into the nutritional phenotype and potential competition between micro-organisms. Given the high levels of inhibition observed, further studies on the modes of action of these BCAs could be conducted, for example on synthesis of anti-germinative compounds by Mycostop®, chitinolytic activities or by-products of microbial detoxification of mycotoxins by Xedavir®, or mycophageous action by Polyversum®.

5. Material and Methods

5.1. Micro-Organisms

5.1.1. *Fusarium* Species

F. graminearum isolate BRFM 1967 and *F. verticillioides* isolate BRFM 2251 were used (CIRM, University of Aix-Marseille, Marseille, France), and chosen for their strong ability to produce their respective mycotoxins. *F. graminearum* strain BRFM 1967, isolated from wheat plant, has a deoxynivalenol (DON/15-ADON) chemotype profile, while *F. verticillioides* BRFM 2251, isolated from maize kernels, has a fumonisins (FB₁/FB₂/FB₃) chemotype profile. All fungi were maintained on potato dextrose agar (PDA; BD Difco, Sparks, MA, USA) under paraffin oil at 4 °C and actively grown on PDA at 25 °C for 7 days for spore production.

5.1.2. Commercial Biological Control Agents (BCAs)

Three commercial biological control agents were selected for their contrasting characteristics. To date, there is little information on their action and biocontrol mechanisms on mycotoxigenic fungi (Table 2).

All strains were isolated from their commercial product with a classical microbial insulation protocol (serial dilution and multiple striation inoculation), on appropriate medium (supplemented with 0.01% of tetracycline for Polyversum only). BCAs were conserved under spore forms in glycerol solution (15%/−80 °C) and in commercial product aliquots (4 °C). The strains were actively grown on ISP4, PDA, and V8, respectively, for Mycostop, Xedavir, and Polyversum at 25 °C for 7 days for spore production. For the rest of the study, the strains isolated from commercial products were referred to

using the following abbreviations: Myco for Mycostop/*S. griseoviridis*, Xeda for Xedavir/*T. asperellum*, and Poly for Polyversum/*P. oligandrum*.

Table 2. Contrasting characteristics of the selected commercial biocontrol agents.

BCAs, Commercial Products	Mycostop	Xedavir	Polyversum
Strain	<i>Streptomyces griseoviridis</i> strain K61	<i>Trichoderma asperellum</i> strain TV1	<i>Pythium oligandrum</i> strain M1/ATCC 38472
Family	Actinomycete	Ascomycete	Pythiaceae
Kingdom	Bacteria	Fungi	Oomycete
Plant/soil of isolation	<i>Sphagnum</i> peat	Root of tomato plant	Sugar beet
Locations of isolation	Finland	Italy	Czechoslovakia
Recommended Use	General soil treatment	General soil treatment	Barley and wheat aerial treatment
Target(s)	Soil-borne pathogen	Soil-borne pathogen	<i>Fusarium graminearum</i>
Commercialization company	Lallemand Plant Care®	Xeda International®	DeSangosse®
Date of marketing	2014	2013	2015
Identified general mechanism of action	Production of antimicrobial compound, mycoparasitism, plant defense induction	Mycoparasitism, plant defense induction	Mycoparasitism, plant defense induction
/on mycotoxigenic fungi	/none	/none	/on <i>F. graminearum</i>

5.1.3. Behaviors on Different Media

To identify the culture media best suited to the dual culture bioassay of each of the BCAs/pathogen, seven culture media candidates were chosen: PDA, Czapeck agar (CZA), Czapeck yeast agar (CYA), and oat meal agar (OMA) for their affinity with pathogens [45–48], and potato dextrose agar (PDA), International *Streptomyces* Project Medium 2 (ISP2), malt yeast agar (MYA), and corn meal agar (CMA) for their affinity with BCAs (respectively all BCAs, Myco [49,50], Xeda [13,51,52], and Poly [53–55]). The compositions of media are available in Figure A1. All isolates were inoculated at the center of Petri dishes ($5 \mu\text{L} \times 10^4$ spores mL^{-1}) and the growth kinetics were measured by image analysis on ImageJ software (area measurement in cm^2 /every two days for 8 days). Produced spores were evaluated by flooding the plates on the final day at the end of kinetics (after 8 days) and counted under an optic microscope with Thoma cell. Mycotoxin analyses were performed for pathogen with the method described below (Section 5.2.2).

This result was used to select two culture media for each BCA/pathogen pair for the dual culture bioassay tests.

5.2. Antagonist Activity *in vitro* on Selected Culture Media

5.2.1. Dual Culture Bioassay and Growth Evaluation

On selected media (Table 1), one BCA and one pathogen were inoculated in a Petri dish, 85 mm in diameter, at the same distance from the center of plate. The pathogens and BCAs were inoculated with the spore suspension except for Poly, which was inoculated with 7-day plugs (5 mm in diameter). Each BCA was inoculated 24 h in advance ($5 \mu\text{L} \times 10^5$ spores mL^{-1} or plug); then the pathogens were inoculated at the opposite side (45 mm from BCA, $5 \mu\text{L} \times 10^4$ spores mL^{-1}). Plates were incubated at 25 ± 2 °C, in the dark, for 12 days. Plates inoculated only with the pathogen were used as a control treatment. Every two days, Petri dish were photographed, and the colony area of each BCA/pathogen were measured in cm^2 by image analysis using ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018). Values were used to create growth curves. Each BCA/pathogen dual culture bioassay and control was set up in triplicate for each analyzed day and two independent repetitions of the test were done.

5.2.2. Mycotoxin Extraction and Analysis

After growth evaluation, each dual culture bioassay plate was submitted to a mycotoxin analysis. The extraction procedure described by Moreau and Levi [56] was used with some modifications (no

purification steps). For both pathogens, half a Petri dish was sampled by cutting along the line formed by the two inoculation points, and finely cut and weighed. For *F. graminearum* tests or controls, 50 mL of acetonitrile/water/acetic acid (79:20:1, *v/v/v*) were added. For *F. verticillioides* tests or controls, 150 mL of water/acetic acid (99.5:0.5, *v/v/v*) were added. All samples were homogenized by mechanical agitation for 20 min. *F. graminearum* samples were preliminarily diluted 1:50 in water/acetic acid (99.5:0.5; mobile phase of analyzer) and filtered with a CA filter (0.45 μm , Carl Roth GmbH, Karlsruhe, Germany); *F. verticillioides* samples were directly filtered, and were ready for injection.

For all samples, mycotoxin detection and quantification were achieved using an Ultra High-Performance Liquid Chromatography (UHPLC, Shimadzu, Tokyo, Japan) coupled with a mass spectrometer (8040, Shimadzu, Tokyo, Japan). LC separation was performed using a Phenomenex Kinetex XB Column C18 (50 mm \times 2 mm; 2.6 μm particles) at 50 °C, with an injection volume of 50 μL . Mobile phase composition was (A) 0.5% acetic acid in ultra-pure water and (B) 0.5% acetic acid in isopropanol (HPLC MS grade, Sigma, St Louis, MO, USA), and the mobile phase flow rate was 0.4 mL min^{-1} . The mass spectrometer was operated in electrospray positive (ESI+) and negative (ESI-) ionization mode, and two multiple reaction monitoring (MRM) transitions for each analyte were monitored for quantification (Q) and qualification (q) (Table 3, Table 4). All data were analyzed using LabSolution Software (v5.91/2017, Shimadzu, Tokyo, Japan, 2017). Limits of detection or quantification (LOD/LOQ in ng mL^{-1} , respectively) for each mycotoxin were: DON (4/14), 15-ADON (10/35), FB₁ (0.03/0.1), and FB₂ (0.01/0.05). Mycotoxin levels were expressed in ng g^{-1} of medium or ng cm^{-2} , respectively, for global and specific mycotoxin production. Values were used to create global and specific mycotoxin production curves. Each BCA/pathogen dual culture bioassay and control mycotoxin analysis was set up in triplicate for each measurement day, and two independent repetitions of the test were done.

Table 3. MS/MS parameters for isotope-labelled internal standard (IS). MRM: multiple reaction monitoring.

IS	Polarity	MRM	EC
DON C13	–	370.3 > 59.0	35
FB ₁ C13	+	756.3 > 356.5	–47

Table 4. MS/MS parameters for mycotoxins. Q: quantification; q: qualification.

Mycotoxin	Polarity	MRM Q	EC MRM Q	MRM Q	EC MRM Q	R ² Calibration Curve	IS
Fumonisin B ₁	+	722.35 > 334.5	–43	722.35 > 352.0	–44	0.9982	FB ₁ C13
Fumonisin B ₂	+	706.2 > 318.4	–37	706.2 > 3336.4	–44	0.9980	FB ₁ C13
DON	–	355.0 > 59.0	35	355.0 > 265.1	35	0.9998	DON C13

5.3. Macroscopic and Microscopic Observations

For the macroscopic observations, plates obtained in the same conditions described previously were used to identify for signs of an inhibition zone between each BCA/pathogen dual culture bioassay or/and the activation of secondary metabolism through the differential presence of pigments compared to the control or/and the BCA overgrowth on the pathogenic colony suggesting mycoparasite activity. The BCA control plates were added, with BCA alone in same conditions.

Microscopic observations were performed with an adapted method from Reithner [57]. On glass slides, 1.5 mL of common medium (CM) for each BCA/pathogens pair (CYA for *F. graminearum* and PDA for *F. verticillioides*) were deposited, inoculated with BCA/pathogen pairs on opposite sides (4.5 cm), incubated at 25 °C on supports in Petri dishes containing wet sterile filter paper, and sealed with parafilm. Glass slides inoculated only with pathogens were used as the control. The evolution of interaction was observed daily until 24 h after contact between BCAs and pathogens (120 h), with a Zeiss PrimoStar microscope. Pictures were taken using a Zeiss Axiocam ERc5s camera (Carl Zeiss Microscopy, Thornwood, NY, USA). The obtained pictures were treated with Photoshop CC Software (19.0, Adobe, San Jose, CA, USA, 2017), using the following pipe-line: contrast/light correction,

filter layer correction, selection of a color/black and white density filter (reduces yellowing of color treatment, provides contrast for the microorganism contours), complementary manual selection of microorganisms, color filling (background transparency and Gaussian blur), and directional gradient in section.

5.4. Nutrient Profiling

Phenotype microarrays plates (Biolog, Hayward, CA, USA) were used for the nutrient profiling of all microorganisms according to the manufacturer's instructions. For the carbon and nitrogen consumption profiling, PM1 and PM3 were used, respectively. Carbon sources in PM1 are in the 5–50 mM range and nitrogen sources in PM3 are in the 2–20 mM range [58]. Inocula were suspended in sterile Biolog FF inoculating fluid and adjusted to a transmission of 81 and 62% at 590 nm for Myco and other micro-organisms, respectively. Plates were incubated in darkness at 25 °C, and growth (optical density) at 750 nm after 0, 12, 18, 24, 36, 42, 48, 60, 66, 72, 84, 90, 96, 114, and 138 h using an Enspire Multimode Reader (Perkin Elmer, Waltham, MA, USA) was monitored. The precise composition of all dual culture bioassay media was established in light of literature and confronted with the nutrient resources of plates.

5.5. Data Expression and Statistical Analysis

With the aim of considering complete growth and/or mycotoxin production kinetics, and not only a final point, the area under the growth/mycotoxin production curves were calculated for the antagonistic test and nutrient profiling test.

Statistical data analysis was performed with R Software (3.4.4, R Foundation for Statistical Computing, Vienna, Austria, 2017). Normality and homogeneity of variances were checked with the Shapiro–Wilk test (with Holm–Bonferroni correction) and Levene's test, respectively. For each pathogen, the effect of BCA treatments was tested with a one-way ANOVA and multiple comparisons of means were done with Tukey's test ($\alpha = 0.05$). For the principal component analysis (PCA), data were standardized, and the analysis was done on the correlation matrix with the FactoMine R package. The first two components were retained in both cases. For the visualization of specific differences between microorganisms nutrient profiling, the negative control was subtracted and a heatmap was built with the ggplot package. The color scale indicates differential nutrient consumption in comparison with other micro-organisms (row comparison).

Author Contributions: L.P., C.S. and S.S.-G. designed the study. N.D. performed mycotoxin analysis via HPLC MS-MS. V.M. participated in the extraction of mycotoxins from culture media. L.P. carried out the entire range of manipulations and experimental approaches and analyzed the data. L.P., C.S. and S.S.-G. interpreted the data. L.P. wrote the original draft. A.F., C.S. and S.S.-G. carefully supervised and reviewed the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A.

Appendix A.1. Medium Composition

CYA - Czapek Yeast Extract Agar	g/L	PDA – Potatoes Dextrose Agar	g/L
Sucrose	30	Dextrose/Glucose	20
Agar ¹	15	Agar ¹	15
Yeast Extract ²	5	Potatoes infusion ³	4
Dipotassium hydrogen phosphate	1		
Sodium nitrate	0.3	MYA – Malt and Yeast Extract Agar	g/L
Potassium chloride	0.05	Agar ¹	20
Magnesium sulphate	0.05	Malt extract ⁴	20
Potassium chloride	0.05	Yeast extract ²	1
Trace elements solution (T.E.S.)	1 ml		
TES:	g/100ml	ISP2	g/L
Iron sulphate	0.1	Agar ¹	20
Zinc sulphate	0.1	Malt extract ⁴	10
Copper sulphate	0.05	Glucose	4
		Yeast extract ²	4
CZA - Czapek	g/L		
Sucrose	30	OMA – Oat Meal agar	g/L
Agar ¹	15	Oatmeal ⁵	20
Sodium nitrate	2	Agar ²	18
Dipotassium phosphate	1	Trace elements solution (T.E.S.)	1 ml
Magnesium sulphate heptahydrate	0.5	TES:	g/100ml
Potassium chloride	0.5	Manganese chloride	0.1
Iron sulphate heptahydrate	0.01	Iron sulphate	0.1
Trace elements solution (T.E.S.)	200 µl	Zinc sulphate	0.1
TES:	g/100ml		
Citric acid monohydrate	5	CMA – Corn Meal Agar	g/L
Zinc sulphate heptahydrate	5	Agar	15
Copper sulphate pentahydrate	0.25	Cornmeal	50
Manganese sulphate monohydrate	0.05		
Boric acid	0.05		
Sodium molybdate dihydrate	0.05		

Figure A1. Detailed composition of culture media. In all tables, principal ingredients for the preparation of media in laboratory are described. Trace element solutions (TES) were added after autoclaving the culture media. For complex ingredients, superscript numbers refer to the main components in C and N identified through the literature. ¹ Agar [59,60]. Major C components: β -D- galactopyrose, α -L-galactopyranose, agarobiose, agaropectine. Major N component: uronic acid. ² Yeast extract [60,61]. Major C component: mannitol. Major N components: alanine, lysine, aspartic acid, glutamic acid. ³ Potato infusion [62,63]. Major C component: starch (glucose + glucose), Major N components: asparagine, glutamine, aspartic acid. ⁴ Malt extract [60,64]. Major C components: maltose, glucose-fructose, dextrine, maltobiose. Major N components: glutamic acid, proline, alanine, leucine, phenylalanine. ⁵ Oatmeal [65–67]. Major C components: starch (glucose+glucose), arabinoxylane, β -glucan. Major N components: phenylalanine, tyrosine, leucine, valine, arginine, lysine, glutamic acid, glycine, alanine, serine.

Appendix A.2. Kinetics of Pathogen Growth and Mycotoxin Inhibition in Dual Culture Bioassay with BCAs (Common Media, CM)

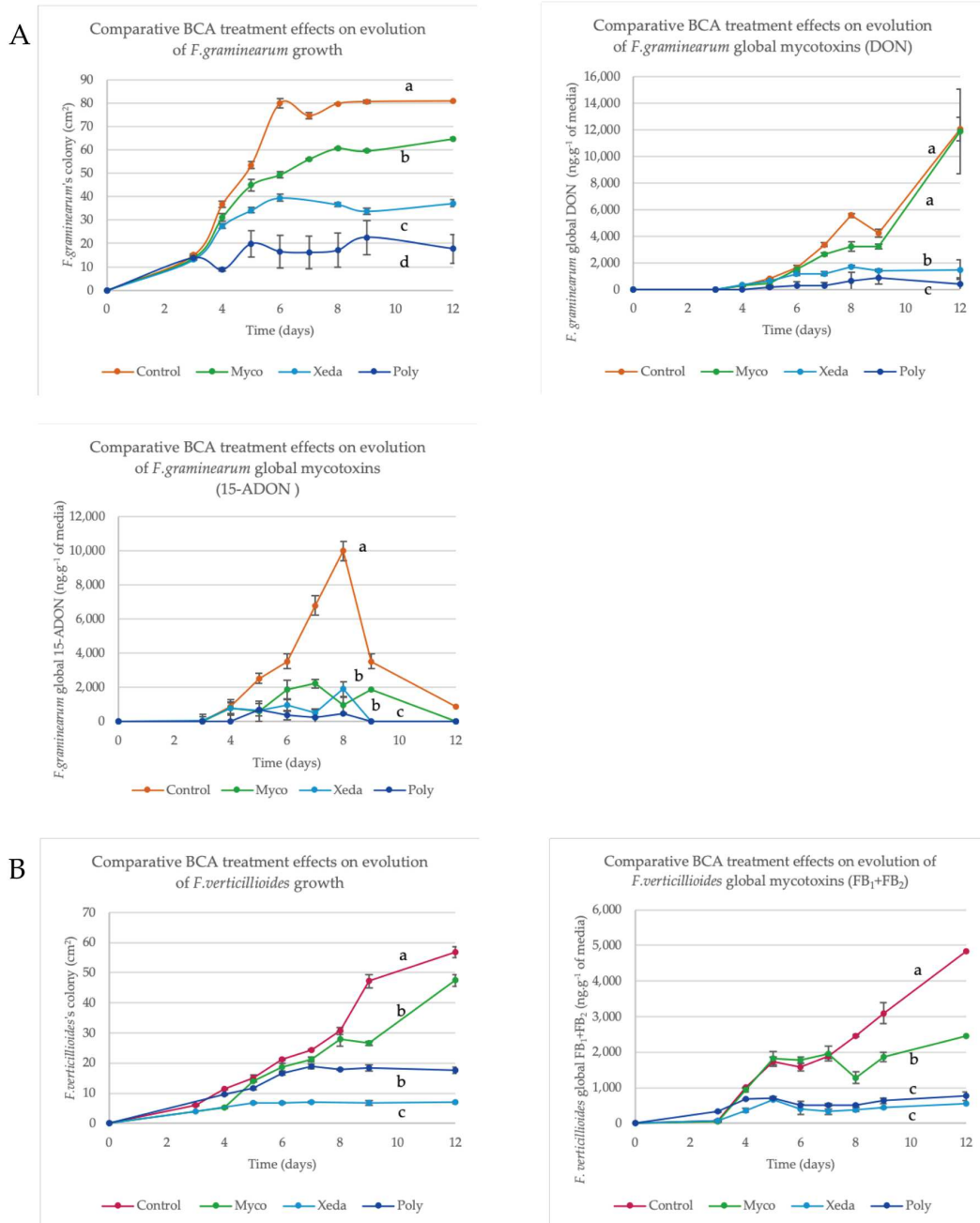


Figure A2. Comparative effects of BCA treatments on the evolution of pathogens. Dual culture bioassays for all BCAs against one pathogen were performed using common medium, CM. (A) *F.graminearum*, on CYA, in orange; (B) *F.verticillioides*, on PDA, in pink, for 12 days. Growth (A1, B1) is expressed in cm², and mycotoxin production levels (A2, A3, B2) are expressed in ng g⁻¹ of media. FB₁ + FB₂ are represented together because the quantity of FB₂ was not significant. ANOVA test, *p*-value < 0.05.

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Partie Expérimentale

Étude n°2

3.2. Étude n°2 :

Décryptage des Mécanismes mis en jeu lors de l'inhibition de pathogènes du genre *Fusarium* par des Agents de Biocontrôle

Cette partie fait l'objet de trois communications écrites, sous forme d'articles de recherche.

Afin de consolider les hypothèses concernant les mécanismes d'action des BCAs émises dans la première partie mais aussi d'explorer les capacités des BCAs pour d'autres modes d'action, nous avons été amenés à rassembler, optimiser et mettre au point un ensemble d'expériences sous forme de toolbox, qui permet d'identifier les différents phénomènes à l'origine de l'inhibition de la croissance et de la production de mycotoxines des pathogènes.

Les deux premiers articles de recherche sont les deux parties d'une même étude portant sur les mécanismes d'actions des BCAs contre les pathogènes à différents stades de l'interaction, soumises dans le journal *Environmental Microbiology*, le 17 juillet 2020 et en révision:

- Biocontrol Agents : Weapons against Mycotoxigenic *Fusarium* (Part 1: Early stage)

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Angélique Fontana¹, Caroline Strub^{1,*} and Sabine Schorr-Galindo¹.

¹ Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Montpellier, France.

- Biocontrol Agents : Weapons against Mycotoxigenic *Fusarium* (Part 2 : Late stage)

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Noël Durand^{1,2}, Angélique Fontana¹, Caroline Strub^{*,1} and Sabine Schorr-Galindo¹.

¹ Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Montpellier, France ;

² CIRAD, UMR Qualisud, F-34398 Montpellier, France.

Un mécanisme d'action complémentaire des BCAs contre les souches toxigènes de *Fusarium* à l'échelle génétique est analysé dans l'article de recherche soumis dans le journal *Biological Control* le 11 août 2020 et en révision :

- *Trichoderma asperellum* and *Pythium oligandrum* can modulate the mycotoxin biosynthesis genes expression in *Fusarium* cereal pathogens during their biocontrol interaction.

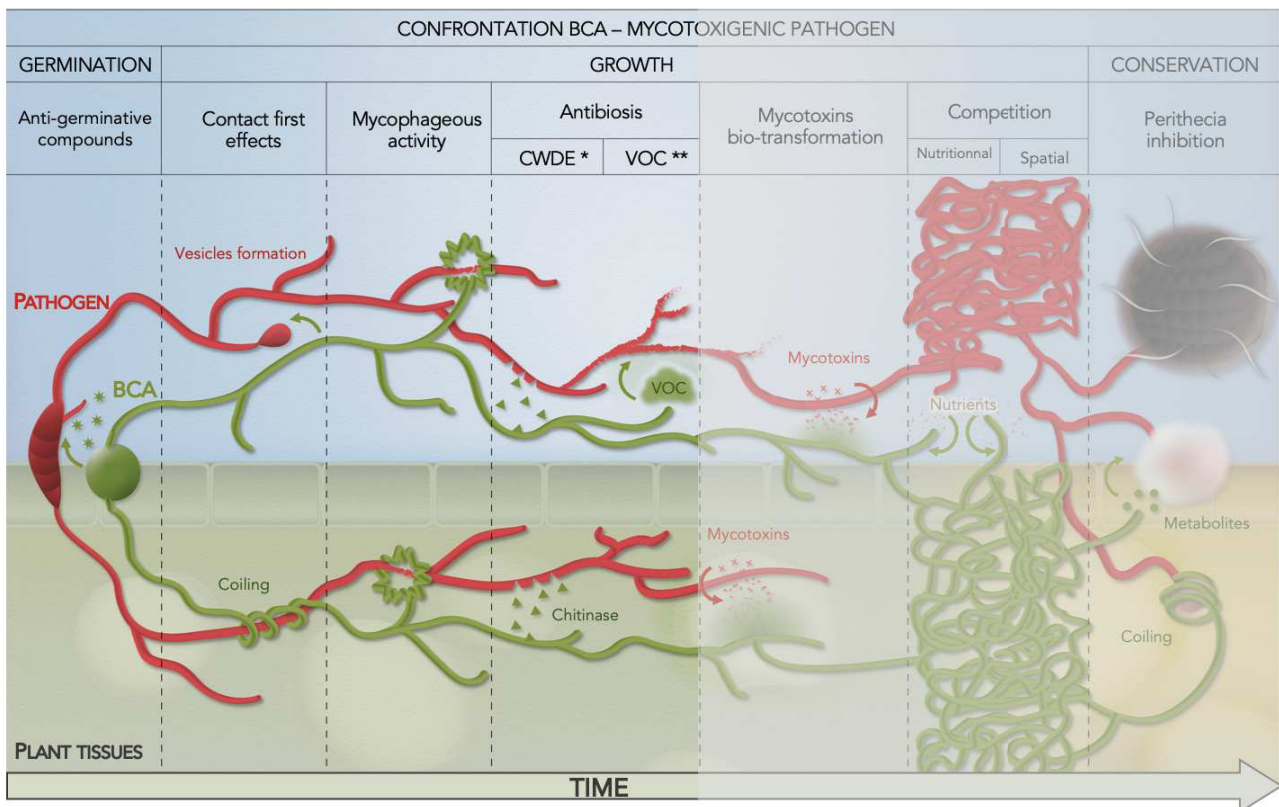
Lucile Pellan¹, Myriam Mamouni¹, Caroline Strub^{1,*} and Sabine Schorr-Galindo¹.

¹ Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Montpellier, France.

3.2.1. Les mécanismes d'action dans les stades précoces de l'interaction

Dans cette première partie de l'étude, les premiers stades d'interaction entre les BCAs et les champignons mycotoxinogènes ont été étudiés. Des échantillons de spores pathogènes et de spores ou extraits de culture des BCAs ont été mis en contact pour évaluer l'impact de composés antifongiques ou d'activités précoces sur la germination des spores pathogène. Un test de mycophage a été mis au point pour pouvoir observer la capacité des agents de biocontrôle à survivre et consommer le mycélium actif du pathogène en l'absence d'autre ressources nutritionnelles. L'activité enzymatique chitinolytique des BCAs a aussi été suivie en simulant ou non la présence de pathogène, et l'impact des composés volatiles a été observé sur la croissance et la production de mycotoxines des agents pathogènes. *S. griseoviridis* (Myc) a produit et sécrété plusieurs composés ayant une activité antifongique et anti-germinative, mais aussi un mélange d'enzymes hydrolytiques, pour attaquer les pathogènes à distance. *T. asperellum* (Xeda) a favorisé des modes d'action par contact avec une forte activité mycophage associée à la synthèse d'enzymes de dégradation, parmi de nombreux autres mécanismes. *P. oligandrum* (Poly) a montré une activité mycophage précoce élevée et sa reproduction a été stimulée par la présence de l'agent pathogène. Les aspects chronologiques, de combinaison de processus et de spécialisation des interactions sont également abordés.

Mot-clé : mycotoxines, contrôle biologique, interaction microbienne, *Trichoderma*, *Streptomyces*, *Pythium*



Résumé Graphique

Biocontrol Agents: Weapons against Mycotoxigenic *Fusarium* (Part 1: Early stage)

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Angélique Fontana¹, Caroline Strub^{1,*} and Sabine Schorr-Galindo¹.

¹Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Montpellier, France;

* Correspondence: Caroline STRUB

UMR Qualisud

Université de Montpellier-Campus Triolet

F-34095 Montpellier Cedex 5

Email: caroline.strub@umontpellier.fr

Tel: +33 67143201

Under review, submitted

Running Title: Biocontrol: Weapons against *Fusarium* (1)

Abstract

The aim of this study was to develop a set of experiments to follow and decipher the mechanisms of commercial Biocontrol Agents (BCAs) against two major mycotoxigenic fungi in cereals, *Fusarium graminearum* and *Fusarium verticillioides*. These two phytopathogens produce mycotoxins harmful to human and animal health and are responsible for massive use of pesticides, for the protection of cereals. It is therefore essential to better understand the mechanisms of action of alternative control strategies such as the use of BCAs in order to optimize their applications. In this first part of study, the early stages of interaction between BCAs and pathogens were investigated from germination of spores to synthesis of antifungal compounds. The analysis of antagonist activities of BCAs revealed different strategies of biocontrol in these early stages. *S. griseoviridis* produced and secreted several compounds with anti-fungal and anti-germination activity, but also a mixture of hydrolytic enzymes to attack pathogens at a distance. *T. asperellum* favoured contact modes of action with high mycophagous activity associated with degradation enzyme synthesis, among many other mechanisms. *P. oligandrum* showed high early mycophagous activity and its reproduction was

stimulated by the presence of the pathogen. Chronological, process combination and specialization aspects of interactions are also discussed.

Originality-Significance Statement

At the crossroads of economic, health care and environmental concerns, studies on the understanding of biocontrol mechanisms are a key step in the implementation of effective alternative solutions for the control of mycotoxin-producing phytopathogens. This study is the first to follows the different mechanisms concomitantly or not, throughout the entire duration of the interaction between mycotoxigenic pathogens and different types of BCAs under the same experimental conditions (from spore germination to pathogen survival). Furthermore, this study provides different experimental design and procedures to analyze these different modes of action.

Introduction

In agricultural ecosystems, many plant pathogens attack crops and cause significant losses in yield and quality. Among them, some fungi such as *Fusaria* are also capable of producing mycotoxins, secondary metabolites that are particularly toxic to humans and animals, with some combining health-related effect (Marasas *et al.*, 2004; Zain, 2011). Climate change in these environments aggravates an already difficult situation with increasing mycotoxin contamination worldwide (Paterson and Lima, 2010). In cereal crops, the first source of consumer exposure to mycotoxins, *F. verticillioides* (fumonisins producer) and *F. graminearum* (trichothecenes producer) infect and colonize the ears during flowering and synthesize toxins in the grains in formation.

The most effective control methods against these toxins are therefore to be implemented in the field before harvesting and transformation of product (Jard *et al.*, 2011). Alternatives to the use of traditional toxic pesticides are being considered, including the use of biocontrol agents (BCAs). These microorganisms implanted in agricultural environments will interact directly with pathogens through various mechanisms to inhibit pathogens colonization (Glick, 2015) and their toxins production. The main mechanisms identified against mycotoxigenic fungus are mainly related to parasitism, antibiosis, bio-competition or biotransformation of mycotoxins. Parasitism is a direct attack of pathogen by the BCA that will degrade or consume the pathogen host until it dies (Kim and Vujanovic, 2016). Antibiosis is attributed to BCAs being able to synthesize secondary antifungal or anti-mycotoxigenic metabolites, such as antibiotics or lytic enzymes (Wilkins, 1996; Reino *et al.*, 2008). Bio-competition may occur when particular ecological niches are simultaneously occupied by pathogens and BCAs, either in terms of nutrients or space (Dawson *et al.*, 2004; Samsudin *et al.*, 2016). The detoxification of mycotoxins includes the degradation, transformation and direct absorption of toxins produced by mycotoxin fungi (Ji *et al.*, 2016; Hassan *et al.*, 2017). Depending on the conditions and type of BCAs considered, one or more of these inter-connected mechanisms may be observed, with variable contributions to the overall reduction of pathogen populations.

In spite of numerous studies and hopeful results, only few BCAs are currently commercialized, and against *Fusarium spp.* in Europe, only *Pseudomonas chlororaphis* and *Pythium oligandrum* are available (Cerall®, Belchim Crop Protection and Polyversum®, Biopreparáty/De Sangosse, respectively). The development of an effective strategy for the use of BCA critically depends on the understanding of a number of key biocontrol processes and their interactions. Without a reliable estimation of these processes, it is therefore impossible to improve BCA application strategies (Xu *et al.*, 2010). Understanding these complex modes of action, and the synergistic effects occurring at different stages of the interaction, is one of the keys to optimize the efficiency of these BCAs. In addition, in majority of pathogen vs. BCAs studies, the major approach is the dual culture assay (Amin *et al.*, 2010; Kunova *et al.*, 2016; El-Debaiky, 2017), which gives a good idea of the impact on growth with a limited investment, but does not systematically consider the synthesis of mycotoxins. This aspect is nevertheless essential in the interaction between BCAs and mycotoxigenic pathogens

because these toxins are naturally synthesized to allow pathogens to protect themselves from competing or invading organisms and can affect the activity of BCAs according to (Quecine *et al.*, 2016). In addition, this method does not allow targeted identification of the mechanisms triggered by BCAs, or the activation of mechanisms requiring specific conditions.

With the objective in mind to establishing an initial mapping of the various impact of BCAs against mycotoxigenic fungi, this study provides an overview of the potential time-series biocontrol action of three commercial BCAs. They were selected for their contrasting uses, demonstrated efficiency, and microorganism types (*T. asperellum*, *S. griseoviridis*, *P. oligandrum*). To detect potential unidentified mode of action of this BCAs, different early stages of pathogens evolution were focused in this first part of study. Various effects like (1) impact of BCAs on pathogens germination, (2) mycophagous activity of BCAs on pathogens, (3) BCAs chitinase activities and (4) antimicrobial BCAs VOCs were analysed. BCA/pathogen selection criteria and descriptions are presented in Experimental Section as well as the detailed devices developed and used to explore first contact aspects of BCAs interactions with mycotoxigenic fungi and identify key weapons used by the BCAs.

Results

1. Impact of BCAs on pathogens germination

During germination test, spores and Cell-Free-Extracts (CFEs) of BCAs were put in contact with spores of two pathogens *F. graminearum* and *F. verticillioides*. The evolution of co-culture was observed to identify the influence of BCAs on the earliest stage of pathogen cycle: the spore germination (Figure 1). Concerning the direct contact between spores of BCAs and macroconidia of *F. graminearum* (Figure 1A), the two BCAs reduce the germination of pathogen throughout the assay with an inhibition of pathogen germinated spores of 50 and 53% for Myco and Xeda respectively. *F. verticillioides* spore germination is less affected by contact with BCAs spores (39 and 22% of inhibition by Myco and Xeda respectively) (Figure 1C). Concerning the contact between CFEs of BCAs and pathogen spores, the germinative ratio of control was higher probably due to higher nutrient availability in liquid medium dedicated to the CFEs. Despite this higher ratio of the control, a more contrasted effect could be observed. Xeda's CFE has stronger effect against germination of *F. graminearum* (82% of inhibition, Figure 1B). After 24h of contact, with almost total inhibition of germination, an increase of pathogen germinated spores could be observed, but the germ tubes remained restricted in size (50-70 μm on average). For *F. verticillioides*, Myco's CFE had more influence on spore germination (76% of inhibition, (Figure 1D)), with atrophied germ tubes and this effect was maintained over time. No effect of CFEs from the Poly culture could be detected on the percentage of germination of pathogenic spores, or even stimulation and the differences with controls were not significant. Nevertheless, length and branching of pathogenic germ tubes in contact with Poly's CFE was more important than control.

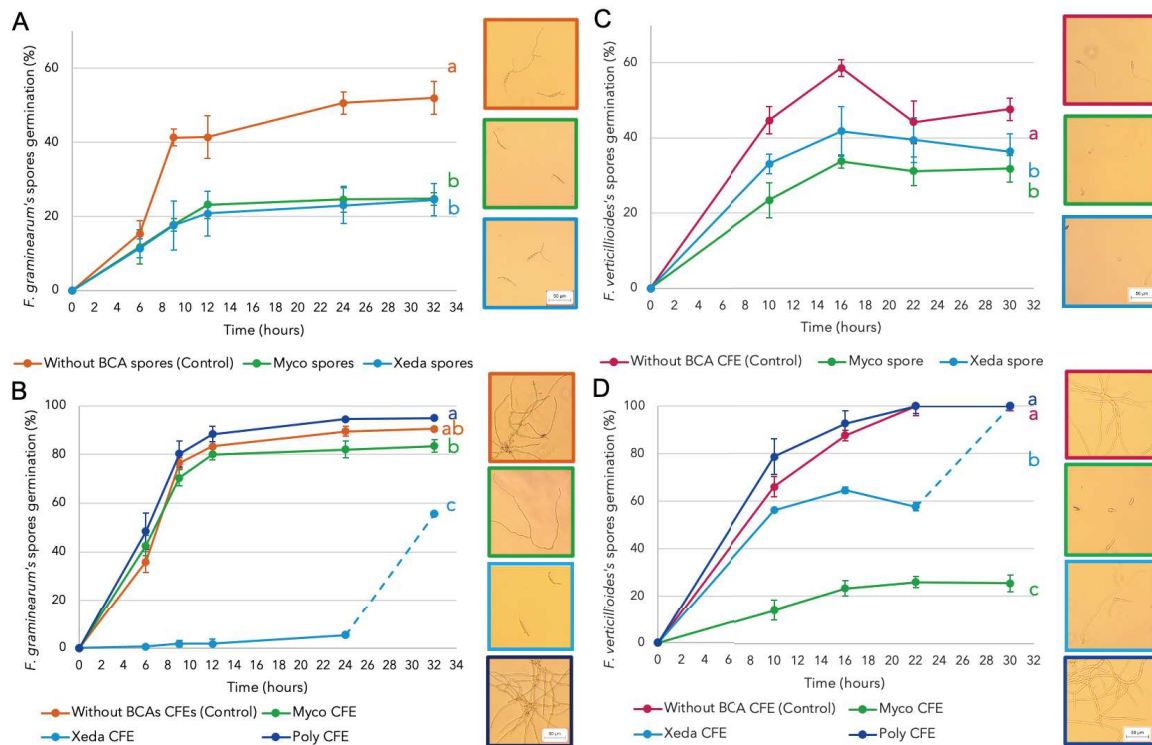


Figure 1: Effect of BCAs on germination of pathogenic spores. *F. graminearum* spores: A. in contact with BCAs spores; and B. in contact with BCAs Cell-Free-Extracts; and on germination of *F. verticillioides* spores: C. in contact with BCAs spores; and D. in contact with BCAs Cell-Free-Extracts. Optical microscope images were taken after 24 hours of contact between pathogen spores and BCAs spores or CFEs at 25°C. Part of curve in dotted line indicate an increase of pathogen spore's germination percentage but with reduced length germ tubes. CFE: Cell-Free-Extract; Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. ANOVA test independent for each competition modality on area under the growth curves (AUGCs), p -value < 0.05.

2. Mycophagous activity of BCAs on pathogens.

At the beginning of contact between alive mycelium of pathogen and spores of BCAs in developed device to reveal mycophagous activity, contrasted observations could be realized (Figure 2). In absence of pathogen, BCAs were unable to develop, but the presence of nearby living mycelium of pathogens allowed the detection of potential mycophagous activity in co-culture system.

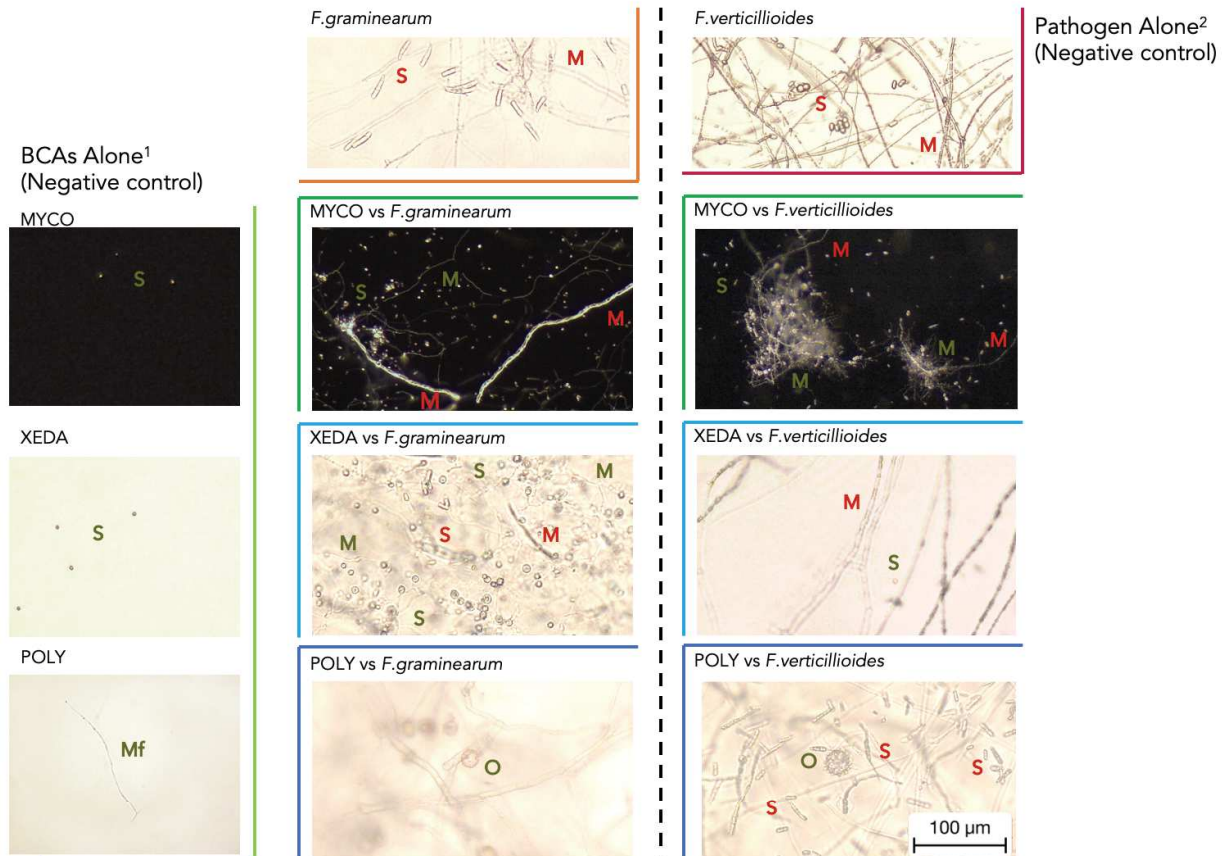


Figure 2: BCAs mycophagous activity against pathogens. Optical microscope images were taken after 20 days of contact between BCAs spore suspensions and alive mycelia of pathogens at 25°C.

¹BCAs Alone: Devices were inoculated with spore suspension only, and the pathogen spore suspension was replaced with water in the culture well. ²Pathogens Alone: Devices were inoculated only with the pathogen spore suspensions in the culture well, the BCA suspension was replaced with sterile water.

S: spores, M: mycelium, Mf: Micro-filament, O: oospores, in green or red for BCA and pathogen respectively. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®.

Myco, was able to grow in contact with the pathogen *F. graminearum*. Spores, produced in large quantities compared to Myco alone control, were accumulated along the pathogenic hyphae. They formed very fine filaments that were able to bind to the pathogen. Myco also had the ability to proliferate in contact with *F. verticillioides* mycelium, even with a greater development of its mycelium/pseudo-mycelium. This one appeared as early as day 10 and formed pellet that enclosed the pathogen filaments. Xeda was also able to grow directly around the spores or filament of *F. graminearum*. Colony of Xeda were frequently observed around deformed *F. graminearum*'s spores or filaments. This colonization had allowed the production of newly generated Xeda's spores then who could form colonies of their own. This ability could be considered early as it was clearly evident on day 6 (data not shown). Nevertheless, no germination of Xeda spores could be observed in contact with *F. verticillioides*. A reduction of quantity of pathogen spores could be observed with Poly. The

action was similar on the two pathogens with a high proliferation of filaments of Poly from day 3 (data not shown). This BCA had progressively invaded the pathogenic mycelium and characteristic reproduction structures, oogonia, could be observed. After fifteen days, matures oogonia containing oospores were starting to come off.

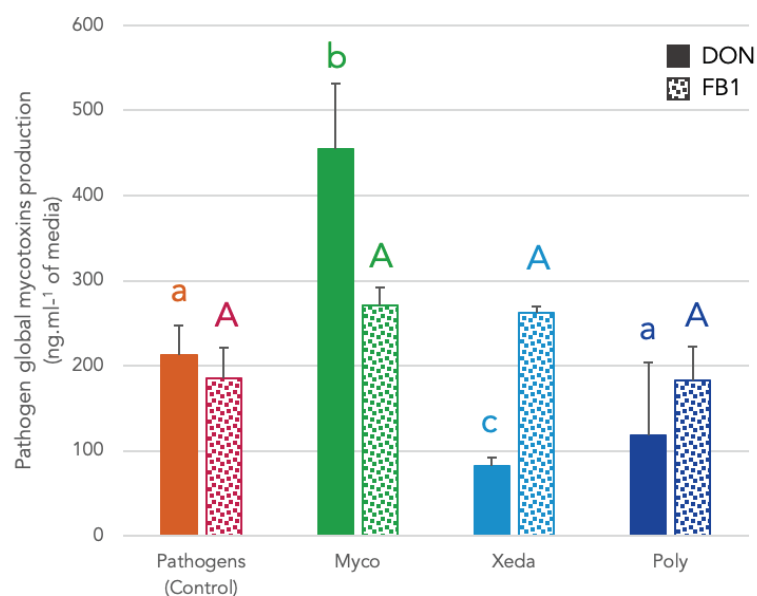


Figure 3: Effect of BCAs mycophagous activity against global production of major mycotoxins of pathogen. Results were obtained after extraction and quantification of mycotoxins in suspension around well, at the end of mycophagy test (after 20 days at 25°C). Different colors indicate treatment and different patterns indicate mycotoxins type: *F. graminearum*, DON (full colors) and *F. verticillioides*, FB1 (small squares pattern). Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. ANOVA test for DON (a, b, c) and FB1 (A), p-value < 0.05.

At the end of experiment (20 days), concentration of mycotoxins diffused on BCAs suspensions were quantified (Figure 3). Although the nutritional conditions of BCAs were very restricted, Xeda was able to reduce DON concentration by 62% compared with control. On the contrary, the presence of Myco strongly stimulated the production of DON by *F. graminearum*. No BCAs allowed the inhibition of FB1 diffusion of *F. verticillioides*.

3. BCAs chitinases activities

During the growth phase of the microorganisms, the ability of BCAs to produce enzymes to degrade pathogen walls was tested, especially chitinases. The results showed how BCAs modulate this activity and their growth according to the presence or absence of pathogens (Figure 4). On Day 7, the presence of the inert pathogen powders stimulated the enzyme activities of exochitinase 1 and exochitinase 2 by Myco, with a 13-fold increase over the control on average of activity of exochitinase 1 and exochitinase 2 (Figure 4A). Xeda had a similar profile with stimulation of exochitinase 1 by *F. verticillioides* powder (9-fold increase) and exochitinase 2 by powders of both pathogens (8.5-fold

increase on average). Myco had the additional ability to produce endochitinases, particularly in the presence of the wall cells of *F. verticillioides*. However, the two BCAs had contrasting growth behavior (Figure 4B). After a short delay (5 days), no significant difference of Myco growth between the modalities with and without the addition of pathogen powders was observed. On the contrary, the growth of Xeda was particularly stimulated by the presence of pathogens (10-fold increase) on average). The enzymatic activity of Poly did not seem to be affected by the presence of pathogens but it showed the ability to produce the three tested chitinases. Its growth was not modulated by pathogenic fungal wall cells.

After ten days, Myco's chitinolytic activities for exochitinases 1 and 2 were stabilized at around 16 and 8 mU.mL⁻¹ on average of all modalities respectively (Figure 4A). For Xeda, enzyme activities exploded in the presence of pathogenic powders (100-fold for exochitinase 1 and 83-fold for exochitinase 2). Polyversum retains approximately the same activity over time with a small stimulation in the presence of pathogen wall cells of *F. graminearum*. To confirm the apparent constitutive synthesis of exochitinase by Poly, supplementary analysis was performed on solid media, and comparable profile was detected.

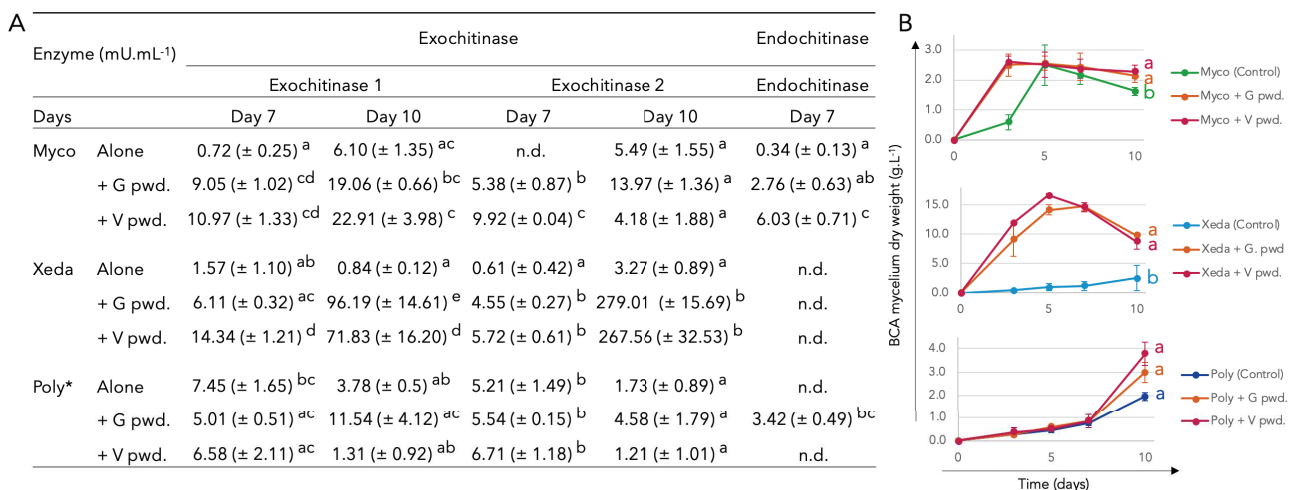


Figure 4: A. BCAs chitinases activities in presence of pathogens mycelium fragments.

Chitinase activity (mU.mL⁻¹) of BCAs alone or in contact with mycelium powder of pathogen at day 7 or 10 (endochitinase was tested only at day 7), B. Dry weight evolution of BCA during chitinase test (g.L⁻¹). Minimum media is liquid and adapted for each BCAs growth. Cultures are carried out at 25°C under agitation of 150 RPM during ten days. Exochitinase 1: chitobiosidase activity, Exochitinase 2: β -N-acetylglucosaminidase activity and Endochitinase: endochitinase activity; G pwd.: *F. graminearum* mycelium powder. V pwd.: *F. verticillioides* mycelium powder; n.d.: not detected. *To confirm the apparent constitutive synthesis of exochitinase by Poly, supplementary analysis was performed on solid media, and comparable profile was detected. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. ANOVA test, between BCAs (all conditions combined (Alone, + G.pwd., + V.pwd.)) for each chitinase (Exochitinase 1, Exochitinase 2, Endochitinase) and day (Day 7 or 10) analysed independently (a, b, c, d), p-value < 0.05.

4. BCAs antimicrobial Volatiles Organic Compounds (VOCs)

BCAs were screened for production of antimicrobial volatile organic compounds against mycotoxigenic *Fusaria* in plates (one turned on top of the other) (Figure 5). In regard to growth effect, only Xeda seemed to produce VOCs able to reduce the growth of *F. verticillioides* by 27% compared to control (Figure 5B). No BCAs influenced the growth of *F. graminearum* (Figure 5A). But it is interesting to note that important effects were found in the specific production of fungal mycotoxins. *F. graminearum* mycotoxins were affected by Poly COVs (67% and 70% of inhibition of DON and 15ADON respectively); but especially by Xeda COVs with 82% and 91% of inhibition of DON and 15ADON respectively (Figure 5C). Myco were able to produce VOCs which can trigger a reduction by half of *F. verticillioides* mycotoxin specific production with 55% and 57% of reduction of FB1 and FB2 respectively. Xeda treatment again induced the lowest quantities of mycotoxins produced by *F. verticillioides* with nearly complete inhibition (95% and 98% of reduction of FB1 and FB2 respectively). During the test, macroscopic observation of reverse side of Petri dish system allowed to observe that the VOCs of Xeda in confrontation against *Fusarium* pathogens caused an important inhibition of the production of pigments by both pathogens.

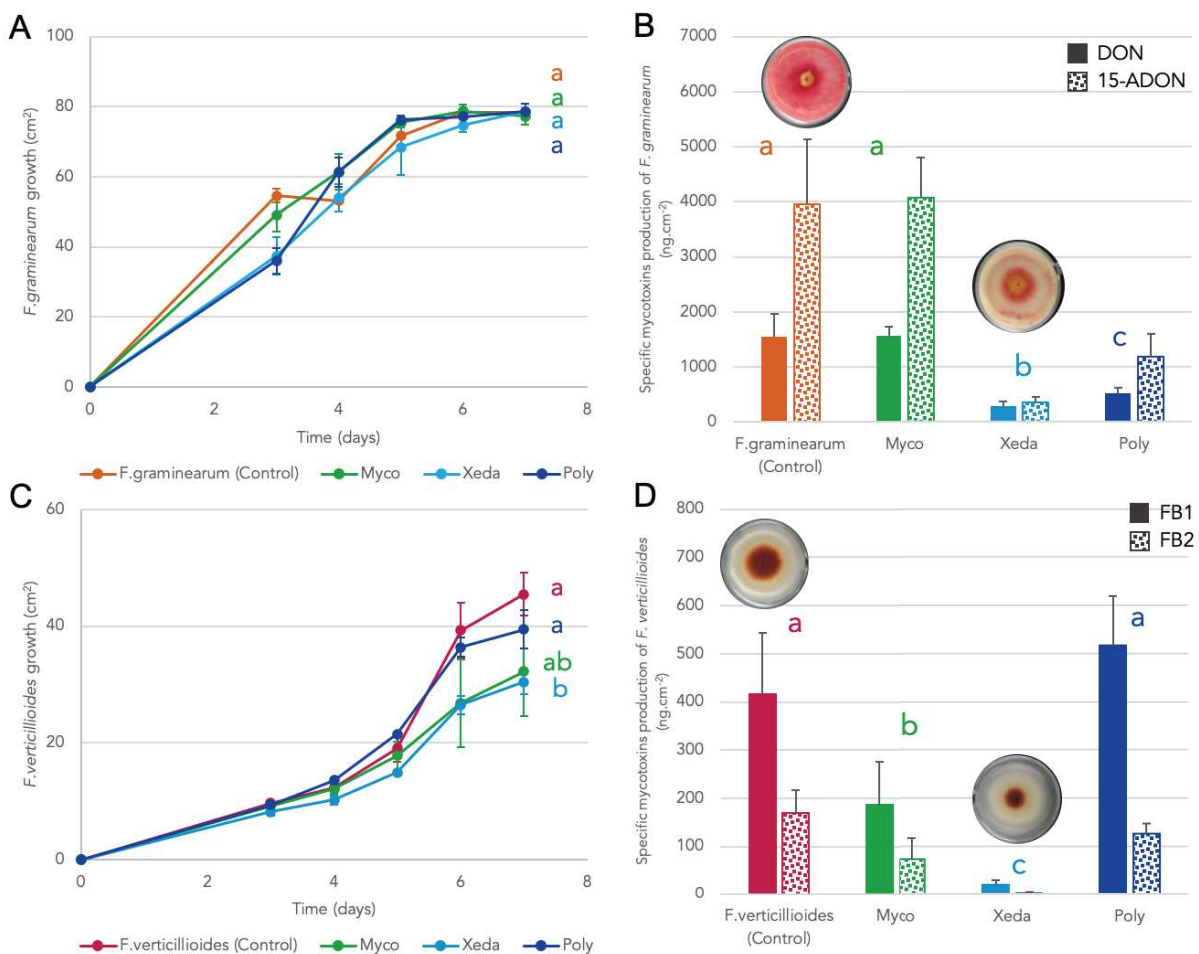


Figure 5: Effect of VOCs from BCAs against growth (A, C) and mycotoxins production (B, D) of pathogens. A,B: Dual culture of BCA-*F. graminearum* confronted without contact on CYA medium during 8 days (A) and at 8 days (B) at 25°C. C,D : Dual culture of BCA-*F. verticillioides* confronted without contact on PDA medium during 8 days (C) and at 8 days (D) at 25°C. Different colors indicate treatment and different pattern indicate mycotoxins type. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. ANOVA test independent for each competition modality on area under the growth curves (AUGCs) (A and C) or Specific mycotoxins production at days 8 (B, D) , p-value < 0.05.

Discussion

The broad reflection presented in this study explored the interaction of mycotoxigenic pathogens and biocontrol agents, the aspects of specialization of certain mechanisms adopted by BCAs and the possible chronology of events during the early stages of the interaction between these two types of microorganisms. This research could lead to the identification of new antifungal activities by the BCAs studied in order to stimulate them, or to detect them in potential new candidates of biocontrol.

Many studies on biocontrol by microorganisms provide a good overview of the different mechanisms that can be implemented by BCAs to prevent or stop the progression of pathogens. It appears that biocontrol is the result of many different types of interactions between microorganisms in a complex synergy (Someya *et al.*, 2001; Fogliano *et al.*, 2002). In general, studies focus on the characterization of mechanisms occurring in different experimental situations and at different stage of pathogen life cycle (Audenaert *et al.*, 2002; Joo, 2005; Ambrico and Trupo, 2017; Tang *et al.*, 2018). In all cases, pathogens are inhibited by the presence and activities of other microorganisms they encounter. But to our knowledge, none of these studies follow the different mechanisms concomitantly or not, in the interaction between different types of BCAs and mycotoxigenic pathogens. Have performed analysis under the same experimental conditions throughout the entire duration of life cycle interaction (from spore germination to pathogen survival), allows to better locate the key moments of the antagonist relationship. This work can be considered as a complementary approach to studies on precise targeted mechanisms involving the characterization of specific genes or molecules, by providing an integrative view of the interaction (Lutz *et al.*, 2003; Reithner *et al.*, 2005).

This first part of study was focused on identifying biocontrol mechanisms in the early phases of the interaction (Figure 6). Concerning the biocontrol global interaction with pathogens studied and the tools developed, the first contact between BCAs and pathogens can occur as early as the spore germination phase. In that way, spore germination inhibitors are frequently investigated as potent BCAs (Miyagawa *et al.*, 2001). Our results show that the susceptibility of pathogens to the types of anti-germinative compounds can be variable. *F. graminearum* was more sensitive to secretions produced by BCA spores, suggesting a very early action of BCAs. *F. verticillioides* is more sensitive to secondary metabolites produced in CFEs, which implies a prior synthesis of anti-germinative

compounds by the BCAs before coming into contact with the pathogenic spores to inhibit their germination. Adhesion phenomena of BCAs spores around pathogenic spores could also be envisaged as it is the case of lactic acid bacteria which adhere to yeast (León-Romero *et al.*, 2016). After this initial contact, the mycophagy test identified BCAs which were able to grow only in the presence of alive and active mycelium of pathogens as source of nutrients. In literature, the mycophagy capacities were investigated mainly in the case of interaction between bacteria and fungus pathogen, notably in Balhausen *et al.* and Rudnick *et al.* studies (Ballhausen *et al.*, 2015; Rudnick *et al.*, 2015), on which our research was based to build the experimental device. When adapting this type of device to study the mycophagous capacity of fungus and oomycete, many adjustments had to be made. Unexpectedly, many microorganisms have the capacity to consume gelling agents composed of complex polymers or very distant from their common consumed compounds (like Phytigel or Kappa). This is why the removal of all external nutrients was a priority in the design of the experiment. The analysis of the mycotoxins present at the end of the test are of course to be nuanced given the critical nutritional conditions of the pathogen, leading to a restricted development (lower control than usual in the confrontation test). BCAs also have critical nutritional conditions because they have no resources apart from the pathogens. In contrast to the few studies that have been conducted on bacterial mycophagy, fungal mycophagy, is very often associated with mycoparasitism. Mycoparasitism is the subject of many research studies (Harman, 2006). Most of them have focused on *Trichoderma species* (Guzmán-Guzmán *et al.*, 2017; ben Amira *et al.*, 2018; Mokhtari *et al.*, 2018). Mycoparasitism can be defined as the ability of a mycoparasite to directly attack another microorganism; which includes all aspects of specialization and trophic mode and was mainly tested under optimal nutritional conditions (Bhat, 2017; Yang *et al.*, 2020). In previous studies, mycoparasitism was broadly divided into two categories, biotrophic and necrotrophic parasitism (Boosalis, 1964). Biotrophic mycoparasites can be further classified into destructive mycoparasites and balanced mycoparasites. Balanced mycoparasites inflict little or no apparent damage to the host, whereas destructive mycoparasites can cause damage or kill the host (Jeffries, 1995). The term microbial predation is mostly used to refer to non-specific interactions (Pal and Gardener, 2006). It would be interesting to continue investigations in order to better understand whether the concepts of microbial predation, mycophagy and mycoparasitism can be considered as the same type of BCA strategy or as different strategy.

During the growth phase of interaction, various weapons were deployed by BCAs to stop the progression of pathogens, including the synthesis of fungal wall degradation enzymes. The capacity of diverse BCAs to produce chitinases in particular is widely known (Swiontek Brzezinska *et al.*, 2014). In our test, the presence of inactivated pathogens in the form of mycelium powder avoids the use of chitin synthetic media and strongly stimulated enzyme activities, because they can be considered as an element of pathogen perception by the BCAs. Indeed, chitinases are inducible and mainly expressed under certain conditions. While chitin is an inducer, some other easily assimilated carbon

source can be a repressor (Sahai and Manocha, 1993), and the composition of pathogenic mycelia can influence the production of BCA enzymes. It might be interesting to introduce this kind of stimulant when formulating BCAs, especially considering that chitin present in mycelium powder can also stimulate plant defences (Katiyar *et al.*, 2015). The simultaneous study of different types of chitinases made it possible to identify the production of enzyme combinations. Endochitinases cleave chitin chains in random locations, generating low molecular weight oligomers and exochitinases. The two exochitinases studied were chitobiosidases, which catalyse progressive release of diacetylchitobiose from terminal nonreducing end and N-acetylglucosaminidases, which cleaves oligomeric products obtained by endochitinases into monomers of N-acetyl glucosamine respectively (Swiontek Brzezinska *et al.*, 2014). These different and complementary modes of action allow maximum anti-fungal efficiency, and synergistic antifungal activity has been observed for endochitinases in association with exochitinase (Someya *et al.*, 2001; Viterbo *et al.*, 2002). But very few studies discuss the differential efficiency of exochitinase intra-family. It seemed that some BCAs produced an exochitinase preferentially to the other one, like Myco; while others strongly reversed the ratios between the two exochitinases studied over time, like Xeda which was able to switch between two chitinase activities. From an ecological point of view, BCAs can have different strategies for the same function: some BCAs grow slowly but produce large amounts of enzyme quickly (like Myco), while others grow rapidly but produce specifically less chitinase (like Poly), as a compensatory phenomenon. Some studies suggest that chitinolytic enzymes could be directly used as a complement to pesticides to increase their efficiency against fungal pathogens, in order to reduce the concentrations required (Dahiya *et al.*, 2006).

In addition to enzymes, BCAs can also produce volatile anti-fungal and/or anti-mycotoxigenic compounds. It has been shown that volatile organic compounds (VOCs) from microorganisms can influence the growth of plant pathogenic fungi (Alstrom, 2001; Giorgio *et al.*, 2015). Most preliminary studies on the effect of VOCs on pathogens are conducted in the same manner as our study (Lahlali and Hijri, 2010; Qualhato *et al.*, 2013). However, for both pathogens, lower levels of mycotoxin production than in direct confrontations were detected, even in controls. For *F. verticillioides*, very different FB1/FB2 ratios compared to direct confrontations (Pellan *et al.*, 2020) can be explained by the sealing of the boxes, creating a microclimate that can disrupt mycotoxin production ratios of fumonisin analogues (Schoeman *et al.*, 2017). Nevertheless, some of the BCAs are able to produce VOCs that inhibit growth and mycotoxins productions (such as Xeda), while others will specifically inhibit mycotoxin production. Without possible contact, it would therefore appear that compounds synthesized by BCAs can have a direct inhibitory action on both pathogen's mycotoxin biosynthesis pathway. In view of the results, the synthesis of volatile compounds could be a major advantage in the strategy of selected BCAs. However, the identification and analysis of the volatile interactions between BCAs, as well as the biotic and abiotic factors that influence these relationships, remains to be elucidated (Dilantha Fernando *et al.*; Campos *et al.*, 2010).

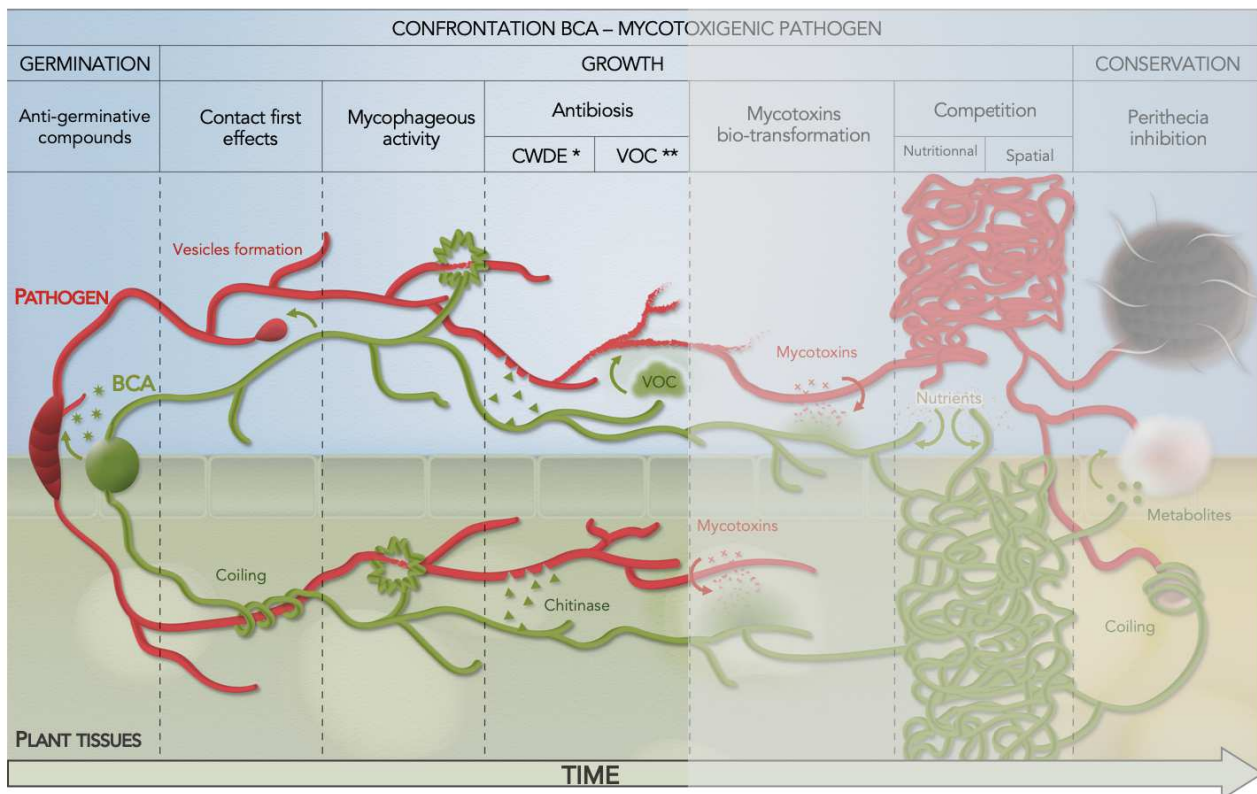


Figure 6: BCAs biocontrol mechanisms activity against pathogenic *Fusaria* over time.

During confrontation between BCA and mycotoxigenic pathogen, a broad range of weapons can be used by BCAs, from the germination of pathogen, during growth phase, to conservation under perithecia structure. Depending on the BCAs considered and at early stage of interaction, anti-germination compounds can be synthesized to inhibit the germination of pathogenic spores. The first contacts between microorganisms causes the formation of particular structures or deformations of pathogenic hyphae. BCAs can coil around the pathogenic mycelium, and block their progression. Even in the absence of nutrients, they may be able to develop colonies around the active pathogen mycelium. BCAs, using a battery of secreted compounds such as Cell Wall Degrading Enzymes (*CWDEs) or antifungal volatile compounds (**VOCs), will limit the spread of the pathogen. The mechanisms involved in later stages will be explored in a second part of the study.

Through the diversity of BCAs selected for the study, it was possible to characterize contrasted strategies during the development of interactions between a BCA and *Fusarium* pathogens.

The first BCA studied, Myco, is an actinobacteria of the species *Streptomyces griseoviridis*. It had a strong impact on spore germination, of *F. verticillioides* in particular (Myco spores and Myco CFEs vs spore of *F. verticillioides*). These conclusions are in line with the anti-germinative effect of Myco against *F. verticillioides* during microscopic observations of the first phases of the interaction between these two microorganisms (Pellan *et al.*, 2020). This effect could be linked to the synthesis of compounds inhibiting the germination of plant pathogen's spores identified with other *Streptomyces spp.*, such as fistupyrone or methyl vinyl ketone (Igarashi, 2004; Wang *et al.*, 2013). *F. verticillioides* is also very sensitive to the mycophagous capacities of Myco, with early development of colonies of

Myco early during the test (starting from days 4-8). Indeed, bacteria have found niches in the use of nutrients derived from fungi, with nutritional strategies from the consumption of mycelial exudates to endosymbiosis and mycophagia (Frey-Klett *et al.*, 2011). Some *Streptomyces* have shown mycophagous ability against *Aspergillus niger* or *Gigaspora gigantea*, through the use of sterile sand tests or microscopic observation of interactions (Leveau and Preston, 2008). Particular attention was paid to the stimulation of the production of mycotoxin DON by *F. graminearum* in the presence of Myco, during the mycophagy test. Microbial or plant interaction can stimulate the mycotoxin production, as it is the case for other pathogens such as *Penicillium* (Paterson, 2007), or when *F. verticillioides* are in interaction with its host plant (Picot *et al.*, 2010). Very few studies have been carried out on the subject, contrary to the impact of abiotic factors, and it seems that this is the first report concerning the stimulation of DON by a biocontrol agent of genus *Streptomyces*. *Streptomyces* are also known for their ability to produce enzymes that can lead to the destruction of fungal cell wall (El-Tarabily, 2003; Gonzales-Franco and Robles Hern, 2009). The ability to rapidly produce endochitinases, exochitinases and chitobiases was an advantage that allowed Myco to achieve complete and efficient hydrolysis of fungal cell wall (Kubicek *et al.*, 2001). VOCs produced by Myco had specific effect against *F. verticillioides*. However, numerous studies showed the capacity of other *Streptomyces* strains to synthesize antifungal and anti-conidial germination's VOCs, efficient against various pathogens like *Aspergillus* and *Fusarium* (Lalitha *et al.*, 2007; Sharma *et al.*, 2020). Globally, in early stage of interactions, Myco produced and secreted one or more compounds with anti-fungal and anti-germination activity, but also a mixture of hydrolytic enzymes to attack pathogens. It could have an effect before contact and during first closer interactions, through its mycophagous capacities. Globally, it activated more different mechanisms against *F. verticillioides* than *F. graminearum*.

The second BCA studied Xeda, is a *Trichoderma asperellum*. It had a major impact on the germination of *F. graminearum* spores, either as spores or CFEs. The reduction in size and deformation of the germ tubes produced has already been identified during the interaction between *F. graminearum* and *Paenibacillus polymyxa*, another biocontrol agent (He *et al.*, 2009). This effect could be related to the synthesis of anti-germinative compounds such as viridin, which has been identified for *Trichoderma spp.* as an inhibitor of spore germination of different pathogens depending on its concentration (Reino *et al.*, 2008). More precisely, another compound secreted by *Trichoderma asperellum*, the 6-pentyl-alpha-pyrone, has shown a higher effect on the conidia germination and tube elongation of *F. graminearum* than *F. verticillioides* (El-Hasan *et al.*, 2007). *F. graminearum* is also very sensitive to the mycophagous capacities of Xeda, confirmed by the temporal aspect of the observations with development of its colonies very early during the test (from 4-8 days). No mycophagous activity can be detected in interaction with *F. verticillioides*, that can be surprising from an ecological point, because the two species of *Fusarium* are close and microbial predation was defined as non-specific (Pal and Gardener, 2006). Concerning *Trichoderma spp.*, the mycophagy and mycoparasitism were considered as same phenomenon, consisting in the recognition and adhesion of *Trichoderma spp.* to

the host cell wall, followed by the hydrolysis of host hyphae by means of lytic enzymes, concluding with the uptake of the host cellular content (Inbar and Chet, 1996). However, the actual importance of mycophagous growth for *Trichoderma spp.* under natural conditions is not known (Höppener-Ogawa *et al.*, 2009). Xeda, even under limited nutritional conditions, still successfully maintains the inhibition of mycotoxins produced by *F. graminearum*, suggesting the use of dedicated mechanisms. During the first contacts with pathogens, Xeda had already demonstrated its ability to dissolve *Fusarium* hyphae through their mycelia (Pellan *et al.*, 2020). The production of chitinases by *Trichoderma spp.* are widely known, and several genes encoding chitin- and β -glucan- degrading enzymes have been identified in different species (Yang, 2014; Ramada *et al.*, 2016). When quantifying the chitinolytic activities of Xeda, exochitinases were massively detected, but not endochitinases, unlike other species of *Trichoderma* able to produce N-acetylglucosaminidases, endochitinases, and chitobiosidases (Haran *et al.*, 1995). However, other strains of *T. asperellum* have shown their ability to produce mainly exochitinases but also small amounts of endochitinase, especially in the contact phase but not in the pre-contact or post-contact phase with the pathogen under study (Guigon-Lopez *et al.*, 2015), which may suggest that chronology plays an important role in the analysis of chitinolytic activities. Another explanation may be related to the repression of chitinase genes by mycotoxigenic *Fusaria* and Deoxynivalenol, as described in the BCA *Trichoderma atroviride* (Lutz *et al.*, 2003). To contribute to its antibiosis action, Xeda has also shown abilities to produce antifungal VOCs, with a very effective inhibition of mycotoxin production by both pathogens. The identification by GC-MS of compounds produced by *Trichoderma spp.*, such as 6-pentyl-alpha-pyrone, viridifungin or dimethyl disulfide (El-Hasan *et al.*, 2009; Chen *et al.*, 2016) could explain the observed effects. In addition, severe discolouration of pathogenic colonies was observed. In *Fusarium*, these pigments like naphthoquinone, may be responsible for phytotoxic, insecticidal, antibacterial, and fungicidal effects (Medentsev *et al.*, 2005). Recently, the synthesis of aurofusarin and the red rubrofusarin, two pigments produced by *F. graminearum*, have been directly correlated with the production of mycotoxins, with health toxicity effects when combined with DON (Cambaza, 2018). Xeda could therefore directly reduce the toxicity of molecules produced by pathogens, through the synthesis of these VOCs. Globally, in early stage of interactions, Xeda can lead anti-germinative and anti-mycotoxins effects against pathogens. Its wide range of mechanisms of action include remote strategies such as antibiosis through the synthesis of VOCs, but especially in direct contact, using its mycophagous/mycoparasite characteristics. Xeda activated more mechanisms against *F. graminearum* than against *F. verticillioides*.

Finally, the last BCA studied Poly, is the oomycete of *Pythium oligandrum*. Although it did not show any ability against pathogenic spore germination, its high colonization capacity allowed it to rapidly divert nutrients from pathogens in the mycophagy test. In addition, a strong stimulation of the sexual reproduction of this BCA was observed in contact with pathogens. The perception of ergosterols from the pathogen could trigger this phenomenon as sterols have been identified as inducers of sexual

reproduction (Hendrix, 1964; Cliquet and Tirilly, 2002), and give to Poly a supplementary advantage. *Pythium oligandrum* has already shown mycoparasitic capacities against fungal and oomycete pathogens (Benhamou *et al.*, 2012). The same questions remain regarding the boundary between mycophagy and mycoparasitism. In terms of Poly's chitinolytic activity tests, it was able to produce all the chitinases tested but constitutively at relatively low levels, under all conditions. These observations are in accordance with the results obtained during microscopic observation of the interaction between Poly and *Fusarium* pathogens, where the connections between Poly and pathogenic hyphae were limited in first steps (Pellan *et al.*, 2020). Despite this, chitinases could play an important role, notably in association with other lytic enzymes to contribute to the mycoparasitism mechanism of this BCA. In molecular study, transcripts encoding cellulases, glucanases, and proteases are produced by *P. oligandrum* which was grown under biocontrol conditions (Benhamou *et al.*, 1999; Horner *et al.*, 2012). Poly can also use its ability to produce VOCs, which have a strong impact on the production of DON mycotoxins. Compared to classical dual culture assay, the effect is strong enough to be considered as part of Poly's overall control strategies against *F. graminearum* (Pellan *et al.*, 2020). But no effect on pathogen growth was detected. Few studies on the production and characterization of VOCs produced by *P. oligandrum* have been performed. However, it appears that *P. oligandrum* is capable of producing non-identified antibiotic and/or antifungal VOCs against different phytopathogens such as *F. oxysporum* or *Phoma medicaginis* (Bradshaw-Smith *et al.*, 1991; El-Katatny *et al.*, 2006). Based on our results without contact, this is the first report of VOCs produced by Poly that could have a direct impact on the mycotoxin biosynthesis pathways of *F. graminearum*. Globally, in early stage of interactions, Poly rapidly inhibits the pathogens progression in a less specific way than other BCAs. It was capable of reproducing rapidly in contact with pathogens and had characteristics of mycoparasitism and microbial predation. It was able to activate fewer mechanisms than others BCAs but their efficiency is higher, allowing it to be a major tool in the fight against mycotoxigenic fungi.

Conclusion

This study allowed the identification of biocontrol mechanisms that occur early in the interactions between BCAs and pathogens. Through dedicated experiments, the results highlighted a variety of primary strategies when contrasting BCAs attack mycotoxigenic pathogens. Some have activated a wide variety of complementary mechanisms, while others have activated fewer modes of action during these first contacts but with greater individual effectiveness. The strategies of biocontrol agents can be very varied. Myco has rapidly established a wide range of pre-contact mechanisms that appear to be mainly related to antibiosis. Xeda was the most multivalent with capabilities in all the mechanisms tested, whether contact or non-contact, enabling it to strongly inhibit the *Fusarium* pathogen early in their life cycle. Poly had the least diverse modes of action, an almost-opportunistic strategy supported

by a strong growth capacity and less specialized with an overall action on both pathogens unlike other BCAs. This study highlights the complexity of the synergy of the modes of action, but also explores many avenues for a better exploitation of these modes of action and the detection of these capacities in other BCAs. The mechanisms involved in the later stages of interaction are the subject of a second part of this study, with certain correlations between the two phases (see Part 2, Late stages). The diversity of modes of action of microorganisms could be more precise or complete by considering an integrative approach combining this approach based on microbiology and biochemistry with other method such as phytopathology and molecular biology, disciplines that will be implemented in future studies.

Experimental Section

1. Microorganisms and culture media

1.1. *Fusaria* species

Fusarium graminearum strain BRFM 1967 and *Fusarium verticillioides* strain BRFM 2251 (CIRM, University of Aix-Marseille, Marseille, France) were selected for their high respective mycotoxins production. *F. graminearum* was isolated from wheat plant and has a deoxynivalenol (DON/15-ADON) chemotype; *F. verticillioides*, was isolated from maize kernels, and has a fumonisins (FB1/FB2/FB3) chemotype. Pure cultures were maintained on potato dextrose agar (PDA; BD Difco, Sparks, MA, USA) at 4 °C under paraffin oil. For spore suspensions production, pathogens actively grown on PDA at 25 °C for 7 days. For *F. graminearum* dual culture assay against BCAs, CYA or CYB (sucrose 30 g; with or without agar 15g; yeast extract (Biokar, Beauvais, France) 5 g; K₂HPO₄·3H₂O 1 g; NaNO₃ 0.3 g; KCl 0.05 g; MgSO₄·7H₂O 0.05 g; FeSO₄·7H₂O 0.001 g; ZnSO₄·7H₂O 0.001 g; CuSO₄·5H₂O 0.0005 g; per liter of distilled water, pH 6.3 ± 0.2) was used, and for *F. verticillioides* dual culture assay against BCAs, PDA or PDB (BD Difco, Sparks, MA, USA) was used. Compartment of these *Fusarium* species and selection of media are detailed in a previous (Pellan *et al.*, 2020).

1.2. Commercial Biocontrol Agents (BCAs)

Three commercial biological control agents (BCAs) were chosen for their contrasting characteristics and their ability to inhibit the two mycotoxigenic fungi. Explanations on how BCAs were selected and more informations about their characteristics and their effects on growth and mycotoxinogenesis of *Fusarium* pathogen in dual culture assays are available in previous work (Pellan *et al.*, 2020). All BCAs were isolated from their commercial formulation with a classical microbial isolation protocol and conserved in commercial product aliquots (4 °C) or under spore forms in glycerol solution (15%/ V:V / -80 °C). Pure cultures were maintained on ISP4 (agar 18g; starch 10g; K₂HPO₄·3H₂O 1g; MgSO₄·7H₂O 1 g; (NH₄)₂SO₄ 1g; CaCO₃ 1g; FeSO₄·7H₂O 0,001g; MgCl₂ 0,001g; ZnSO₄·7H₂O 0.001g; per liter of distilled water), PDA, and V8 juice agar (V8 juice 200ml (Campbell's, Camden, NJ,

USA); agar 15g; CaCO₃ 3g; per 800ml of distilled water), respectively, for Mycostop, Xedavir, and Polyversum at 25 °C for 7 days for production of spore suspensions or inoculation plug concerning Polyversum. For the rest of the study, the isolates isolated from commercial formulation were referred to using the following abbreviations: Myco for Mycostop/*S. griseoviridis* (Lallemand Plant Care®), Xeda for Xedavir/*T. asperellum* (Xeda International®), and Poly for Polyversum/*P. oligandrum* (DeSangosse®).

2. Toolbox (early stage)

2.1. Impact of BCAs on pathogen germination

Cell-Free-Extracts (CFEs) of BCAs were prepared as follow. Liquid culture of all BCAs in CYB and PDB was incubated at 25°C for ten days at 175 RPM (Unitron INFORS, Bottmingen, Suisse). The microbial culture was centrifugated (10,000 RPM – 4°C – 10min; Centrifuge 5804 R, Eppendorf, Hamburg, Germany) and filtered with sterile PES filter (0.22 µm, Sartorius, Göttingen, Germany) to remove filaments and spores produced by BCAs and to sterilize CFEs. Spore suspensions of two BCAs Myco and Xeda were prepared from solid medium as pure cultures in sterile water and filtered with carded cotton and standardized at 1x10⁵ spores. mL⁻¹. Spore suspensions of pathogen were prepared (1x10⁴ spores.mL⁻¹) in the same way. In microtube, 250 µL of spore suspensions or CFEs of BCAs have been mixed with 250 µL of pathogens spore suspensions and incubated for 30 hours. Microscopic observations and counts of germinated spores were conducted on sample of 10µL, every 3 to 12 hours depending on germination speed of pathogens with a Zeiss PrimoStar microscope. The images were taken using a Zeiss Axiocam ERc5s camera (Carl Zeiss Microscopy, Thornwood, NY, USA). For BCAs spore suspensions modality, H₂O was used as control and for BCAs CFEs modality, PDB and CYB was used as control for *F. graminearum* and *F. verticillioides* spores respectively. Pathogen spores vs BCAs spores or CFEs mixes and controls was set up in triplicate for each analysed time point and 200 spores on average were considered per replicate to calculate the percentage of pathogen germinated spores. Average values were used to create percentage of pathogen spore germination curves.

2.2. Mycophagous activity of BCAs on pathogen.

Specific device was developed based on experiments of Balhausen (Ballhausen *et al.*, 2015) (Figure 7). A well consisting of a microtube cap (Eppendorf, Hamburg, Germany), containing culture medium was deposited in the center of an empty Petri dish. Pathogen spore suspension was added in culture medium well 48h before the beginning of the experiment (10 µL - 1x10⁴ spores.mL⁻¹). CYA and PDA was used for *F. graminearum* and *F. verticillioides* respectively. Then, BCAs spore suspensions (30mL - 1x10² spores.mL⁻¹) was drop off around a culture medium well. To ensure that BCAs had no longer sufficient nutrients to grow in the absence of pathogens, various improvements were made

(Figure 8). The main problem was the elimination of nutritional resources for BCAs in media around the well. Different gelling agents were tested: Agar, Phytigel, Kappa, (5 and 3 g.L⁻¹). Finally, as all gelling agent tested allowed the growth of BCAs, the spores were only diluted in sterile water. Moreover, the spores were previously washed 5 times to remove any residues from the culture medium used to obtain them. To complete the experimental device, a cellophane sheet was layed around the well at spore suspension surface. After incubation of 20 days at 25°C, pathogen was gradually coming out of the well and colonizing BCA suspensions thanks to the cellophane sheet support. If BCA presents a mycophagous activity, BCA colony could be observed at contact with alive pathogen. Observations were realized with a Zeiss PrimoStar microscope and pictures were taken using a Zeiss Axiocam ERc5s camera (Carl Zeiss Microscopy, Thornwood, NY, USA). Phase contrast was used only with Mycostop because its spores are very small. Each BCA/pathogen confrontation and controls were set up in triplicate. To validate the results, only modalities where the three triplicates showed growth of BCAs were considered as positive. Control without pathogen (First negative control) and control without BCAs (Second negative control) were realized. After 20 days, 10mL of spore suspensions of BCAs were sampled and the mycotoxins produced by pathogens were extracted and quantified as described in previous study (Pellan *et al.*, 2020).

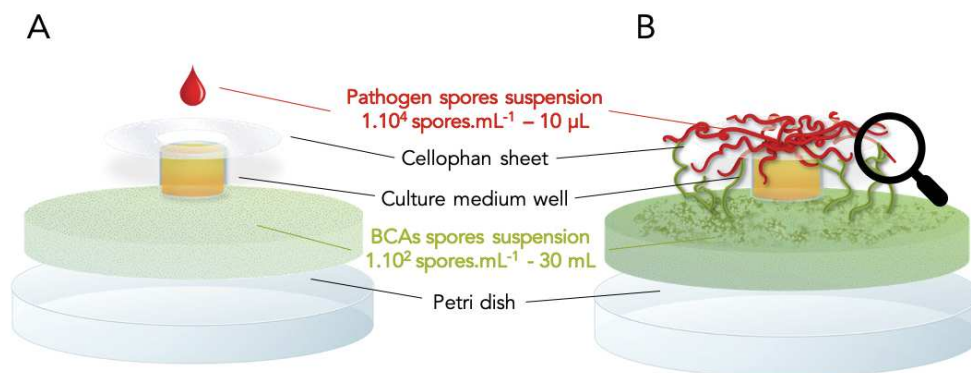


Figure 7: A: Experimental device at beginning of test: In Petri dish, a washed BCAs spore suspension was drop off around a culture medium well. CYA and PDA was used for *F. graminearum* and *F. verticillioides* respectively. A cellophane sheet was layed around the well at surface of the BCAs suspension. Pathogen spore suspension was added in culture medium well at the beginning of experience; B: Experimental device after incubation of 20 days, pathogen was gradually coming out of the well and colonizing BCA suspension thanks to the cellophane sheet support. If BCA present a mycophagous activity, BCA colony could be observed at contact with alive pathogen. Magnifying glass indicate the global position of optical microscopic images.

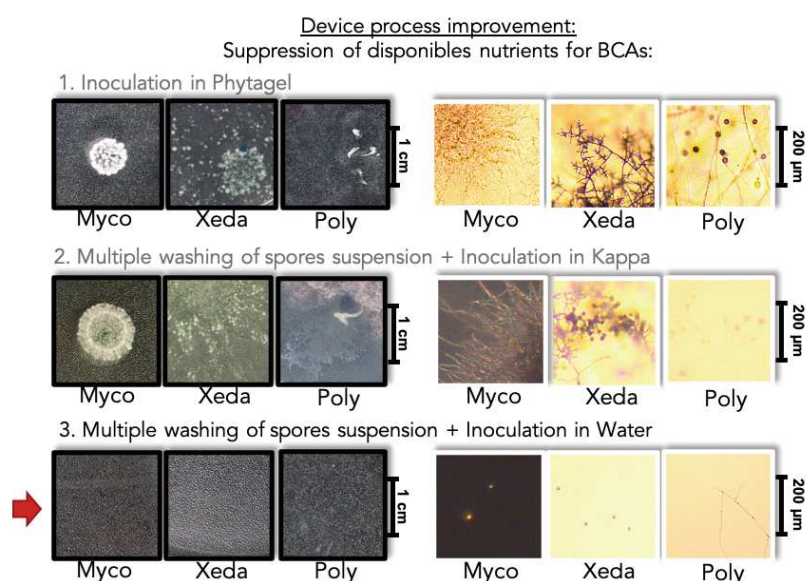


Figure 8: Experimental device process improvement.

To ensure that BCAs no longer had sufficient nutrients to grow in the absence of pathogens, various improvements were made based on devices proposed by Balhausen (2015). All BCAs were directly inoculated on different poor media without pathogen to verify the absence of growth (1. Phytigel, 2. Kappa, 3. Water). Optical microscope images framed in black were obtain at macroscopic scale and those framed in white was obtain at microscopic scale after 20 days of incubation. Only (3:) Multiple washing of spore suspension followed by inoculation in water had permitted to note the absence of development of BCAs without nutrients. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®.

2.3. Chitinases activities of BCAs

In 250 ml Erlenmeyer, 100 ml of minimum liquid medium specific for each BCAs was prepared as described in literature: ISP4 liquid for Myco (Shepherd *et al.*, 2010), MSM for Xeda (Mallikharjuna Rao *et al.*, 2016) and MMPO for Poly (Al-Rawahi and Hancock, 1997), supplemented or not (Control) with 1% (w/v) of cell wall powder of *F. graminearum* or *F. verticillioides* (El-Katatny *et al.*, 2006) (abbreviated G pwd. and V pwd. respectively), i.e. three combination per BCAs. BCAs were inoculated with spore suspensions (5mL - 1×10^5 spores.mL⁻¹), except for Poly, which was inoculated with 7-days plugs (5 mm in diameter) in Erlenmeyer flasks and incubated at 25°C and 150 RPM (Unitron INFORS, Bottmingen, Suisse) for ten days. Liquid cultures were centrifugated (5 min – 10,000 RPM), the supernatant was used as the crude enzymes extract and stored at -20°C until used to assay enzyme activity. Pellets were used to determinate corresponding dried weight of BCAs, values were used to create BCAs dried weight curves.

Chitinase activity was assessed using a Chitinase assay kit (Sigma-Aldrich, St Louis, MO, USA), according to the manufacturer's instructions. The kit provides three substrates: 4-Nitrophenyl N,N'-diacetyl-β-D-chitobioside (exochitinase 1 activity detection : chitobiosidase activity), 4-Nitrophenyl N-acetyl-β-D-glucosaminide (exochitinase 2 activity detection : β-N-acetylglucosaminidase activity) and

4-Nitrophenyl β -D-N,N',N''-triacetylchitotriose (endochitinase activity detection). Cleavage of these substrates release p-nitrophenol which has been quantified at 405 nm by an Enspire Multimode Reader (Perkin Elmer, Waltham, MA, USA). One unit had released 1.0 μ mole of p-nitrophenol from the appropriate substrate per minute at pH 4.8 at 37 °C. *Trichoderma viride* chitinase (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control. Assays were carried out in triplicate, and the results were expressed as mean \pm SD (Standard deviation). To verify the apparent constitutive synthesis of exochitinase by Poly, supplementary analysis was performed on minimal solid medium.

2.4. Antimicrobial BCAs Volatiles Organics Compounds (VOCs)

Petri dish containing CYA or PDA medium were inoculated in the center with a spore suspension (10 μ L - 1×10^4 spores. mL^{-1}) of *F. graminearum* or *F. verticillioides* respectively. The three BCAs were inoculated in the center (10 μ L - 1×10^5 spores. mL^{-1} , except for Poly, which was inoculated with 7-days plugs (5 mm in diameter)) in separate plates containing the same media. The lids were removed and two plates containing each one pathogen and one BCA, were placed face to face (El-Katatny *et al.*, 2006). The set-up has been sealed with a double layer of parafilm (Parafilm M, Amcor, Warmley, UK). A plate containing medium without BCAs was placed face to face of pathogen for the control. The plates were incubated at 25°C during 8 days. After 3 days then every day of co-culture, Petri dish were photographed, and the colony area of pathogen were measured in cm^2 by image analysis using ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018). Average values were used to create growth curves. At the end of experiment, half a Petri dish of both pathogens was sampled, and the mycotoxins produced by pathogen were extracted and quantified as described in preliminary study (Pellan *et al.*, 2020). Each BCA/pathogen dual culture and control was set up in triplicate.

Data expression & Statistical analysis

Statistical data analysis was achieved with R Software (3.4.4, R Foundation for Statistical Computing, Vienna, Austria, 2017). Normality and homogeneity of variances were verified with the Shapiro–Wilk test (with Holm–Bonferroni correction) and Levene's test, respectively. For each pathogen, the effect of BCAs treatments was tested with a one-way ANOVA and multiple comparisons of means were done with Tukey's test ($\alpha = 0.05$). To consider complete evolution of interaction, and not only a final point, the area under the curves were calculated for the germination test, the evolution of weight during chitinase test and the evolution of mycelial area during COV test.

Author contributions: L.P., C.S. and S.S.-G. designed the study. C.A.T.D. participated in execution of main experimental procedure. N.D. performed mycotoxin analysis via HPLC MS-MS. L.P. carried out the entire range of manipulations and experimental design approaches and analysed the data.

L.P., C.S. and S.S.-G. interpreted the data. L.P. wrote the original draft. A.F., C.S. and S.S.-G. carefully supervised and reviewed the paper. All authors have read and agreed to the published version of the manuscript.

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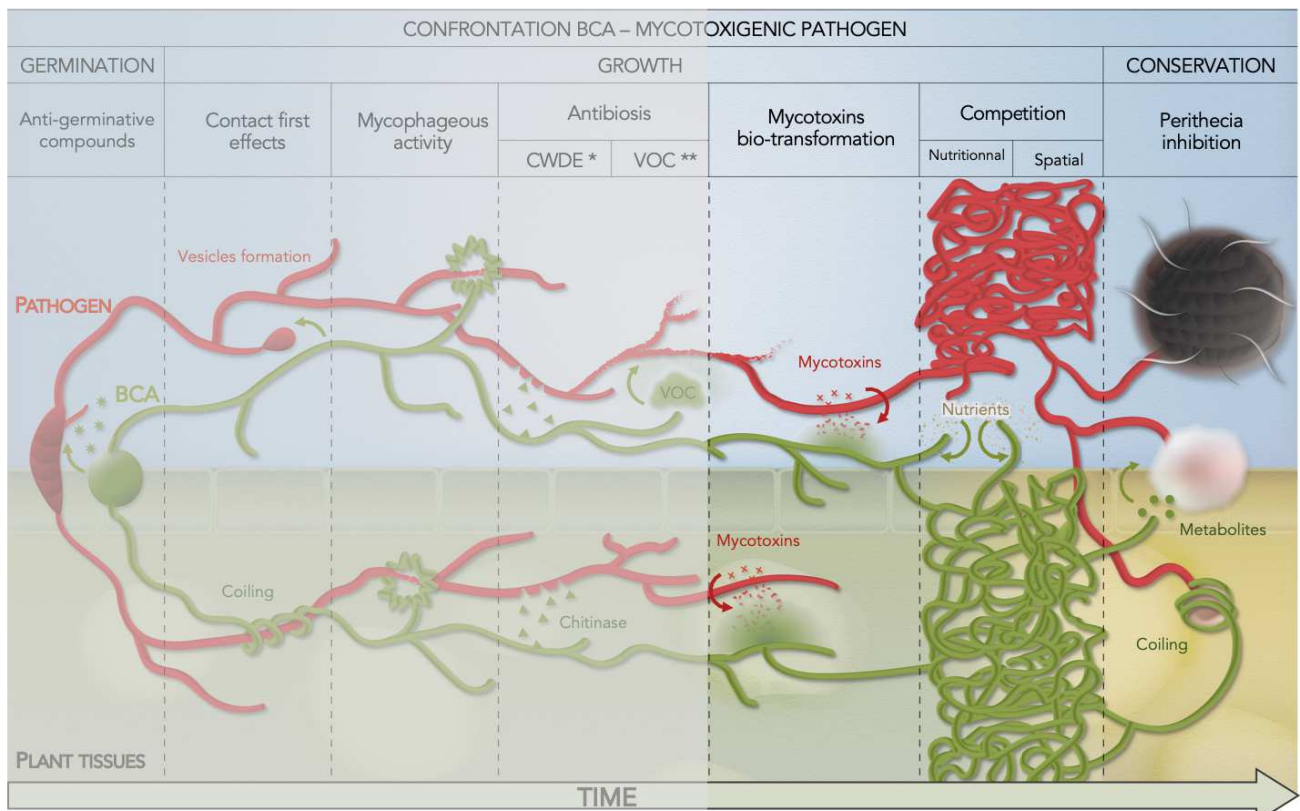
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3.2.2. Les mécanismes d'action dans les stades tardifs de l'interaction

Cette deuxième partie se concentre sur les étapes ultérieures de l'interaction. Les mycotoxines de *F. graminearum* ont été produites en milieu liquide, isolées puis mises en culture avec les BCAs afin d'identifier leurs capacités de bio-dégradation des mycotoxines produites par le pathogène. Différents modes de compétition entre les micro-organismes bénéfiques et les deux *Fusarium* pathogènes ont été envisagés notamment la compétition spatiale en milieu non-limitant, et la compétition nutritionnelle pour les divers composés potentiellement présents dans et à proximité de la plante hôte. L'impact des BCAs sur le nombre et le contenu des périthèces formés en fin de cycle de vie de *F. graminearum* a également été évalué. Les activités antagonistes des BCAs ont été confirmées et mises en relation avec les différentes phases d'interaction lors du cycle de vie des micro-organismes. La spécificité et les actions synergiques ont là aussi été discutées. *S. griseoviridis* (Myco) a de bonnes capacités en termes de compétition nutritionnelle potentielle sur le matériel végétal. Cependant, sa stratégie principale est basée sur l'antibiose, qui compense un déficit important en termes de capacité de colonisation spatiale. *T. asperellum* (Xeda) est capable d'activer un très large éventail de défenses et d'attaques combinant la synthèse de divers composés antifongiques (métabolite, enzymes, COV), avec différentes cibles (spores, mycélium, mycotoxines), et une action directe par mycoparasitisme. Concernant (*P. oligandrum*), son efficacité est principalement due à sa forte capacité à coloniser l'environnement, avec une action directe via la prédation microbienne et la réduction de la formation de périthèces.

Mot-clé : mycotoxines, contrôle biologique, interaction microbienne, *Trichoderma*, *Streptomyces*, *Pythium*



Résumé Graphique

Biocontrol Agents: Weapons against Mycotoxigenic *Fusarium* (Part 2: Late stage)

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Noël Durand^{1,2}, Caroline Strub^{*1} and Sabine Schorr-Galindo¹.

¹ Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Montpellier, France;

² CIRAD, UMR Qualisud, F-34398 Montpellier, France

* Correspondence: Caroline STRUB

UMR Qualisud

Université de Montpellier-Campus Triolet

F-34095 Montpellier Cedex 5

Email: caroline.strub@umontpellier.fr

Tel: +33 67143201

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Running Title: Biocontrol: Weapons against *Fusarium* (2)

Abstract

The aim of this study was to complete a set of experiments to decipher the mechanisms of commercial Biocontrol Agent (BCAs) against major mycotoxigenic fungi of cereals, *Fusarium graminearum* and *Fusarium verticillioides*. In the first part of the study, the early stages of interaction were investigated from germination of spores to synthesis of antifungal compounds. The second part focuses on later stages of interaction from bio-degradation of mycotoxins to effects on perithecia (survival form). Antagonistic activities of BCAs have been confirmed and linked to the different phases of interaction according to life cycles of the microorganisms. Specificity and synergic actions were discussed. *S. griseoviridis* has good abilities in terms of nutritional competition. However, its main strategy is based on antibiosis, which compensates for an important deficit in terms of spatial colonization capacity. *T. asperellum* is capable of activating a very wide range of defences and attacks combining the synthesis of various antifungal compounds (metabolite, enzymes, VOCs), with different targets (spores, mycelium, mycotoxins), and direct action by mycoparasitism. Concerning *P. oligandrum*, its efficiency

is mainly due to its strong capacity to colonize the environment, with a direct action via microbial predation and the reduction of perithecia formation.

Originality-Significance Statement

At the crossroads of economic, health care and environmental concerns, studies on the understanding of biocontrol mechanisms are a key step in the implementation of effective alternative solutions for the control of mycotoxin-producing phytopathogens. This study is the first to follows the different mechanisms concomitantly or not, throughout the entire duration of the interaction between mycotoxigenic pathogens and different types of BCAs under the same experimental conditions (from spore germination to pathogen survival). Furthermore, this study provides different experimental design and procedures as a toolbox to analyse these different modes of action.

Introduction

At the intersection between the health protection requirement of consumers and producers, economic issues of intensive production, and those related to the environment such as climate changes or limiting our impact on biodiversity, the management of mycotoxigenic fungi are of serious concern (Zain, 2011; Wu and Mitchell, 2016). Indeed, some *Fusaria* that attack cereals are capable of producing toxic secondary metabolites, such as *Fusarium graminearum* and *Fusarium verticillioides*, producers of trichothecenes and fumonisins respectively. After the infection and colonization, the pathogens are able to persist in the crop debris and constitute an important source of inoculum for the following seasons (Goswami and Kistler, 2004). In order to limit their presence and mycotoxins throughout the food chain, the best way is to inhibit their progression in the field, notably with alternative methods to chemical pesticides such as biocontrol (Legrand *et al.*, 2017). In particular, the use of antagonistic microorganisms that prevent or reduce the spread of plant pathogens was investigated and numerous biocontrol agents have been identified (Nourozian *et al.*, 2006). However, few of them are marketed and many factors still need to be studied to ensure effective implementation (Nicot *et al.*, 2012). Notably, understanding the mechanisms that govern the interaction of BCAs and pathogens over time is of particular interest (Xu *et al.*, 2010a). Several possible biocontrol mechanisms have been suggested to operate on mycotoxigenic fungi: hyper-parasitism and predation mechanisms, antibiosis (production of an inhibitory metabolites, lytic enzymes or antibiotics), nutritional competition (intrinsic ability to utilize the nutrients released by the host plant), or spatial competition, but also detoxification of mycotoxins produced by pathogen (degradation, transformation or direct fixation of toxins) (Vujanovic and Goh, 2011; Samsudin *et al.*, 2016; Hassan *et al.*, 2017). The most efficient biocontrol agents simultaneously employ a combination of different types of interactions for controlling pathogens (Pal and Gardener, 2006). Each of these modes of action can be characterized at different levels (e.g. antibiosis can be demonstrated by the presence of inhibition halos of the growth of the pathogen in dual culture assay but also by precise analysis of the metabolite(s) responsible for this inhibition). They also can be related to each other (e.g. the secretion of degradation enzyme can be one of the components of the overall parasitism strategy, or several independent modes of action can have synergic effect, such as nutritional competition and antibiosis). The complexity of these mechanism and their synergy makes their analysis difficult. Hence experimental devices need to be developed to target a particular mode of action, at different stages of pathogen evolution.

This study provides an integrative picture of the potential chronological biocontrol modes of action of three commercial BCAs selected for their contrasting uses, demonstrated efficiency, and microorganism types diversity (*T. asperellum*, *S. griseoviridis*, *P. oligandrum*). As the production season for many cereals is long and environmental conditions are subject to constant fluctuations, further research is needed to link the life cycle of mycotoxigenic pathogens and BCAs in order to find

the optimal application timing and dosage ratios at key stages of the evolution of pathogen spread (Medina *et al.*, 2017). Different effects of BCAs during early stages were investigated in the first part of study (see Biocontrol Agents: Weapons against Mycotoxigenic Fusarium (Early stage, Part 1)). To identify potential unknown mechanisms of these BCAs, different later stages of pathogens evolution were considered in this second part of study. Various effects like (1) Bio-transformation of mycotoxin by BCAs, (2) Nutritional competition, (3) Spatial competition and (4) Impact of BCAs on perithecia production by *F. graminearum* were analysed. BCA/pathogen selection criteria and descriptions are presented in Experimental Section as well as the detailed devices developed and used to explore advanced stage of BCAs interactions with mycotoxigenic fungi and identify key weapons used by the BCAs.

Results

1. BCAs bio-transformation of pathogen mycotoxins

During growth phase, BCAs have shown their ability to reduce pathogenic mycotoxin concentrations, even independently of growth reduction as detailed in a previous study (Pellan *et al.*, 2020). An experimental device was developed to identify the capacity of BCAs to degrade, transform or bind mycotoxins. After 12 days of growth (Figure 1A), it appeared that Xeda is the only one to act directly on mycotoxin reduction with nearly 55% of mycotoxin degraded or transformed (respectively 13%, 32% et 55% of reduction of mycotoxins after 5, 7 and 12 days of growth, data not shown). Poly showed a 17% reduction but this effect was not significant (respectively 0%, 2% et 17% of reduction of mycotoxins after 5, 7 and 12 days of growth, data not shown). These two BCAs had a good ability to colonize and cover the enriched medium part of Petri dish. Black droplets were observed on the Xedavir colony that covered the mycotoxin medium part (Figure 1B). A diffusion of mycotoxins from the enriched medium part to the unenriched part was observed in all plates. However, the presence of Xeda also limited the amount of mycotoxins in the free-mycotoxins part of media (compared to control where diffusion was observed), in addition to reduced amount of mycotoxins in the enriched part. In Xeda samples, main DON by-products from microbial transformation known in the literature were searched (DOM-1, 3-epi-DON, 3-keto-DON,(Venkatesh and Keller, 2019)). None of these molecules could be detected in the 5, 7 or 12-day samples despite the significant reductions over time.

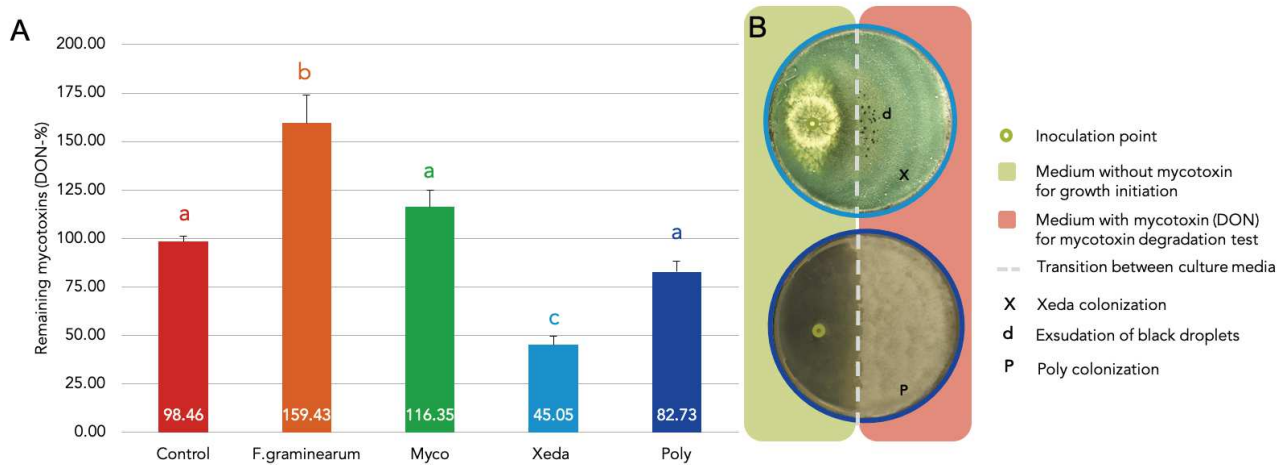


Figure 1: BCAs biotransformation of mycotoxin (DON). A. Remaining mycotoxin of *F.graminearum* (DON) in CYA medium (whole dish extraction), $15,000 \text{ ng.g}^{-1}$ of media = 100%, after 12 days of growth of BCAs at 25°C . Control corresponded to Petri dish without inoculation of micro-organisms. B. Dispositive and visual aspect of Xeda and Poly colonization during biotransformation test. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. ANOVA test, p-value < 0.05.

Considering the similar condition of bio-transformation of mycotoxin test and *in vitro* dual culture assays (1), it was possible to hypothesize the importance of mycotoxin bio-transformation proportion over global mycotoxin reduction. In order to try to have explanation, the data of both kinetics of the two BCAs previously considered (Poly and Xeda) were compared. It would appear for Xeda modalities that the percentage of bio-transformation of mycotoxin (respectively 13%, 32% et 56% of reduction of mycotoxins after 5, 7 and 12 days of growth) was an important contribution to global reduction of mycotoxin (respectively 17%, 64% et 81% of global reduction after 5, 7 and 12 days of growth) and thus as early as the beginning of interaction. On the contrary, regarding Poly modalities, large differences between the results obtained from the global mycotoxin reduction during dual culture assay respectively 75%, 92% et 81% of global reduction after 5, 7 and 12 days of growth) and the mycotoxin bio-transformation test could be observed.

2. Nutritional competition between BCAs and pathogens

The capacity of microorganisms to consume different sources of nitrogen and carbon was assessed (Figure 2). A selection of major compounds from wheat and maize has been compiled from literature analysis. These compounds are expected to be those for which pathogen and BCA compete in the plant. Some compounds can be found as carbon and nitrogen sources because they have been used either as a source of carbon or as a source of nitrogen (supplemented with glucose).

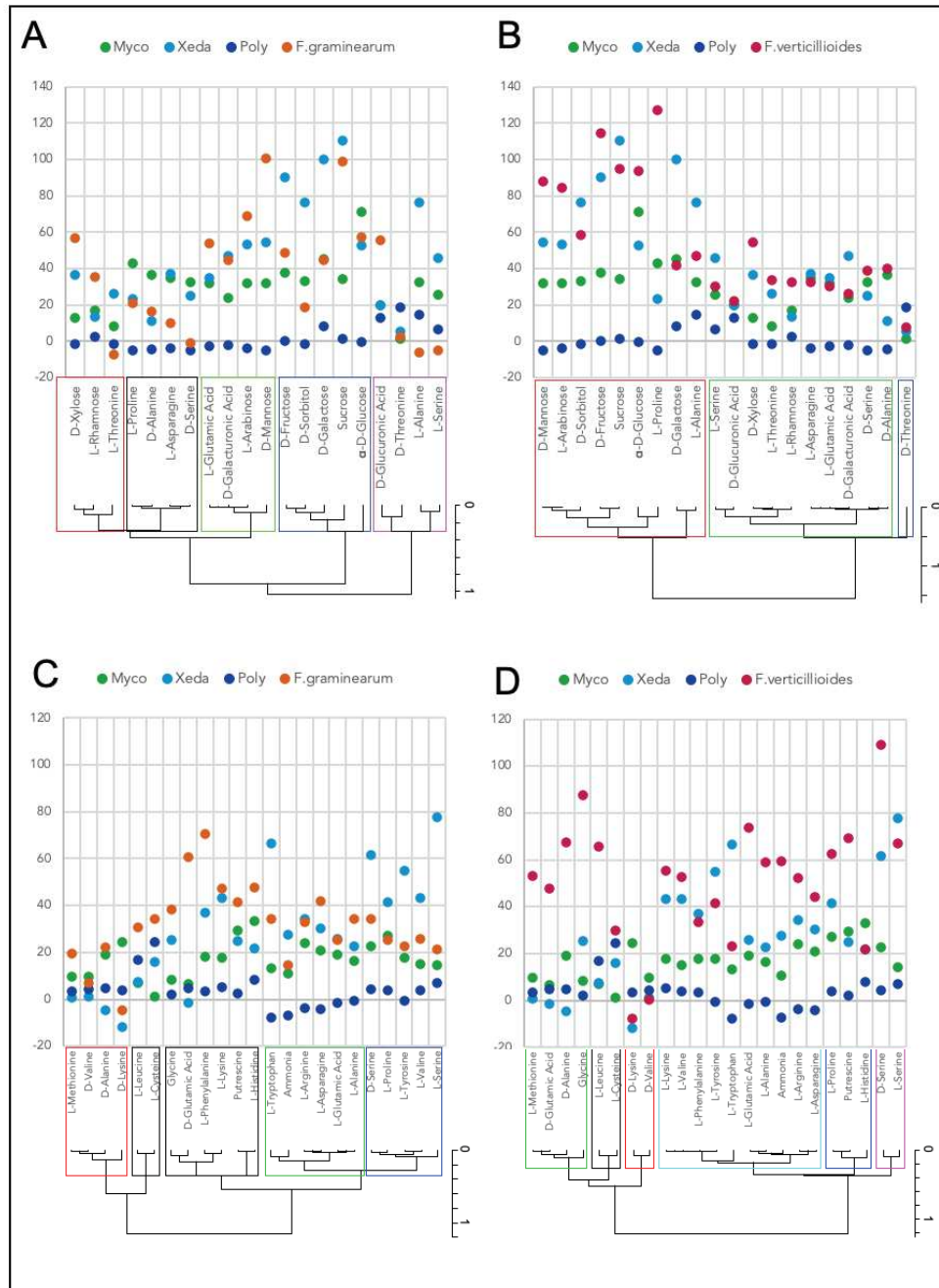


Figure 2: Nutritional profiling of BCAs and *Fusaria* pathogens for cereals compounds (carbon or nitrogen sources).A,B: *F. graminearum* and *F. verticillioides* respectively vs all BCAs (Mycos, Xedas and Polys) for carbon compounds; C,D: *F. graminearum* and *F. verticillioides* respectively vs all BCAs (Mycos, Xedas and Polys) for nitrogen compounds.

Both panels show optical density measurements over a 138-h time course at 25°C in phenotype micro-arrays (Area under growth curve). Major elements found in cereals (wheat and maize for *F. graminearum* and *F. verticillioides* respectively) are available in literature (Audenaert et al., 2002; Joo, 2005; Ambrico and Trupo, 2017; Tang et al., 2018). Hierarchical classification and clustering were done with all microorganisms independently. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®.

When comparing the nutrient profiles of BCAs and the phytopathogen *F. graminearum*, the carbon sources were divided into 5 clusters with 30% of dissimilarity (Figure 2A). The black cluster included compounds averagely consumed and mostly by only Myco, or Xeda and Myco. The green and blue clusters grouped many compounds for which *F. graminearum* and Xeda seemed to compete with high consumption. Increased growth was observed for *F. graminearum* (green cluster, notably arabinose and mannose) and Xeda (blue cluster, mainly sucrose, in direct competition with *F. graminearum*, galactose and sorbitol). The last group of carbon sources contained the best sources for the growth of Poly, with in contrast a low average growth of *F. graminearum* (notably threonine, alanine and serine). For the comparison of the nutrient profiles of BCAs and *F. verticillioides*, the different nutrient sources were grouped into three clusters (50% of dissimilarity (Figure 2B)). The red cluster grouped compounds that are highly consumed by all microorganisms except Poly. In this cluster, mainly *F. verticillioides* and Xeda were in competition, with once again a stronger growth of Xeda with sucrose (also widely consumed by *F. verticillioides*), galactose and sorbitol. The green cluster included moderately consumed carbon sources, with a branch of compounds mainly to the advantage of *F. verticillioides* and one branch of compounds mainly to the advantage of Myco and/or Xeda BCAs. Threonine remains the compound which alone most stimulates the growth of Poly compared to other compounds and microorganisms (blue cluster). The *Fusarium* pathogens could have very different nutritional patterns. For example, proline strongly stimulated the growth of *F. verticillioides* but less the growth of *F. graminearum*, or the opposite with glucuronic acid.

The same analysis was performed on the nutrient profiles of microorganisms in the presence of different nitrogen sources (Figure 2 C and D). When comparing the growth of BCAs and *F. graminearum* (Figure 2C), some groups of compounds were on average more strongly assimilated by BCAs, Myco and Xeda, and the pathogen (pink, green and blue clusters, 40% of dissimilarity); in contrast to Poly which has the best capacity in assimilating nitrogen sources belonging to the red and black clusters. The pink cluster contained the compounds that strongly stimulated the growth of *F. graminearum* in contrast to the blue cluster that contained the sources that stimulated the growth of Xeda. Figure 2D shows the comparison of the consumption profiles of different nitrogen sources according to microorganisms (BCAs and *F. verticillioides*), and few similar patterns were observed (20% of dissimilarity between clusters). Globally the first two groups show compounds highly consumed by *F. verticillioides* (green and black) in opposition to the BCAs Myco and Xeda which consume them less. Opposite profile could be observed in the red cluster. The second branch of the dendrogram (yellow, blue and pink group) shows more competition between BCAs and *F. verticillioides* (particularly Xeda, especially on compounds such as phenylalanine, tyrosine or serine). It should be noted that some compounds such as lysine or valine had reduced the growth of both pathogens compared to Myco.

3. Spatial competition between BCAs and pathogens

By providing more time and space for the microorganisms to grow, spatial competition between BCAs and pathogens was tested in non-limiting nutritional conditions and contrasted profiles could be observed (Figure 3). In competition with *F. graminearum*, Myco is widely outdistanced in terms of its ability to colonize the environment, alone or in association with the pathogen (Figure 3A). The pathogen outperformed Xeda. However, this BCA allowed a significant reduction of 30% in the progression of the pathogen from the contact point (Day 10, Figure 3B). Poly was able to colonize the environment faster than *F. graminearum* whether alone or in confrontation. It also allowed a significant reduction in pathogen development from the point of contact (-45%). At the end of kinetics (20 days), Poly continued to progress over the pathogen's mycelium (Figure 3C). In competition with *F. verticillioides*, Myco was overtaken in spatial competition. However, a slight inhibition of the pathogen before contact during co-culture was observed (Figure 3D). Xeda and Poly were able to grow faster than the pathogen and won the spatial competition in both cases (alone or confronted to pathogen). When co-cultured, they reduced pathogen colonization by 70 and 67% respectively Figure 3 D and E). Overall, the spatial competition induced by BCAs was more effective on *F. verticillioides* than on *F. graminearum*.

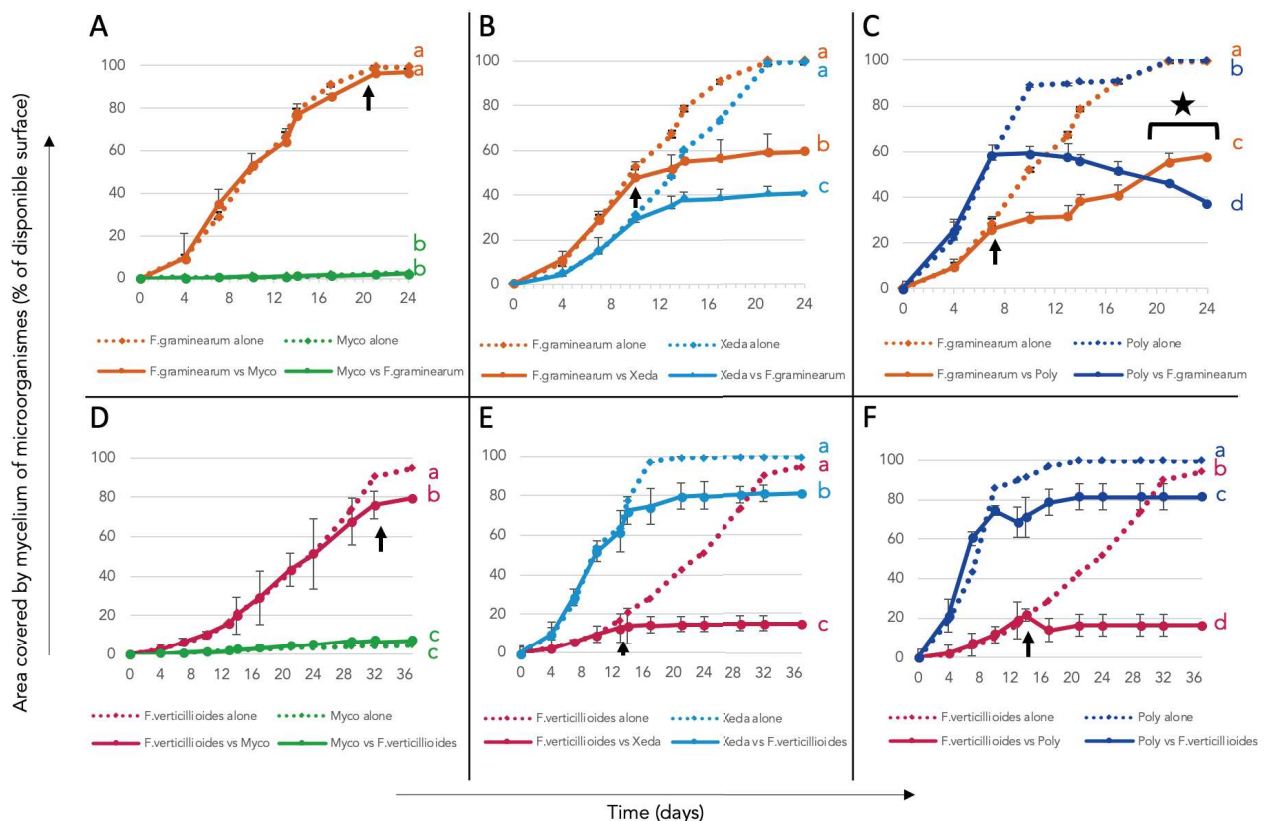


Figure 3: Spatial competition between BCAs and pathogens. A,B,C: CYA medium non-limitant with *F. graminearum* in competition against Myco, Xeda and Poly respectively or each ones alone, during 25 days at 25°C. D,E,F: PDA medium non-limitant with *F. verticillioides* in competition against Myco, Xeda and Poly respectively or each ones alone, during 40 days at 25°C. Arrow indicates first contact between BCA and

pathogen and star indicate overgrowth of BCA on pathogen colony; on confrontation curves. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. ANOVA test independent for each competition modality on area under the growth curves (AUGCs), p-value < 0.05.

4. Perithecia production

At the end of its life cycle, *F. graminearum*, produces dark morphological structures with ascospores enclosed, called perithecia, to assure its conservation. The potential of BCAs to inhibit the synthesis of perithecia was evaluated (Figure 4). *F. graminearum* (Control) had produced many perithecia, averaging 0.08 mm in diameter, but all three BCAs strongly inhibited the formation of its survival structures. Myco caused a slight re-emergence of pathogenic filaments that did not change into perithecia but were tangled with its small colonies. It strongly inhibited the production of perithecia, with an average reduction of 81%, and those that did form were quickly covered with thin Myco filaments. Xeda showed a 75% inhibition of perithecia with a reduction of their development, and a large decrease in the size of perithecia formed. Poly showed the strongest impact with 88% fewer perithecia, the oomycete covered the perithecia from the beginning of their formation and prevented their evolution. Moreover, the small amount of formed structures was reduced in size.

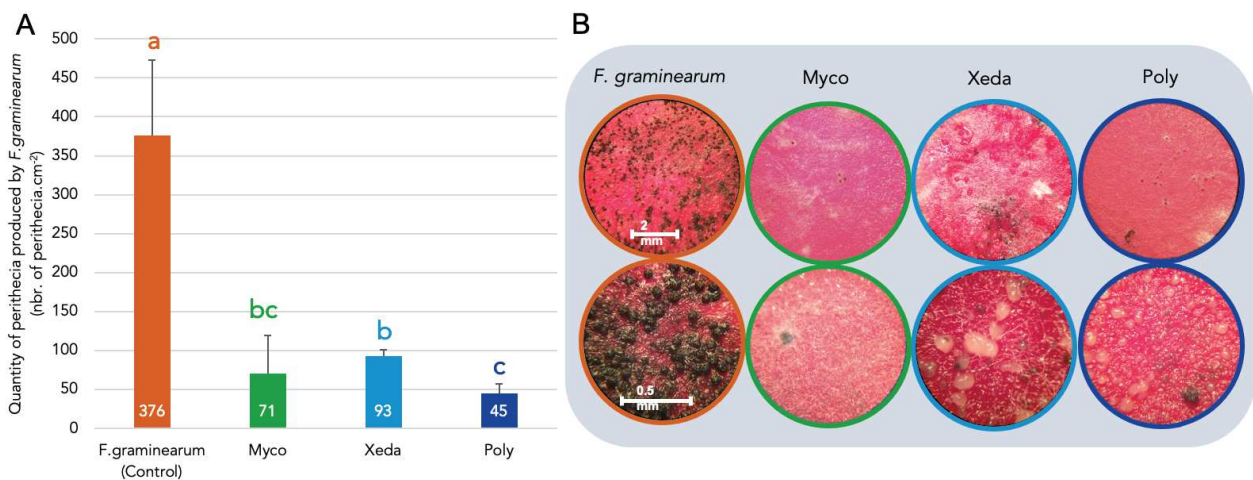


Figure 4: Effect of BCAs on perithecia production of *F. graminearum*. A. Quantity of perithecia produced by *F. graminearum* on carrot agar after 6 days of contact with BCAs at 22°C, B. Visual aspect of treated or control perithecia test, microscopic images were taken with binocular loupe (x20 and x150 enlargement). Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. ANOVA test, p-value < 0.05.

Discussion

The large investigation presented in this study explored the interaction of mycotoxigenic fungi and biocontrol microorganisms, the specialization of some mode of action adopted by BCAs, and the potential chronology of events during the later phases of the interaction between these pathogens

and BCAs. After describing the biocontrol effects of BCAs in the early phases of interaction with pathogens, this second part focuses on the establishment of mechanisms taking place later in the pathogen development cycle. This complementary approach allows to complete the vision provided during the analysis of the early phases, and possibly propose solutions when the pathogen is already present (curative rather than preventive action).

Concerning the biocontrol global interaction with pathogens studied and the tools developed, this second part of study focused on identifying biocontrol mechanisms in the later phases of the interaction (Figure 5). After a variable contact time, some BCAs are capable of bio-transforming the mycotoxins produced by *F. graminearum* (McCormick, 2013). This mode of action includes degradation, binding and transformation or conjugation of toxins. Detoxification has been identified in Lactic acid bacteria and *Pseudomonas spp.* (Juodeikiene *et al.*, 2017; ben Taheur *et al.*, 2019), and many studies consider similar the reduction of mycotoxin in dual culture assays and the detoxification and degradation of mycotoxins (Zhu *et al.*, 2017). In our case, significant reductions in the major mycotoxins were observed in the bio-transformation test of toxins previously isolated from the pathogens. The common by-products resulting from the microbial action on these mycotoxins (e.g. DOM-1, 3-epi-DON and 3-keto-DON (Venkatesh and Keller, 2019)) were searched by HPLC-MS/MS. However, none of them could be detected. This phenomenon could be explained by the timing of transformation (mycotoxins could be quickly transformed into these compounds and then into simpler molecules before analysis at 5, 7 and 12 days), or mycotoxins could be transformed into new molecules from other mechanisms of bio-transformation. This is only a part of the process and it is also necessary to ensure that the transformation products are not toxic, before an application can be considered in the mitigation of mycotoxin contamination (Zhu *et al.*, 2017). In addition, it is important to mention that the test has been designed to be comparable with the results obtained in "conventional" dual assays. Indeed, the chosen mycotoxin concentration as well as all the characteristics of the inoculation and growth have been preserved (position, inoculum concentration, culture medium, temperature, etc.). For this reason, it is possible to estimate the proportion of degradation phenomenon in the overall aspect of mycotoxin reduction by BCAs.

During their growth phase, BCAs can also compete with pathogens for different ecological resources, such as nutrients or space. These mechanisms have been notably identified in yeasts or yeast-like fungi, that use competition for substrate or amino acids as a major biocontrol mechanism (Magallon-Andalon *et al.*; Bencheqroun *et al.*, 2007). Moreover, BCAs that possess this ability were more effective in reducing disease development than BCAs with either mycoparasitism or antibiosis as its mechanism (Xu *et al.*, 2010b). Research on the composition of wheat and corn has allowed the isolation of major compounds for which there could potentially be nutritional competition during contact between BCA and the pathogen on the plant. The analysis makes it possible to measure the compromise between the demand of this microorganism for a compound and the potential competition with other microorganisms for this compound. In general, regarding carbon compounds, pathogens

have highly consumed the essential structural compounds of cereals such as: cellulose (amylopectin α -L-rhamnose, acid α -D-galacturonic acid, mannose), β -glucans (glucose) and arabinoxylans (arabinose and xylose). These profiles were opposite to BCAs, which do not attack the plant directly, but have actually shown an ability to compete with pathogens, notably on soluble sugars known to be diverted by the pathogen (sucrose, glucose, etc.) (Fernandez *et al.*, 2014), but also released naturally by the plant and used through symbiosis (e.g. mycorrhizae). This could be the case with the studied BCAs. Moreover, pathogens were generally good competitors for the plant's nitrogen compounds, for many amino acids and forms of nitrogen that circulate in the plant (nitrate and nitrite). Such a diversity of plant sugar access mechanisms appears to reflect some of the differences in nutritional strategies between symbiotic and pathogenic fungi (Bonfante and Genre, 2010). Other types of devices have been developed to measure nutrient competition but did not provide such a broad view of the compounds in competition (Celar, 2003; Liu *et al.*, 2013).

Competition can also translate in terms of space. The space competition test allows to assess the capacity of pathogens or BCAs to colonize the culture medium, under non-limiting growth condition. The assay provides a good estimation of the specific growth rates of microorganisms and the impact of the confrontation between these microorganisms on this capacity of progression. This mechanism of action is often associated with the production of degradation enzymes needed to colonize the medium, (such as the chitinases identified in Par 1). It is also associated with nutritional competition, but this link remains unclear. Indeed, the ability to transform various substrates to grow is not necessarily linked to the ability to colonize and spread over a surface, specific to the species. In order to better understand this mode of action, the spatial competition test could be improved to visualize the colonization of microorganisms on more complex matrices by using GUS-transformation (Thrane *et al.*, 2000; Gardiner *et al.*, 2009) or by quantifying specific biomarkers by qPCR for example (Matarese *et al.*, 2012).

At the end of the life cycle of the pathogen, and in particular for *F. graminearum*, the pathogen produces ascospores enclosed in dark structures called perithecia. Plant infections by ascospores are known to contribute significantly to the severity of head blight *F. graminearum* epidemics (Fernando *et al.*, 1997), and residues of infected crops are the main source of inoculum for epidemics (Luongo *et al.*, 2005). Despite this, little research is conducted on the effect on perithecia of potential or commercially available BCAs for other stages of pathogen development. For many years, the traditional carrot agar medium of Klittich and Leslie (Klittich *et al.*, 1988) has been used to induce sexual reproduction by Fusarium. Some BCAs have shown their ability to reduce perithecia (Bujold *et al.*, 2001), notably *Clonostachys rosea* (Xue *et al.*, 2014). It is interesting to note that an additional effect could be that repeated foliar applications of BCAs in order to increase its presence on debris buried in the soil and help reduce the inoculum of pathogens (Köhl and Fokkema, 1998).

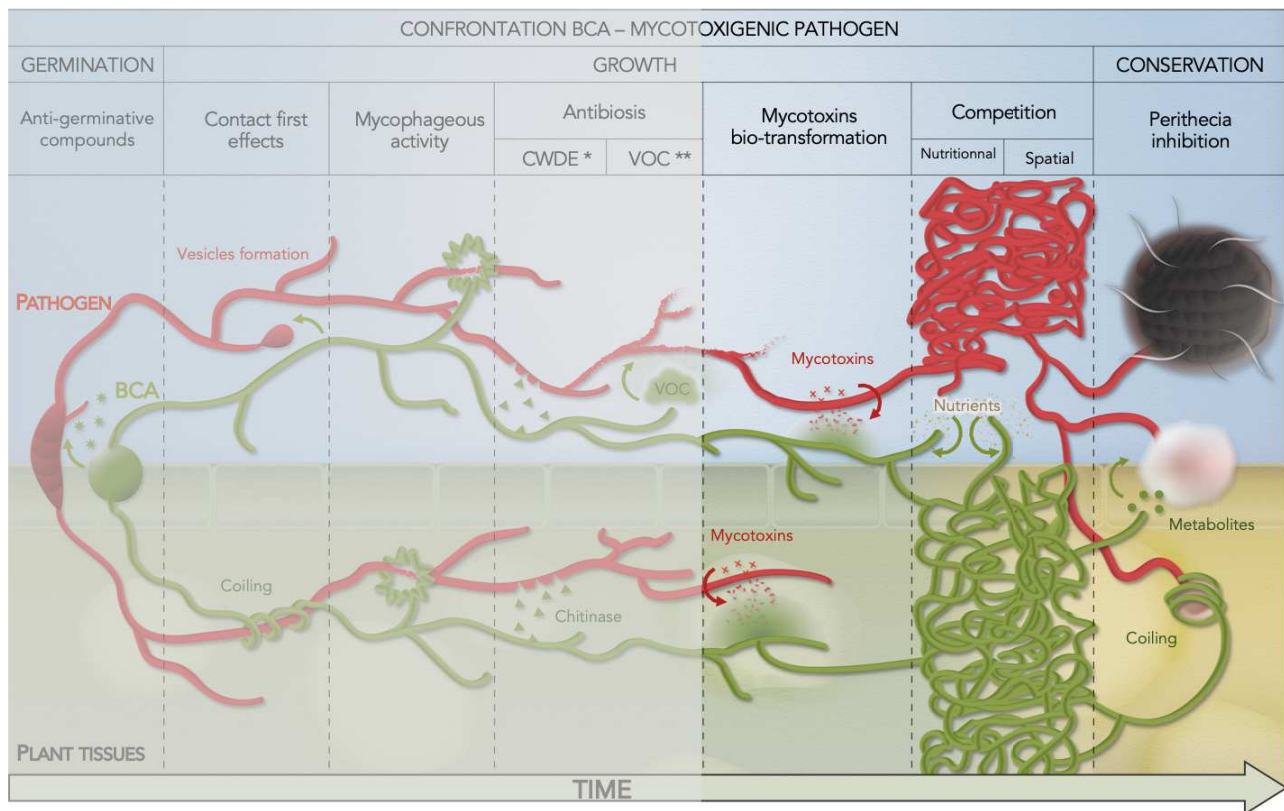


Figure 5: BCAs biocontrol mechanisms activity against pathogenic *Fusaria* over time.

During confrontation between BCA and mycotoxigenic pathogen, a broad range of weapons can be used by BCAs, from the germination of pathogen, during growth phase, to conservation under perithecia structure. Depending on the BCAs considered, and at later stages of interaction, they can be excellent allies in the particular case of the control of mycotoxigenic fungi. It would appear that they can directly degrade the mycotoxins produced or inhibit all or part of the biosynthesis pathway of these toxins in pathogens. Moreover, when the development of microorganisms is more advanced, competitions may occur. BCAs are able to attract the nutrients necessary for the growth of the pathogen, but also to colonize more space quickly to block the progression of the *Fusarium*. At the end of the life cycle of pathogens, BCAs can also prevent the formation of conservative structure, the perithecia, or interfere with them after they are formed. The mechanisms involved in early stages of interaction was presented in the first part of the study.

Through the diversity of BCAs selected for the study, it was possible to characterize contrasted strategies throughout the development of interactions between a BCA and *Fusarium* pathogens during later stage.

The first BCA studied, Myco is an actinobacteria, of the species *Streptomyces griseoviridis*. It did not exhibit the ability to bio-transform trichothecenes produced by *F. graminearum*, however this strain is capable of degrading OTA, a mycotoxin produced by other fungal pathogens of *Penicillium* genera (Campos-avelar *et al.*, 2020). In addition, other actinomycetes have been shown to degrade other toxic molecules such as carbazole, naphthalene, anthracene and atrazine (Hu *et al.*, 2012). Myco is also among the BCAs that have shown the ability to compete with pathogens in terms of nutrients,

particularly on soluble sugars that are also consumed by pathogens. Myco was able to consume a wide variety of compounds, but has a lower specific growth rate. Actinomycetes are also known to produce siderophores that can compete for iron uptake (Zeng *et al.*, 2018). In the space competition test, Myco induces a slight inhibition of *F. verticillioides* growth prior to contact, which supports the hypothesis of antimicrobial compound diffusion, such as when inhibiting the germination of pathogenic spores (Part 1). Among actinobacteria, *Streptomyces* genus has a complex life cycle and secretes various secondary metabolites of diverse nature and function (Challis and Hopwood, 2003). Different antibiotics have been identified and used for the management of plant pathogens. Most of these compounds could be developed as targeted-fungicides with minimal side effects on agro-systems (Sharma *et al.*, 2020). Some are even marketed as blasticidin or kasugamycin (Law *et al.*, 2017). However, specific growth rate of Myco remained low on the culture media used, limiting its capacity for spatial competition. At the end of the cycle, Myco reduced strongly the formation of perithecia. To our knowledge, this is the first report of direct observation of the action of *Streptomyces spp.* on the perithecia of *F. graminearum*. Overall, Myco's biocontrol strategy is based on remote action *via* the synthesis of enzymes and antigerminative and antifungal compounds that inhibit the progression of pathogens. It has good nutritional competition capacity for wheat and maize compounds, suggesting a good establishment in the natural environment, but with limited capacity for spatial competition. Despite this, having been originally isolated from the soil, its use could be recommended against perithecia of *F. graminearum*.

The second BCA studied Xeda, is a *Trichoderma asperellum* strain. It was particularly able to degrade, bind or transform mycotoxin. It is still the most effective in the reduction of mycotoxins, which supports the observations made during tests to identify particular mechanisms (see mycophagy, VOCs, Part.1). This mode of action also appears to play an important role in global reduction of mycotoxin. Recently, *Trichoderma* strains have shown their ability to detoxify DON via glycosylation, or zearalenone, which supports its interest in detoxification (Tian *et al.*, 2016, 2018). Xeda has also shown the ability to compete with pathogens for the consumption of soluble sugars known to be redirected by pathogens for their benefit, notably sucrose was identified as a key element in the symbiotic association between *Trichoderma virens* and maize plants (Vargas *et al.*, 2009). Other *Trichoderma spp.* also produced siderophores for the iron competition (Renshaw *et al.*, 2002), like the strain studied (growth on CAS medium and halo formation, data not shown). In association with other *Fusarium* species, competition for carbon, nitrogen and iron plays an important role during the interactions between beneficial and phytopathogenic fungi, and is linked to the biocontrol mechanisms of *Trichoderma* species (Vinale *et al.*, 2014). In space competition experiments, the pathogens in confrontation with Xeda did not show pre-contact growth inhibition, which supports the hypothesis of preferential direct mechanisms (in correlation with the microscopic observations at first contact (Pellan *et al.*, 2020) and the strong enzyme synthesis during the perception of the pathogens, Part 1). It has a good specific growth rate which allows it to limit the space available for pathogens. Xeda strongly

limits the development of perithecia during the survival phase of *F. graminearum*, which may also be related to the competition for ecological niches discussed above (Luongo *et al.*, 2005). It is also able to directly attack the formed mature perithecia. Co-culture of *F. graminearum* perithecia with other *Trichoderma* species also showed decreases in pigment production, and the outer wall of the perithecia are deformed (Inch and Gilbert, 2011). Interestingly, an additional effect could be that repeated foliar applications of *Trichoderma* to enhance its occurrence on soil-buried debris and help to reduce the inoculum of pathogens (Köhl and Fokkema, 1998). Overall, Xeda will be able to set up a large panel of mode of action with various targets (spores, mycelium or mycotoxins). Direct mechanisms such as the synthesis of anti-germinating compounds, the production of enzymes or volatile or non-volatile anti-fungal compounds have been identified. It also acts as short-range antagonists, like many *Trichoderma spp.*, via coiling, mycophagy or spatial competition, which support its necrotrophic mycoparasite character.

Finally, the last BCA studied Poly, is an oomycete of *Pythium oligandrum* specie. In an unprecedented way, it seems to be able to transform the mycotoxins of *F. graminearum*. Despite a strong effect on overall mycotoxin reduction in dual culture assays, the specific degradation activity is weaker and later than other BCAs. These patterns seemed to indicate a less important part of bio-transformation process in the interaction of pathogens. This difference could be explained by the combination of several mechanisms by Poly to reduce the amount of mycotoxins: slight detoxification but also, direct action on the biosynthesis pathways of mycotoxins. Recently, a study has shown that microorganisms communication in wheat microbiome regulates epigenetic modifiers that in turn control mycotoxin biosynthesis (Chen *et al.*, 2018). Concerning its nutritional profile, and according to our previous investigations, Poly will privilege complex media, therefore its average growth is lower in the presence of a single source of carbon or nitrogen. Poly has a very high specific growth rate, yet it does not have a strong action in terms of nutritional competition. It seems that for this organism, the study of nutritional competition would be more adapted on a complex medium (Benhamou *et al.*, 2012). In the spatial competition test, Poly does not allow inhibition of pathogens prior to contact, which supports the hypothesis of preferential direct mechanisms (Part 1, (Pellan *et al.*, 2020)). However, it has a very strong capacity for colonization alone or in confrontation with *Fusarium* pathogens. This property allows it to be “rhizosphere competent for space”, (i.e. root zone niches) when observed with other plant pathogens. A parallel can be envisaged with the way *P. oligandrum* interacts with plant root tissues, one of the most significant effects was the quick colonization of cortical and vascular root areas (le Floch *et al.*, 2005). Curiously, an unusual feature of this interaction probably concerns the unexpected degradation of invasive oomycete hyphae soon after penetration into root tissues, oomycete cells progressively degenerate while a typical oogonia appears (Benhamou *et al.*, 2012). Finally, during the last phases of the *F. graminearum* cycle, Poly severely limits the formation of perithecia and covers those that have been formed, mainly due to its strong expansion capacity. This species has already shown its ability to colonize and inhibit the germination of other survival

structures, such as the sclerotia of the pathogen *S. sclerotiorum* (Madsen and de Neergaard, 1999). However, this is the first time that it is being considered for the treatment of *F. graminearum* perithecia in soil, which would make it a BCA suitable for all phases of the pathogen's life cycle (good colonization of aerial parts). Globally, Poly has a very strong capacity for spatial competition and environmental colonization, which is a major advantage in the interaction with pathogens., which is essentially based on direct contact between mycoparasitism and microbial predation. Its strong capacity to inhibit pathogens occurs throughout the pathogen cycle via mycophagy mechanisms, stimulation of self-reproduction, limitation of growth and mycotoxin production, and perithecia control. However, it presents little aspect of specialization for the pathogens studied and is an aggressive parasite with a wide host range

Conclusion

To conclude this study, it is important to recall that even today, after years of research, the lack of appropriate selection methods and understanding of the mechanisms of action remains one of the main obstacles to the identification of new BCAs (Pliego *et al.*, 2011). The analysis presented could therefore represent important advances as well as efficient tools to improve these aspects. Discussing the BCAs effects on pathogen allows us to highlight various strategies partially interconnected, with their advantages and disadvantages: Mycoparasitism is more specific, antibiosis can generate the production of compounds that are too damaging to ecosystems, and spatial and nutritional competition seems to be more adapted to implantation in natural environments (Xu *et al.*, 2010a). Degradation can be a double-edged mechanism depending on the toxicity of the by-products. The best results are observed when a single BCA combines several modes of action, and many synergies have already been mentioned (Saravanakumar *et al.*, 2016). Specificity is an interesting aspect to propose targeted BCA without side effect on the microbial community of the ecosystem. It could also be interesting to validate the observations with a wide variety of strains representative of the pathogenic agent, which has very rarely been tested with the exception of Palazzini *et al.* study (Palazzini *et al.*, 2017). This integrative approach could be complemented by molecular biology and phytopathology assays. An antagonistic interaction implies specific recognition of the target pathogen by BCAs followed by genetic reprogramming of their gene expression and subsequently affects the pathogen physiology. Further studies on *in situ* or *in planta* interaction could validate the BCAs activities.

Experimental procedures

1. Micro-organisms et culture media

1.1. *Fusarium* species

Fusarium graminearum strain BRFM 1967 and *Fusarium verticillioides* strain BRFM 2251 (CIRM, University of Aix-Marseille, Marseille, France) were selected for their high respective mycotoxins production. *F. graminearum* was isolated from wheat plant and has a deoxynivalenol (DON/15-ADON) chemotype; *F. verticillioides*, was isolated from maize kernels, and has a fumonisin chemotype (FB1/FB2/FB3). Pure cultures were maintained on potato dextrose agar (PDA; BD Difco, Sparks, MA, USA) at 4 °C under paraffin oil. For production of spore suspensions, pathogens actively grown on PDA at 25 °C for 7 days. For *F. graminearum* dual culture assay against BCAs, CYA or CYB (sucrose 30 g; with or without agar 15g; yeast extract (Biokar, Beauvais, France) 5 g; K₂HPO₄·3H₂O 1 g; NaNO₃ 0.3 g; KCl 0.05 g; MgSO₄·7H₂O 0.05 g; FeSO₄·7H₂O 0.001 g; ZnSO₄·7H₂O 0.001 g; CuSO₄·5H₂O 0.0005 g; per liter of distilled water, pH 6.3 ± 0.2) was used, and for *F. verticillioides* dual culture assay against BCAs, PDA or PDB (BD Difco, Sparks, MA, USA) was used. Compartment of these *Fusariums* species and selection of media are detailed in a previous study (Pellan *et al.*, 2020).

2.5. Commercial Biocontrol Agents (BCAs)

Three commercial biological control agents (BCAs) were chosen for their contrasting characteristics and their ability to inhibit the two mycotoxigenic fungi. Explanations on how BCAs were selected and more informations about their characteristics and their effects on growth and mycotoxinogenesis of *Fusarium* pathogen in dual culture assays are available in previous work (Pellan *et al.*, 2020). All microorganisms were isolated from their commercial formulation with a classical microbial isolation protocol and conserved in aliquots (4 °C) or under spore forms in glycerol solution (15%/ V:V / -80 °C). Pure cultures were maintained on ISP4 (agar 18g; starch 10g; K₂HPO₄·3H₂O 1g; MgSO₄·7H₂O 1 g; (NH₄)₂SO₄ 1g; CaCO₃ 1g; FeSO₄·7H₂O 0,001g; MgCl₂ 0,001g; ZnSO₄·7H₂O 0.001g; per liter of distilled water), PDA, and V8 juice agar (V8 juice 200ml (Campbell's, Camden, NJ, USA); agar 15g; CaCO₃ 3g; per 800ml of distilled water), respectively, for Mycostop®, Xedavir®, and Polyversum® at 25 °C for 7 days for production of spore suspensions or inoculation plug concerning Polyversum®. For the rest of the study, the microorganism isolated from commercial formulation were referred to using the following abbreviations: Myco for Mycostop®/*S. griseoviridis* (Lallemand Plant Care), Xeda for Xedavir®/*T. asperellum* (Xeda International), and Poly for Polyversum®/*P. oligandrum* (DeSangosse).

2. Toolbox (later stage)

2.1. Bio-transformation of *F. graminearum* mycotoxins

In order to evaluate the capacity of BCAs to transform, degrade or fix *F. graminearum* mycotoxin (DON), minimum liquid medium (CZB) was inoculated with 7-days plugs (1 cm in diameter) of *F. graminearum* in Erlenmeyer flasks and incubated 10 days at 25°C. Liquid cultures were filtered with PES filter (0.22 µm, Sartorius, Göttingen, Germany) to remove mycelium and spores produced by

BCAs and to sterilize medium enriched with mycotoxins. A volume of 10 mL was sampled for quantify the mycotoxin concentration. In parallel, half Petri dish of medium without mycotoxins (CYA) were inoculated with BCAs or *F. graminearum* alone (control of mycotoxins non-degradation by the pathogen) with 5 μ L of 1x10⁵ spores. mL⁻¹, except for Poly, which was inoculated with 7-days plugs (5 mm in diameter) or H₂O (inoculation control). After 3 days, corresponding to initiation of growth of BCAs, the empty half Petri dish were completed with enriched mycotoxin medium (CYA with DON content adjusted to 15,000 ng.g⁻¹ of media, equate to 100% of mycotoxin content) and incubated 12 days at 25°C (same condition as dual culture assays in previous study). Samples were analyzed on days 5, 7 and 12 for all modalities. BCAs colony had gradually colonized the mycotoxin-enriched part. At the end of experiment, in each half a Petri dish, the remaining mycotoxins were extracted, quantified and summed as in (Pellan *et al.*, 2020), and compared to control plates without microorganisms (mycotoxin content control, 100% of mycotoxin content). The experiment was performed only with the mycotoxins produced by *F. graminearum* because the *F. verticillioides* strain used did not produce enough mycotoxins in liquid medium. The main known degradation products resulting from the microbial transformation of mycotoxins were investigated (McCormick, 2013; Tian *et al.*, 2018; Venkatesh and Keller, 2019): DOM-1, 3-epi-DON and 3-keto-DON; and particular attention was paid to the identification of peaks that appeared on chromatogram when mycotoxin amount was reduced, because they could correspond to new product of degradation.

2.2. Nutritional competition between BCAs and pathogens

Phenotype MicroArrays plate (Biolog, Hayward, CA, USA) measure the carbon consumption profile (PM1), and the nitrogen consumption profile (PM3) of strains for 96 sources, and were used according to the manufacturer's instructions. Carbon sources in PM1 are in the 5– 50 mM range and nitrogen sources in PM3 are in the 2–20 mM range. Standardized cell suspensions were prepared in sterile Biolog FF inoculating fluide (transmittance of 81% and 62% at 590 nm for Myco and other microorganisms, respectively), and inoculated into the PMs. PMs were incubated in dark at 25°C, and growth (optical density) at 750 nm was monitored after 0, 12, 18, 24, 36, 42, 48, 60, 66, 72, 84, 90, 96, 114, and 138 h using an Enspire Multimode Reader (Perkin Elmer, Waltham, MA, USA). Compounds present in wheat or maize have been identified through the literature (Wang *et al.*; Englyst, 1989; Bach Knudsen, 1997; Kim *et al.*, 2003; Bader UI Ain *et al.*, 2019), and the consumption of these compounds by different microorganisms have been compared to highlight a potential nutritional competition on these natural substrates.

2.3. Spatial competition between BCAs and pathogens and potential antimicrobial metabolites diffusion by BCAs

In square Petri dish, CYA or PDA medium for *F. graminearum* conditions or *F. verticillioides* conditions respectively, were deposited in non-limiting quantity (300 mL) representing a total area of 900 cm². In order to identify the most competitive micro-organisms between pathogen and BCA, each BCA and

pathogen were inoculated alone or in competition (10 μL - 1×10^4 spores. mL^{-1} , except for Poly, which was inoculated with 7-days plugs (5 mm in diameter)), and incubated for 25 or 40 days at 25°C, for *F. graminearum* test or *F. verticillioides* test respectively (i.e. until full coverage of the available space). During test, Petri dish were photographed, and the colony area of pathogens and BCAs were measured in cm^2 by image analysis using ImageJ software (1.52a, Wayne Rasband National Institute of Health, Bethesda, MD, USA, 2018). Average values were used to create growth curves. Particular attention was taken to the contact time and potential overgrowth of microorganisms on each other.

2.4. Impact of BCAs on perithecia production of *F. graminearum*

The production of perithecia was set up based on existing protocol (Urban *et al.*, 2003; Cavinder *et al.*, 2012; Laurent, 2016). *F. graminearum* was inoculated on solid Carrot agar medium and incubated at 22°C. When the mycelium had covered the whole area, its aerial part was scraped off with a sterile spreader. After 24 hours, the same process was repeated. Then TWEEN20® at 2.5% (V :V) was supplemented with BCAs spores (1×10^4 spores. mL^{-1}), excepted Poly which was added with microfilaments. A volume of 1 mL of this spore's suspension with TWEEN20® was deposited and spread in Petri dish, inducing stress and leading to the formation of perithecia. TWEEN20® at 2.5% (V:V) was used as control.

3. Data expression & Statistical analysis

Statistical data analysis was achieved with R Software (3.4.4, R Foundation for Statistical Computing, Vienna, Austria, 2017). Normality and homogeneity of variances were verified with the Shapiro–Wilk test (with Holm–Bonferroni correction) and Levene's test, respectively. For each pathogen, the effect of BCA treatments was tested with a one-way ANOVA and multiple comparisons of means were done with Tukey's test ($\alpha = 0.05$). To consider complete evolution of interaction, and not only a final point, the area under growth curves were calculated for the spatial and nutritional competition test. For the visualization of specific differences between microorganisms nutrient profiling, the negative control was subtracted and a hierarchical classification and clusterisation was carried out with the FactoMineR package. Absorbance values of the negative control were subtracted from the measured values for the different modalities.

Author contributions: L.P., C.S. and S.S.-G. designed the study. N.D. performed mycotoxin analysis via HPLC MS-MS. C.A.T.D. participated in execution of main experimental procedure. L.P. carried out the entire range of manipulations and experimental design approaches and analyzed the data. L.P., C.S. and S.S.-G. interpreted the data. L.P. wrote the original draft. A.F., C.S. and S.S.-G. carefully supervised and reviewed the paper. All authors have read and agreed to the published version of the manuscript.

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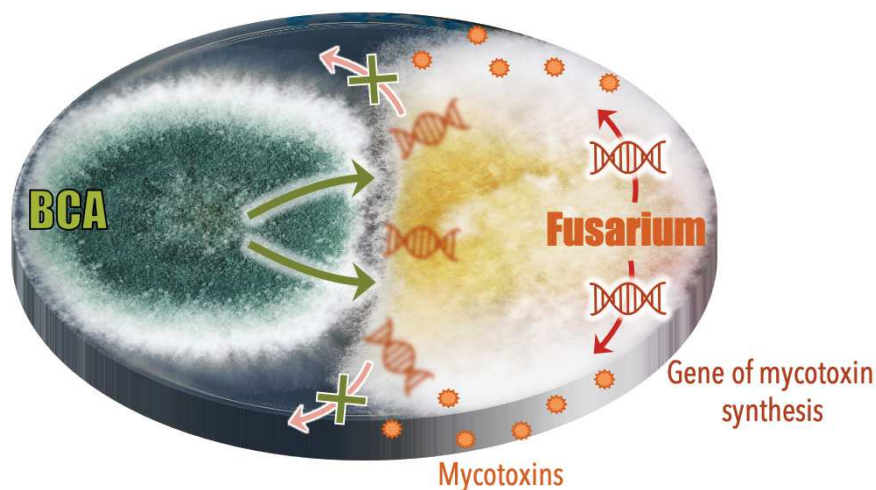
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3.2.3. L'impact des BCAs sur l'expression des gènes de mycotoxinogénèse

Dans cet article complémentaire, un mécanisme potentiel des BCAs contre les souches toxigènes de *Fusarium* à l'échelle génétique lors des confrontations a été évalué. En effet, la biosynthèse des mycotoxines par les pathogènes est gouvernée par des clusters de gènes assez bien identifiés, que ce soit chez *F. graminearum* pour la synthèse des trichothécènes (gènes *Tri*), ou chez *F. verticillioides* pour la synthèse de fumonisines (gènes *Fum*). Donc pour compléter l'approche en aval sur la bio-transformation des mycotoxines, cette étude a pour objectif d'évaluer l'impact des deux BCAs, les plus prometteurs en termes de réduction des mycotoxines, sur l'expression de différents gènes impliqués dans la biosynthèse des mycotoxines chez chacun des champignons pathogènes. D'importantes inhibitions de l'expression des gènes ont pu être observées et quantifiées durant les tests en confrontation, *via* une PCR quantitative, précédée d'une transcription inverse. *T. asperellum* (Xeda) a montré une inhibition des gènes *Tri* de 56% en moyenne, et de 39% des gènes *Fum* en moyenne, ce qui suggère une capacité d'inhibition intermédiaire des gènes. *P. oligandrum* a été capable de réduire de 85% et 55% en moyenne les gènes de biosynthèse des trichothécènes et des fumonisines respectivement, ce qui en fait un puissant inhibiteur de l'expression des gènes à l'origine de la production des mycotoxines. Avec ces profils contrastés, les niveaux de corrélation entre la synthèse des mycotoxines, la croissance et la production de mycotoxines ont également été analysés, et mis en perspectives avec d'autres mécanismes comme la bio-transformation.

Mots-clé : interaction microbienne, mycotoxines, expression des gènes, modulations épigénétiques.



Résumé graphique



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Trichoderma asperellum and *Pythium oligandrum* can modulate the mycotoxin biosynthesis genes expression in *Fusarium* cereal pathogens during their biocontrol interaction.

Lucile Pellan¹, Myriam Mamouni¹, Caroline Strub^{1,*} and Sabine Schorr-Galindo¹.

¹ Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Montpellier, France.

* Correspondence: Caroline STRUB

UMR Qualisud

Université de Montpellier-Campus Triolet

F-34095 Montpellier Cedex 5

Email: caroline.strub@umontpellier.fr

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Abstract:

Mycotoxins are toxic molecules produced by fungi that can attack cereal production and cause significant qualitative and quantitative damage. Instead of conventional pesticides, crop protection through the use of antagonistic microorganisms (BCAs) can limit the proliferation of pathogens while respecting the environment and the health of consumers. During this study, two selected BCAs, *Trichoderma asperellum* and *Pythium oligandrum* showed their ability to interact directly with the mycotoxin biosynthesis pathways of two major cereal plant pathogens, *Fusarium graminearum* and *Fusarium verticillioides*. Important inhibitions of the expression of genes positioned at different levels of the synthesis of these toxins could be observed and quantified by reverse transcription followed by quantitative PCR. *Trichoderma asperellum* showed 56% of inhibition of *Tri* gene on average, and 39% of inhibition of *Fum* gene on average, suggesting an intermediate gene inhibition capacity. *Pythium oligandrum* was able to reduce by 85% and 55% on average the biosynthetic gene of trichothecene and fumonisin respectively making it a strong inhibitor of the expression of mycotoxigenic genes. With this contrasted profile, correlation levels between repression of mycotoxin synthesis, growth and mycotoxin production were also analyzed.

Key words: microbe interaction, mycotoxins, gene expression, epigenetic modulations

1. Introduction

Mycotoxins are toxic secondary metabolites synthesized by some fungi contaminating the food matrixes from their production to their consumption, through all phases of storage and processing of these products (Gruber-Dorninger et al., 2019). Notably, production of two major groups of mycotoxins, the fumonisins and the trichothecenes, by *F. verticillioides* or *F. graminearum* during anthesis of maize and wheat respectively, affect significantly cereal food and feed quality (Jard et al., 2011). The major trichothecenes that affects product quality is deoxynivalenol (DON) and its acetylated forms such as 15-ADON. The most widespread fumonisins in cereals are the fumonisins B, including fumonisin B1 (FB1, 70-80% of the total fumonisins), fumonisin B2 (FB2) and fumonisin B3 (FB3) (Rheeder et al., 2002). Indeed, after entering the product processing chains through field infection, they persist in food products until consumption. They can cause symptoms ranging from food poisoning and vomiting to disruption of reproduction and endocrine systems, neurological disorders or death (Barac, 2019).

The application of biological control agents (BCAs) is well-known as a potentially, environmental-friendly and sustainable alternative to excessive use of synthetic chemical pesticides (le Mire et al., 2016). In this context, a better understanding of mycotoxin biosynthesis regulation by antagonist microorganisms may lead to innovative strategies to control the production of toxins by fungi in cereals, and thus can reduce the risk that it represents to human and animal health.

The biosynthesis of trichothecene mycotoxins is determined by 15 TRI genes, twelve of which are encoded by a gene cluster in *F. graminearum*. The gene *Tri5* (or *Tox5*) encodes a trichodiene synthase, which cyclizes farnesyl pyrophosphate to trichodiene, at beginning of trichothecene biosynthesis pathway (Alexander et al., 2009), and is one of the most largely studied. The inactivation of *Tri5* results in mutants that are unable to perform the first biosynthesis step and therefore cannot produce DON, acetylated DON or any trichothecene biosynthetic intermediates (Proctor et al., 2002). Once formed, trichodiene undergoes a series of four oxygenation reactions, involving the expression of *Tri4*, followed by a long series of reactions including non-enzymatic steps, and the expression of other genes of the TRI cluster (including regulatory factors) that lead to the formation of 3,15-acetyl deoxynivalenol (3,15-diADON) (Alexander et al., 2009). *Tri8* encodes a trichothecene C-3 esterase and is involved in the formation of either 3-ADON or 15-ADON (McCormick et al., 2011) in last step of trichothecene formation. Concerning biosynthesis of fumonisin, a single cluster includes 17 *FUM* genes encoding biosynthetic enzymes but also regulatory and transport proteins (Choi and Shim, 2008). In particular, *Fum1* plays a crucial role in biosynthesis because its product is implicated at early stages in the assembly of fumonisins B (Boja, 2004). This gene expression allows the synthesis of Fum1 protein which catalyzes the condensation of nine acetate and two methyl units to form a linear 18-carbon-long polyketide. *Fum8* encodes an aminoacyl transferase that leads to the formation

of the complete C20 molecule by completing the polyketide skeleton generated by *Fum1*. It has been shown that the mutation of these fumonisin genes causes a blockage in the synthesis of mycotoxins (Desjardins et al., 1992).

Despite the large amount of information available on BCAs activities, few studies are conducted to prevent mycotoxigenic risk, and even less to measure the direct impact of antagonistic microorganisms on the biosynthetic pathways of mycotoxins, especially trichothecenes and fumonisins (Samsudin et al., 2017). Furthermore, previous study showed that *T. asperellum* and *P. oligandrum*, two commercial BCAs, have strong impact on global growth and mycotoxins production of *F. graminearum* and *F. verticillioides* pathogens (Pellan et al., 2020), so they are ideal candidates for identifying their potential modulation of biosynthetic pathways of mycotoxins by BCAs.

In this study, we determined the inhibition capacities of *T. asperellum* and *P. oligandrum* on DON/15-ADON and FB1/FB2 production of *F. graminearum* and *F. verticillioides* respectively. A reverse transcription and quantitative PCR assay were developed to detect and monitor the level of expression of *Tri5* and *Tri8* genes of *F. graminearum*, and *Fum1* and *Fum8* genes of *F. verticillioides*. This analysis was subsequently used to investigate the effects of two BCAs on the expression of synthesis pathway genes in dual culture of the microorganisms.

2. Material and methods

2.1. Fungal material

F. graminearum strain BRFM 1967 and *F. verticillioides* strain BRFM 2251 were used (CIRM, University of Aix-Marseille, Marseille, France). They present deoxynivalenol (DON/15-ADON) chemotype and fumonisins (FB1/FB2/FB3) chemotype respectively, and were selected for their high production of mycotoxins.

Two commercial biological control agents (BCAs) were chosen for their contrasting types and characteristics and their ability to inhibit the two mycotoxigenic fungi. Explanations on how BCAs were selected and more information about their characteristics and the effects of BCAs on growth and mycotoxigenesis of Fusarium pathogen in dual culture assays are available in previous work (Pellan et al., 2020). They were isolated from their commercial formulation with a classical microbial isolation protocol. For the rest of the study, the strain isolated from commercial product were referred with the following abbreviations: Xeda for Xedavir®/*T. asperellum* (Xeda International®), and Poly for Polyversum®/*P. oligandrum* (DeSangosse®).

2.2. Dual culture assays

One BCA and one pathogen were inoculated in a Petri dish at the same distance from the center of plate. Spore suspension or plug of BCAs ($5 \mu\text{L} \times 10^5 \text{ spores.mL}^{-1}$ or 7 day-plug, respectively for Xeda and Poly) and spore suspension of pathogen ($5 \mu\text{L} \times 10^4 \text{ spores.mL}^{-1}$) were inoculated at the opposite side (at 45 mm from BCA). Each BCA was inoculated 24 h in advance; then the pathogens were

inoculated at the opposite side. Potato Dextrose Agar medium (PDA; BD Difco, Sparks, MA, USA) was used for *F. verticillioides* confrontation and CYA medium (sucrose 30 g; agar 15g; yeast extract (Biokar, Beauvais, France) 5 g; K₂HPO₄·3H₂O 1 g; NaNO₃ 0.3 g; KCl 0.05 g; MgSO₄·7H₂O 0.05 g; FeSO₄·7H₂O 0.001 g; ZnSO₄·7H₂O 0.001 g; CuSO₄·5H₂O 0.0005 g; per liter of distilled water, pH 6.3 ± 0.2) was used for *F. graminearum* confrontation. Plates were incubated at 25 ± 2 °C, in the dark, for 12 days. Plates inoculated only with pathogens were used as a control.

2.2.1. Growth and mycotoxin analysis

Mycelium area evolution of each BCA/pathogen were monitored overtime and measured in cm² by image analysis using ImageJ software. For all samples, mycotoxin extraction, detection and quantification were achieved as described in a previous work (Pellan et al., 2020) using optimized extraction method then an Ultra High-Performance Liquid Chromatography (UHPLC, Shimadzu, Tokyo, Japan) coupled with a mass spectrometer (8040, Shimadzu, Tokyo, Japan). Each BCA/pathogen dual culture bioassay and control analysis of mycotoxins was set up in triplicate for each measurement day. To consider complete evolution of interaction, data collected were used to determinate the area under growth or mycotoxins production curves for each pathogen/BCAs dual culture assays. Significant differences were assessed with a one-way ANOVA and multiple comparisons of means were done with Tukey's test ($\alpha = 0.05$).

2.2.2. RNA extraction, primers, and RT-qPCR assay

A sample of 100 mg of pathogen mycelium was collected from the confrontation line with BCAs. Mycelia were grinded using GenoGrinder (SpexSamplePrep, NJ, USA) with steel grinding balls. Total RNA was extracted using NucleoSpin RNA (Macherey-Nagel, GmbH & Co. KG, Germany) according to manufacturer's instructions. Amount of RNA extracted was estimated by determining the absorbance at 260 nm, and concentration was standardized at 100 ng.µL⁻¹. For RT, RNA was reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions (Mudge et al., 2006), and the resulting cDNA was diluted 1:20 with water. Primers used for expression analysis are summarized in Table 1. β -tubulin gene was used as a housekeeping gene because it is involved in basic cellular functions and its expression is constitutive and constant over time (Pinson-Gadais et al., 2008). PCR was prepared in 384 wells microplate using pipetting robot with a total volume of 1.5 µL (final volume of 1µL SYBR GREEN PCR master mix (Applied Biosystems, Scoresby, Vic., Australia), 0.5 µL cDNA and final concentration of 600 nM of each forward and reverse primer). For each reaction, Tri/Fum cDNA and β -tubulin cDNA were coamplified. The concentration of primers and reagents were optimized by selecting the combination which gave the lowest value of quantification cycle in the amplification plots, and the highest fluorescent signal for a fixed concentration. Optimal cycling conditions were determined by testing several annealing temperatures (60, 66, 68°C). The final real-time PCR was performed using the following parameters for the two groups of primer sets (*Tri5* or other primers): first pre-incubation at 95°C for 3 min, 45

amplification cycles with first denaturation at 95°C for 10 s, annealing at 68°C or 60°C (*Tri5* or other primers respectively) for 10 s, extension at 72°C for 10 s. The final cycle of melting curve using following parameters: 95°C for 5 s, 65°C for 1 min and up to 97°C before cooling at 40°C. Data was analyzed using the LightCycler® 480 Software, version 4.0 to calculate internal PCR amplification efficiencies and relative gene expression values normalized with β -tubulin.

Table 1: Primer pairs characteristics used for relative quantification by RT-qPCR

Gene	Primer Sequence	Position	Amplicon size (bp)	Targeted organism	Source
<i>Tri5</i>	F GATCTGATGACTACCCTCAATTCCTT	518-544	416	<i>F. graminearum</i>	(Marín et al., 2010a)
	R GCCATAGAGAAGCCCCAACAC	960-981			
<i>Tri8</i>	F GCTACTTTGGACTIONCAATTCG	517-537	98	<i>F. graminearum</i>	(Lee et al., 2014)
	R CATACTGTACYGCAAGTTCTG	635-656			
β Tubulin	F GGTAACCAAATCGGTGCTGCTTTC	37-61	595	<i>F. graminearum</i>	(Jiao et al., 2008)
	R GATTGACCGAAAACGAAGTTG	264-275			
<i>Fum 1</i>	F ACACCAAAGCCTCTACAGGTGA	574-591	128	<i>F. verticillioides</i>	(Visentin et al., 2012)
	R AGGTATCGGGCACCGCT	668-689			
<i>Fum 8</i>	F AGCACAGACGGCGGAGAAGTT	624-645	315	<i>F. verticillioides</i>	(Visentin et al., 2012)
	R TGAGTTGTCGCTCGCTTGTTG	960-981			
β Tubulin	F CAGCGTTCCTGAGTTGACCCAACAG	852-877	101	<i>F. verticillioides</i>	(Choi and Shim, 2008)
	R CTGGACGTTGCGCATCTGATCCTCG	978-1003			

3. Results and discussion

3.1. BCAs inhibition of *Tri5* and *Tri8* genes of trichothecenes biosynthesis pathway.

The comparative analysis of effect of BCA treatments on *F. graminearum* trichothecene gene expression during interaction was presented in Figure 1. *Tri5* et *Tri8* (respectively in the first and last steps of the trichothecenes biosynthesis pathways) showed similar pattern expression curves in the different samples. An expression peak was recorded at 5 days in the control samples, underlining the importance of studying expression kinetics over time. The observed gene expressions are consistent with the behavior of *F. graminearum* under natural conditions, which will tend to produce mycotoxins in the early stages of colonization of plant substrates (Boedi et al., 2016). Two significant different types of inhibition by BCAs could be observed with an intermediate inhibition of the expression of the two genes studied by Xeda (60% and 52% inhibition of *Tri5* and *Tri8* expression respectively) and a strong inhibition by Poly (77% and 93% inhibition of *Tri5* and *Tri8* expression respectively).

Tri8 is mainly expressed in controls while it is almost absent in samples treated with Poly, which appears to act mainly on the late stages of the trichothecene biosynthetic pathways.

The modulation of these genes expression against biotic factors has already been evaluated in the case of bacterial interactions and the authors showed the ability of *Pseudomonas spp.* to alter histone acetylation and thus block DON synthesis (Chen et al., 2018). However, few other studies have

examined the impact of biocontrol agents on gene expression. Most research has focused on the analysis of DON regulation by signals generated during the host plant-*F. graminearum* interaction (Mudge et al., 2006), but also by abiotic factors such as temperature, nutrients, pH, and light (Gardiner et al., 2009; Llorens et al., 2004; Merhej et al., 2010).

Poly has the strongest impact on gene expression, which supports the hypothesis considered in a previous study (Pellan, 2020b) suggesting that Poly could have upstream effects of mycotoxin production. Indeed, during dual culture assays, Poly caused a strong overall reduction of mycotoxins, but had a low bio-transformation capacity for isolated DON. In contrast, Xeda, which also caused a strong overall reduction of mycotoxins, was able to transform mycotoxins but had a less upstream effect on mycotoxin expression. These two mycotoxin-limiting modes of action were complementary and more or less preponderant in each of the BCAs considered.

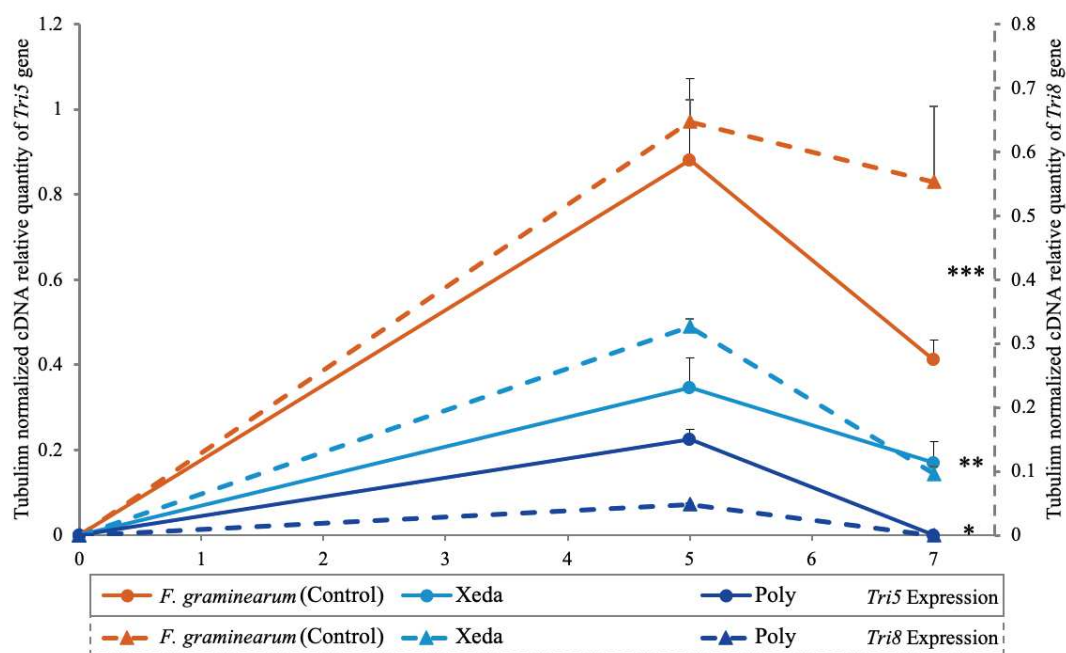


Figure 5 : Comparative effects of BCA treatments on *F. graminearum* trichothecene gene expression during interaction. Dual culture bioassays of BCAs against pathogen were performed on CYA for 7 days. Gene expression of *Tri5* appears in solid line (—) and gene expression of *Tri8* appears in dotted line (---), and are expressed in β -Tubulin normalized cDNA relative quantity. Xeda: Xedavir®, Poly: Polyversum®. ANOVA test independent for each gene was made, p-value < 0.05.

3.2. BCAs inhibition of *Fum1* and *Fum8* genes of fumonisin biosynthesis pathway

When performing similar analysis of the expression profiles of mycotoxin biosynthesis genes of *F. verticillioides* in presence of BCAs, a completely different behavior was observed (Figure 2). Indeed, the expression of *Fum1* and *Fum8* (respectively implicated in the first and later steps of the fumonisin biosynthesis pathways) increased over time in the control samples. Significant inhibitions were

observed during the interaction of *F. verticillioides* with BCAs. Xeda caused a 37% and 41% decrease in the expression of *Fum1* and *Fum8* respectively, with few differences between the two days after inoculation analyzed, suggesting that a plateau was reached. This profile suggests that Xeda may have more resilient gene inhibition activity over time. Poly caused a 52% and 58% decrease in *Fum1* and *Fum8* expression respectively with a similar increasing curve pattern to the control.

As with *F. graminearum*, the majority of studies focused their analyses on the influence of abiotic factors on the expression of fumonisin production genes (Marín et al., 2010b; Picot et al., 2010). Only few studies have analyzed the impact of biotic factors on gene expression involved in mycotoxin biosynthesis. A recent study showed the impact of biocontrol actinomycetes on *F. verticillioides* transcriptome (Strub et al., 2019). Several genes including *Fum 8* involved in fumonisin biosynthesis pathway and in fumonisin regulation of *F. verticillioides* were found down-regulated, but *Fum1* was not affected. Another research group also showed the ability of fungal and bacterial microorganisms, notably the mycoparasitic fungus *Clonostays rosea*, to interact and inhibit the mycotoxin biosynthesis pathways of *F. verticillioides* (Samsudin and Magan, 2016). Assays were conducted *in vitro* or on maize cobs at different ripening stages (Samsudin et al., 2017), and this time a decrease in the expression of *Fum1* in the presence of BCA was observed.

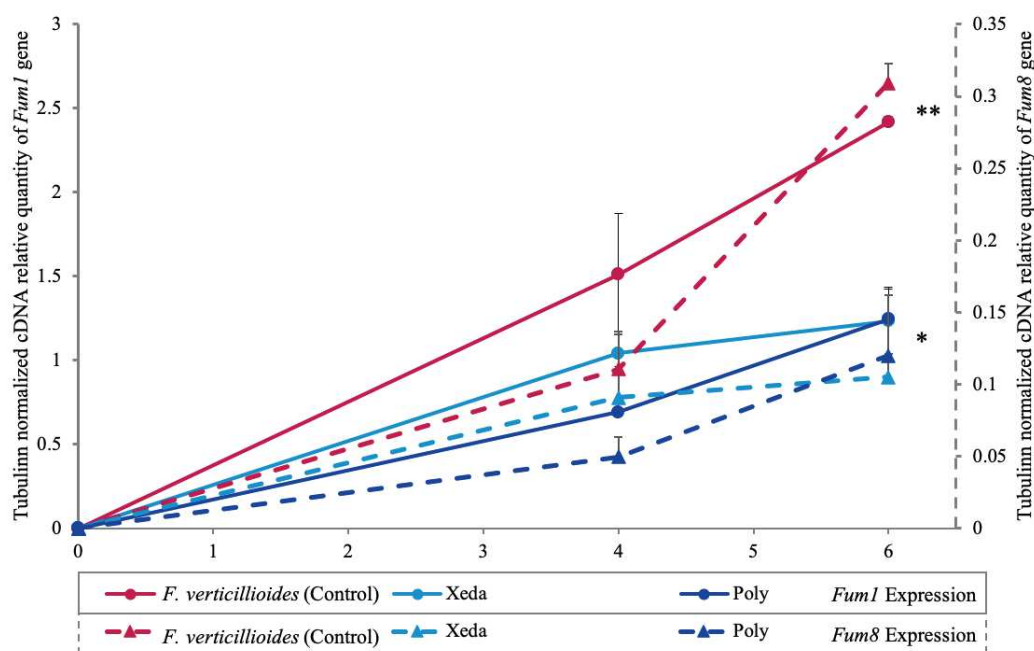


Figure 2 : Comparative effects of BCA treatments on *F. verticillioides* fumonisin gene expression during interaction. Dual culture bioassays of BCAs against pathogen were performed on PDA for 7 days. Gene expression of *Fum1* appears in solid line (—) and gene expression of *Fum8* appears in dotted line (---), and are expressed in β -Tubulin normalized cDNA relative quantity. Xeda: Xedavir®, Poly: Polyversum®. ANOVA test independent for each gene was made, p-value < 0.05.

3.3. Relationship between expression level of mycotoxin biosynthesis genes, growth, and mycotoxin production during interaction *Fusarium*-BCAs.

Principal Component Analysis (PCA) was performed to visualize the correlation between mycotoxin gene expression, growth and mycotoxin production of two pathogens in the presence of BCAs. For both biplots, the variables are well projected, and the first two components explain 93.5% and 90.6% of the distribution of the data for *F. graminearum* (Figure 3) and *F. verticillioides* (Figure 4), respectively.

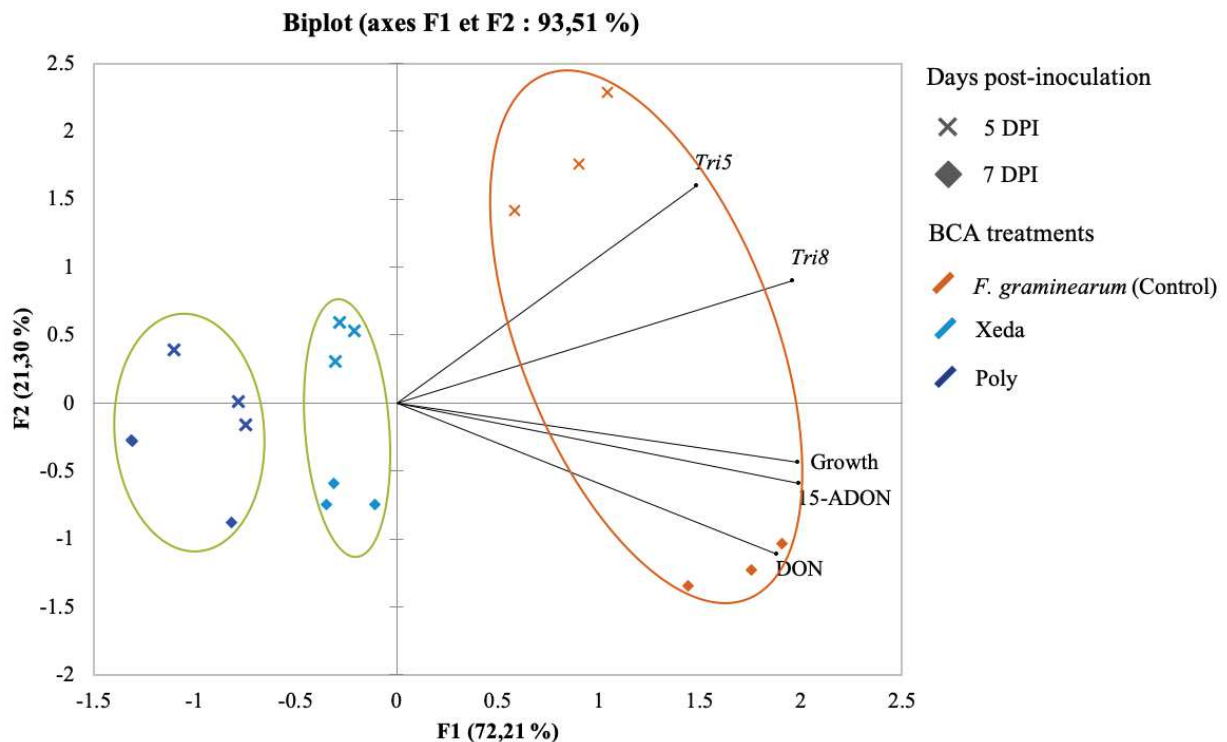


Figure 3: Principal component analysis (PCA) of correlation bi-plot with respect to the expression of gene of trichothecene biosynthesis (*Tri5*, *Tri8*), growth and mycotoxin production (DON, 15-ADON) data set (—) for *F. graminearum*, obtained after BCA confrontation (indicated by colors) at different time after inoculation (DPI, indicated by forms). Xeda: Xedavir[®], Poly: Polyversum[®].

F. graminearum analysis presented a strong correlation between the different pathogenicity related-traits of the pathogen, in particular the mycotoxin gene expression of *Tri5* and *Tri8* with Pearson's coefficients (P_c) of 0.87 on the one hand and the growth and production of both mycotoxins on the other hand (P_c : 0.84 on average). Control samples from culture of *F. graminearum* grown alone express mycotoxin genes more strongly at 5 DPI, while the distribution of samples at 7 DPI are more strongly related to growth and mycotoxin production variables (Figure 3).

This phenomenon could be quite consistent with the complex lifestyle of the pathogen: a first invasive phase consists in synthesizing mycotoxins (biotrophy) and then, once well established, a saprophytic phase that will encourage growth rather than the production of defensive toxins (Boedi et al., 2016).

Xeda and Poly samples are regrouped in the left part of graph, suggesting strong inhibition to the studied pathogen factor (growth and trichothecenes production). For the Xeda-confronted samples, inhibition of pathogenic factors appears to follow the chronology of pathogen development (first on mycotoxin biosynthesis pathway and then on growth and mycotoxin production). Global antagonist activity of Poly against *F. graminearum* was higher and less focused on pathogen development.

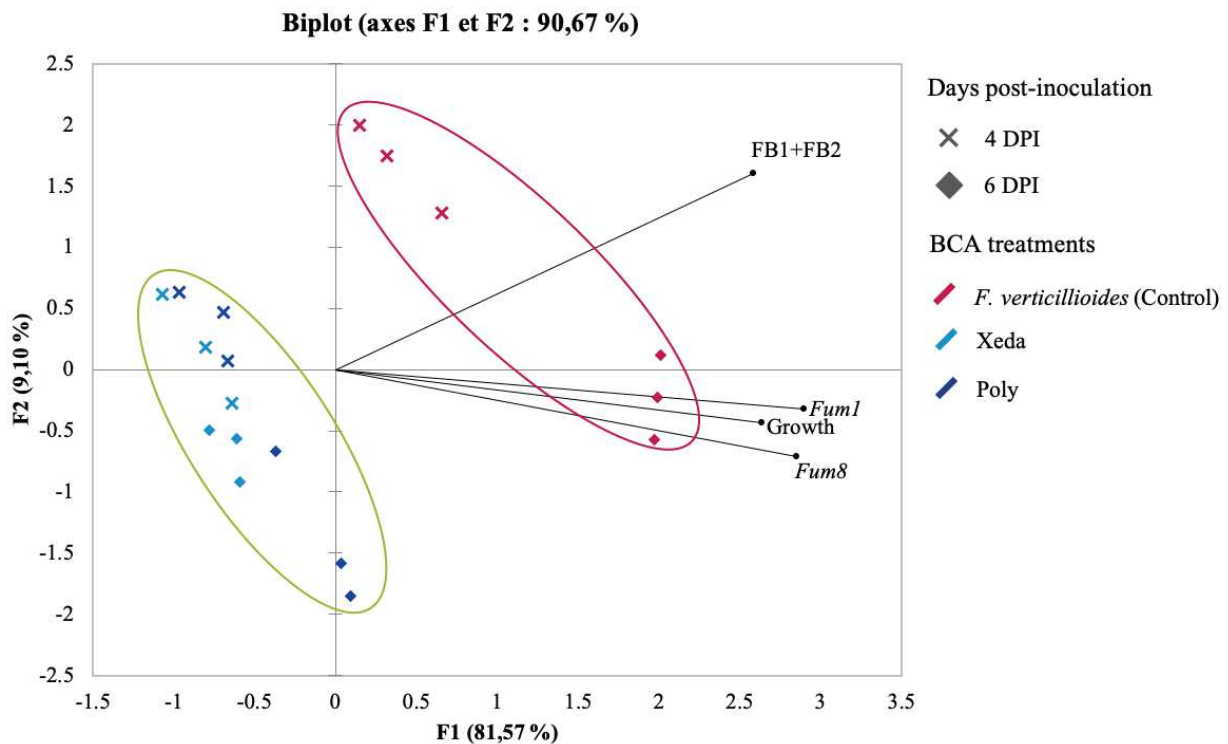


Figure 4 Principal component analysis (PCA) of correlation bi-plot with respect to the expression of gene of fumonisin biosynthesis (*Fum1*, *Fum8*), Growth and Mycotoxin production (FB1+FB2) data set (—) for *F. verticillioides*, obtained with BCA dual culture assays (indicated by colors) at different time after inoculation (DPI, indicated by forms). FB1 + FB2 are represented together because the quantity of FB2 was not significant. Xeda: Xedavir®, Poly: Polyversum®.

Considering the potential links between fumonisin biosynthesis (*Fum1*, *Fum8*), growth and mycotoxin production (FB1+FB2) of *F. verticillioides* (Figure 4), PCA analysis showed differential profile compared to *F. graminearum* analysis. Indeed, for this pathogen, even if the virulence factors all increased over time, a stronger correlation existed between the expression of the *Fum1* and *Fum8* genes and the growth of the pathogen (P_c : 0.74 on average) associated with late samples (7 DPI), distinct from the evolution of mycotoxin production (FB1+FB2) which was high for the two days of growth considered. This phenomenon would seem to indicate that for this pathogen, mycotoxins were synthesized at the beginning of the interaction but the expression of the genes implicated in this mycotoxin formation continued to increase over time and after the establishment of the pathogen,

suggesting an aggressive behavior. However, these results are in contradiction with the mode of life of *F. verticillioides* in major environmental condition, rather described as a biotrophic parasite because it is associated with maize as a symptomless endophyte, Bacon 1996, and that fumonisins are not required for the effective infection (Desjardins et al., 2002). Nevertheless, although the role of fumonisins in the virulence of *F. verticillioides* remains to be clarified, it is possible that they enhance its the virulence on some other host plants (Proctor et al., 2002). In addition, the biological mechanisms involved in the development of virulence factors of the pathogen (such as mycotoxin production) may be very strongly affected by the matrices on which the test is performed, which may also explain this apparent difference in behavior during *in vitro* dual culture assay or in natural conditions (Fanelli et al., 2012; Jian et al., 2019; Medina et al., 2013). Xeda and Poly samples are regrouped in the left part of graph, suggesting strong opposition to *F. verticillioides* control sample without strong difference in effect unlike previously for *F. graminearum*. Both BCAs exhibit strong inhibition of pathogenic factors by reducing the expression of fumonisin biosynthesis (*Fum1*, *Fum8*), growth and mycotoxin production (FB1+FB2).

4. Conclusion

During this study, the two selected BCAs showed significant inhibition of the expression of genes positioned at different levels of mycotoxin synthesis, thus contributing to a better understanding of the biological modes of action of these antagonistic microorganisms against mycotoxigenic *Fusarium*. Indeed, Poly (*Pythium oligandrum*) and Xeda (*Trichoderma asperellum*) could has different strategies to reduce the trichothecenes risk. Poly induced strong upstream inhibition impact on mycotoxigenesis gene for both pathogens studied, while Xeda combined this lowest gene inhibition with downstream bio-transformation of mycotoxins. These capacities could be a major asset in the pre-harvest mitigation of toxins, before the products enter the food processing industry. It would be interesting to also examine which other genes are affected by the presence of BCAs in order to better understand the link between identified mechanisms of action and genome expression. Similarly, genes can be repressed in BCAs by the synthesis of mycotoxins and limit their action (Lutz et al., 2003), so the expression of their genes could also be monitored.

Author contributions: L.P., C.S. and S.S.-G. designed the study. M.M. toked part to the realization of extraction and amplification procedures. L.P. carried out the entire range of manipulations and experimental design approaches and analysed the data. L.P., C.S. and S.S.-G. interpreted the data. L.P. wrote the original draft. C.S. and S.S.-G. carefully supervised and reviewed the paper. All authors have read and agreed to the published version of the manuscript.

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3.2.4. Aller plus loin sur l'identification des composés organiques volatiles (COVs) antifongiques ou anti-mycotoxinogènes

Suite aux résultats intéressants obtenus à l'issue de l'analyse des effets inhibiteurs des composés volatils des BCAs sur la croissance et la production de mycotoxines des pathogènes, un dispositif a été mis au point pour suivre l'évolution et caractériser les profils des métabolites volatils produits lors de l'interaction sans contact entre BCAs et pathogènes. Cette piste, bien qu'encore au stade expérimental pourrait être poursuivie à la suite de ces travaux grâce au travail préliminaire suivant.

Matériels et Méthodes

- Le dispositif expérimental

L'objectif était dans un premier temps de reproduire le montage expérimental réalisé dans les premiers tests, c'est-à-dire une double culture sans contact et hermétique, tout en permettant de capter et identifier les composés volatils produits par les micro-organismes au cours du temps. Pour cela, un dispositif en verre autoclavable a été conçu puis réalisé par un verrier, afin d'ajouter une membrane permettant d'accéder à l'espace de tête des composés volatils dans le dispositif. La micro-extraction en phase solide en espace de tête (HS-SPME) couplée à la chromatographie en phase gazeuse et à la spectrométrie de masse (GC-MS) a été retenue pour analyser les composés volatils produits car elle permet de suivre l'évolution des composés fongiques sans destruction de la culture puisqu'aucune utilisation de solvant n'est nécessaire.

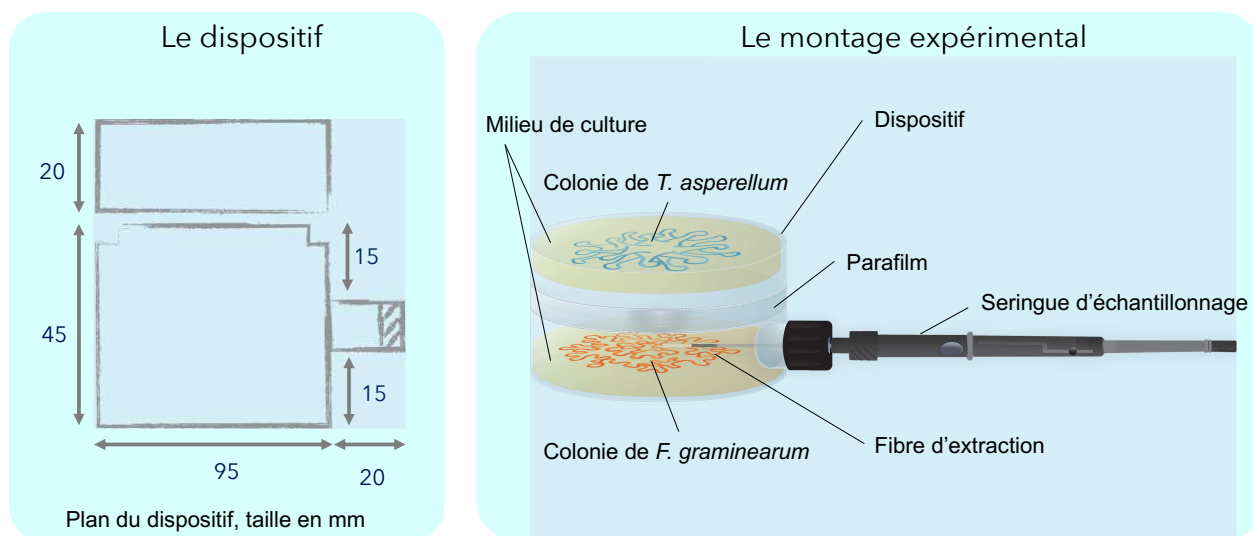


Figure 1: Présentation du dispositif d'incubation et d'extraction des COVs

Les COVs sont ensuite identifiés par comparaison des spectres de masse et des indices de rétention linéaire (LTPRI) avec les références de la base de données et les indices de rétention linéaire publiés dans la littérature.

- La réalisation des essais

Culture des champignons et inoculation du dispositif

F.graminearum et *T. asperellum* (Xedavir, choisi pour son fort impact sur la production de mycotoxine du pathogène), ont été cultivé sur PDA à 25 °C pendant 7 jours pour produire des suspensions de spores à 1.10^4 spores.mL⁻¹ et 1.10^5 spores.mL⁻¹ respectivement. La base et le couvercle du dispositif sont remplie avec 20 mL de CYA (milieu de culture favorable à *F. graminearum*). Les suspensions de spores des micro-organismes sont ensemencées au centre de chaque parties et couvertes par un couvercle en verre durant 24 h à 25 °C. Lorsque les gouttes d'inoculum sont absorbées par les milieux de culture, les couvercles en verre sont retirés et le dispositif est monté et scellé avec trois tours de parafilm. Les dispositifs sont ensuite incubés pendant 14 jours, et des extractions de COVs sont réalisées tous les 4 jours. En plus des échantillons biologiques (*F. graminearum* seul, *T. asperellum* seul, *F. graminearum* – *T. asperellum*), un dispositif contenant uniquement du milieu CYA (témoin : blanc de dispositif) doit être préparé et analysé pour exclure toute fausse identification de composés libérés par le milieu de culture, verrerie, septa en silicone, environnement du laboratoire, etc.

Extraction des composés

Avant toute extraction, la fibre est choisie et conditionnée : pour cette étude, une fibre DBV-CAR-PDMS (Divinylbenzene/Carboxene/Polydimethylsiloxane) de 30-50 µm a été choisie pour optimiser la diversité des COVs captés (Kluger et al., 2013), et conditionnée pendant 1 h à 270 °C (maximum de température, température opératoire 230-270°C). La fibre est montée dans l'échantillonneur, qui la protège des pollutions extérieures, si la fibre a été contaminée, cet exercice de conditionnement peut être répété pour nettoyer thermiquement la fibre.

La fibre est ensuite insérée dans le dispositif (profondeur de la fibre de 35 mm) pour une micro-extraction en phase solide de 30 min, à température ambiante sans chauffage qui pourrait altérer la culture ou la physiologie des micro-organismes, avant d'être désorbée dans l'analyseur GC-MS. Des optimisations de durée d'extraction ont été réalisées au préalable pour obtenir un maximum de composés sur les chromatogrammes.

Analyse GC-MS

La fibre est donc insérée dans le port d'injection de la GC-MS, et les composés volatils sont désorbés de la fibre durant 4 min dans l'injecteur (40 °C, profondeur de la fibre de 25 mm). La fibre est ensuite nettoyée par traitement thermique pendant 3 min (250°C). L'hélium est utilisé comme gaz vecteur à un débit constant de 1 ml/min. Les COVs désorbés sont alors analysés à travers une colonne DB-Wax 30 m x 0,25 mm x 0,25 mm (de phase PEG (polyéthylèneglycol) de haute polarité, recommandée pour l'extraction de composés à bas point d'ébullition et permettant une large gamme de détection de COVs) dans les conditions suivantes optimisées :

- Programme de température : Température du four : 40 °C (maintenir 2 min), 10 °C/min à 200 °C, 25 °C/min à 260°C (maintenir 5 min)
- Spectromètre de masse : ionisation électronique (EI) à 70 eV, source 230°C, quadripôle 150°C, balayage complet (m/z 36-350).

Au cours des expérimentations, la viabilité de la fibre est testée à l'aide d'un mélange de composés aromatiques dont la composition est connue (ici : APR-1 Kemal, 1 mg.mL⁻¹, 10 µL), déposé sur le milieu de culture à la place de l'inoculum pour vérifier que la méthode d'analyse détecte toujours le bon nombre de composés, avec des temps de rétention cohérents et avec une amplitude similaire au cours du temps (Figure).

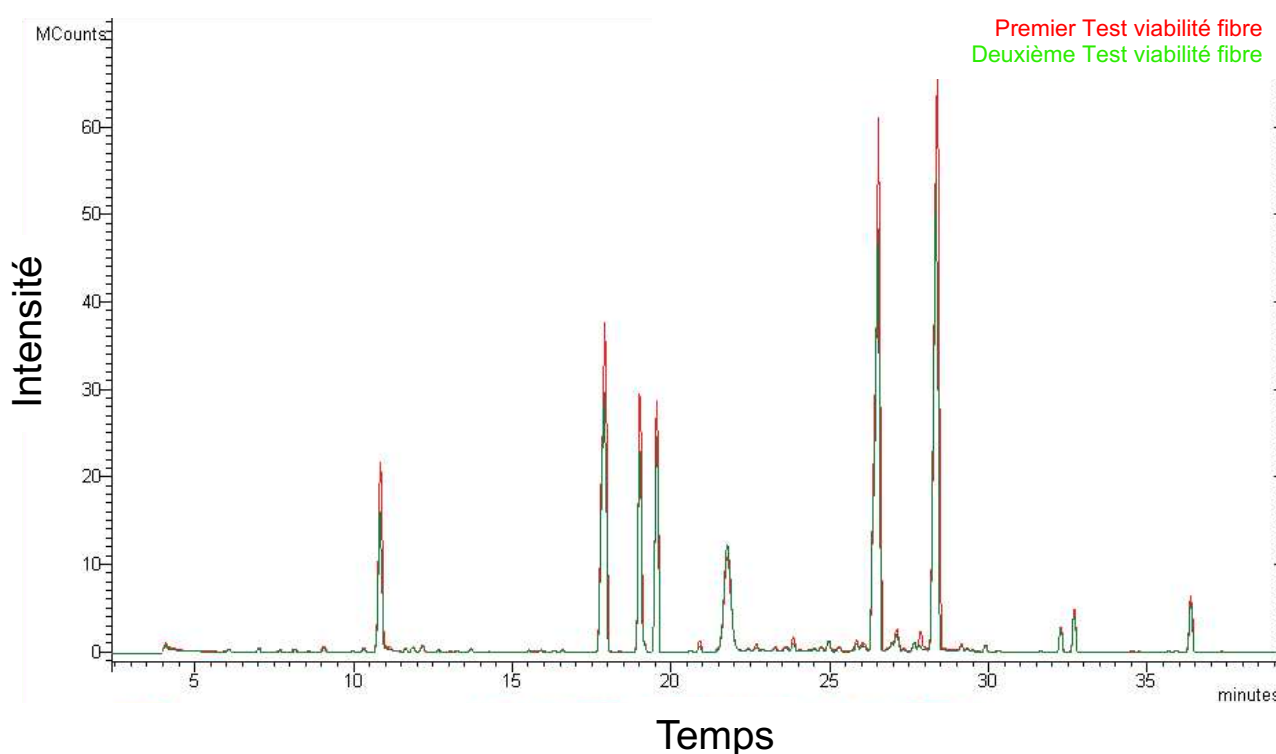


Figure 2: Chromatogramme de surveillance de la viabilité de la fibre, intensité en fonction du temps de rétention.

Afin d'estimer au mieux les indices de rétention (LTPRI) des composés volatils présents dans le dispositif d'étude, des solutions d'alcane standards doivent être analysées dans les mêmes conditions et avec la même méthode de GC-MS que les échantillons fongiques. Il est nécessaire de connaître leurs temps de rétention pour déterminer les valeurs LTPRI des métabolites fongiques.

Pour cela, deux solutions complémentaires d'hydrocarbures sont injectées (C7 à C17, puis C13 à C20, Sigma) et les spectres sont compilés et analysés pour calculer la droite de régression linéaire des indices de rétention.

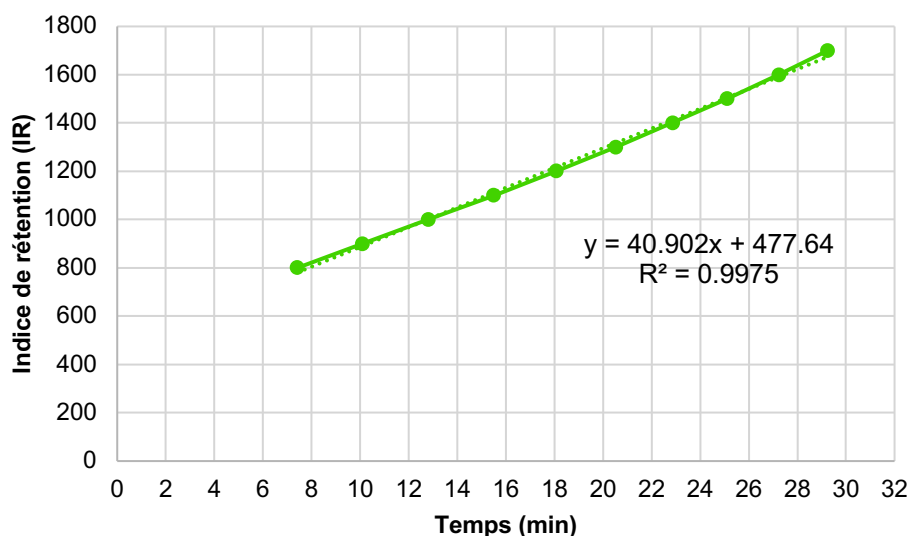


Figure 3: Indice de rétention des hydrocarbures

Les COVs sont ensuite identifiés par comparaison de deux paramètres, les indices de rétention linéaire (LTPRI) et les spectres de masse, avec les entrées de base de données et les indices de rétention linéaire publiés dans la littérature dans les mêmes conditions.

Ces valeurs de LTPRI ont été calculées selon la formule proposée par Van den Dool et Kratz en 1963 :

$$LTPRI = 100 \times \left(\frac{t_R(A) - t_R(n)}{t_R(n+1) - t_R(n)} \right) + 100n$$

$t_R(A)$: Temps de rétention du métabolite

$t_R(n)$: Temps de rétention de l'alcane élué juste avant le métabolite.

$t_R(n+1)$: Temps de rétention de l'alcane élué juste après le métabolite.

n : nombre d'atome de carbone de l'alcane élué juste avant le métabolite.

L'identification des composés grâce au logiciel WorkStat se fait par comparaison automatique des spectres de masse à la base de données NIST et les Indices de Rétention calculés ($\pm 2\%$) sont également comparés avec les IR publiés dans nos conditions d'analyse. Les composés sont considérés comme identifiés lorsque les deux informations sont convergentes.

Résultats préliminaires :

Voici les types de chromatogrammes que l'on peut obtenir après 7 jours de confrontation entre les deux micro-organismes.

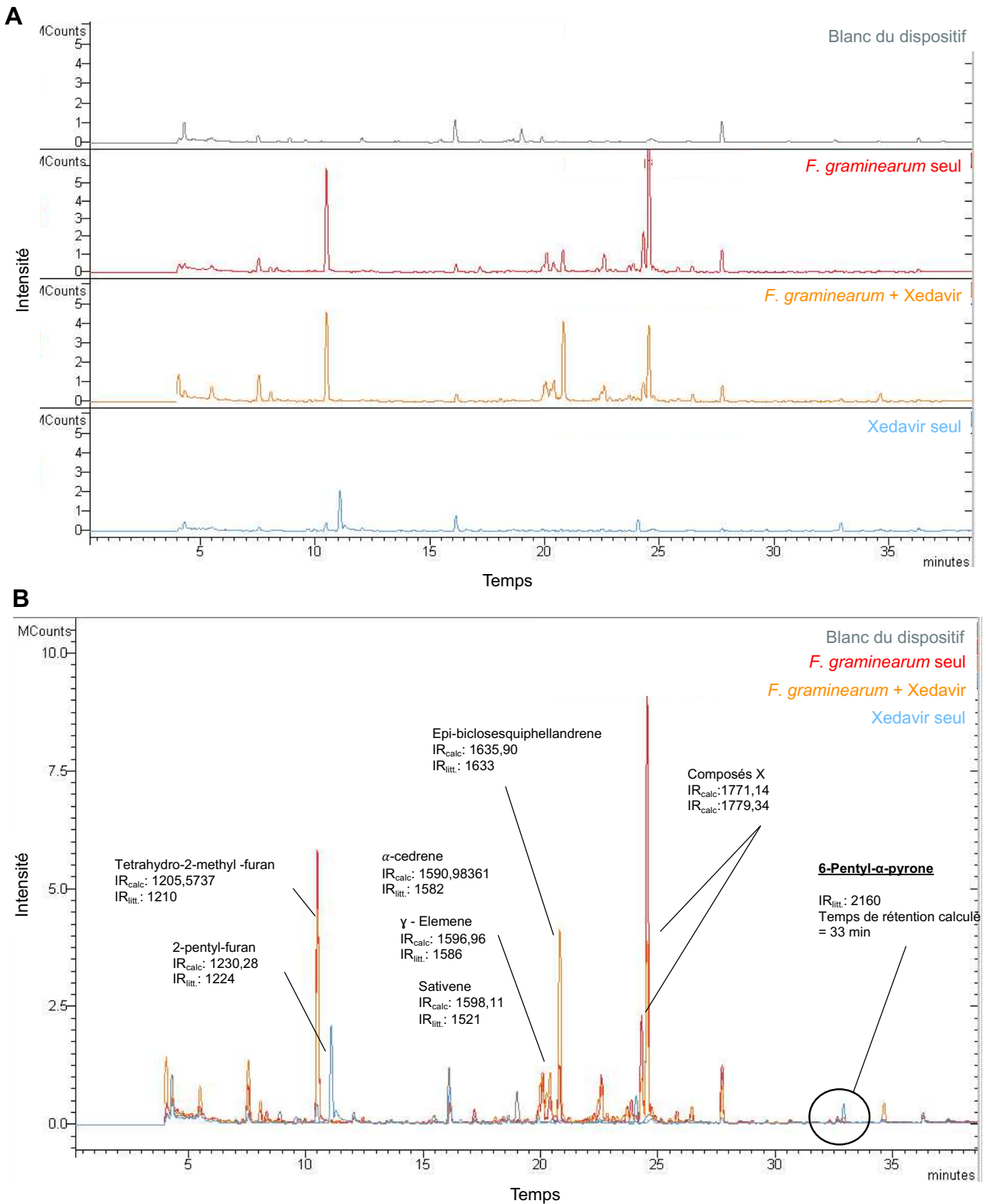


Figure 4 : A. Exemple de chromatogrammes obtenus après 7 jours d'incubation sur CYA à 25°C des différents dispositifs (non-inoculé ou blanc de dispositif, *F. graminearum* seul, Xedavir seul, *F. graminearum* – Xedavir) B. Chromatogrammes superposés avec quelques exemples d'identification spectrale.

En superposant les spectres de chromatographie, nous avons pu observer différents profils et composés, et cibler parmi ce métabolome volatil, des composés dont le profil peut paraître intéressant. Du côté du pathogène, certains composés représentatifs ont pu être identifiés comme des sesquiterpènes (α -cedrene, γ - Elemene ou Sativene). Certains composés sont fortement synthétisés par le pathogène lorsqu'il est seul mais réduit lors de la confrontation, comme par exemple le Tetrahydro-2-méthyl-furan, qui est identifié comme toxique. D'autres composés présentent ce type de profil mais n'ont pas pu être identifiés encore. Côté BCA, des composés qu'il est le seul à synthétiser ont été identifiés comme le 2-pentyl-furan, qui a des propriétés anti-fongiques connues (Wu et al., 2015; Zhang et al., 2020). Certaines molécules antimicrobiennes comme la 6-Pentyl- α -pyrone identifiée chez une autre souche de *Trichoderma asperellum* (Hamrouni et al., 2019; Morath et al., 2012), peuvent être recherchées à partir des Indices de Rétention calculée dans ces autres études en conditions similaires (ici il devrait apparaître autour de 32-34 minutes). Dans cette première approche le pic reste petit et indétectable mais il pourrait s'intensifier au cours de l'étude cinétique).

Conclusion :

Ce type de matériel et de démarche expérimentale, qui est en cours au laboratoire, pourrait apporter des réponses intéressantes sur les COVs des pathogènes comme des BCAs, notamment fongiques mais plus largement microbiens, mais aussi sur leur spécificité comme marqueurs de mycotoxinogénèse pour les pathogènes ou à activités anti-mycotoxinogènes pour le BCAs. Les COVs antifongiques identifiés pourraient ainsi être mis en relation avec des effets observés sur lors d'autres expérimentations *in vitro* (inhibition de germination, de croissance, de mycotoxines).

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Partie Expérimentale

Étude n°3

3.3. Étude n°3:

Évaluation *in situ* des effets des Agents de biocontrôle sur *Fusarium graminearum*

Cette partie fait l'objet d'une communication écrite sous forme d'article de recherche soumise dans le journal *Microbiological Research* le 1^{er} septembre 2020 et en révision:

How Biocontrol Agents disturb life cycle interconnections of mycotoxigenic *Fusarium graminearum* and wheat ?

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Noël Durand^{1,2}, Sabine Schorr-Galindo¹ and Caroline Strub^{1,*}

¹Qualisud, Univ. Montpellier, CIRAD, Montpellier SupAgro, Univ.d'Avignon, Univ.de La Réunion,

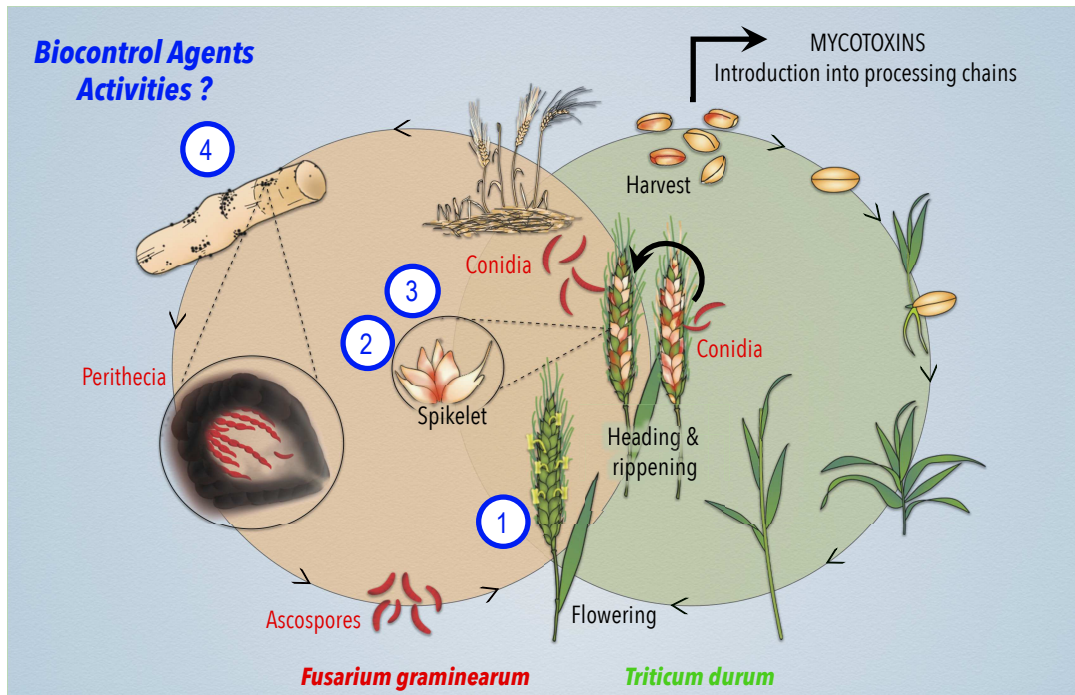
²CIRAD, UMR Qualisud, Maison de la Technologie, 73, avenue Jean-François Breton. F-34398 Montpellier, France

* Correspondance : Caroline STRUB : caroline.strub@umontpellier.fr

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Dans cette troisième partie, l'impact des BCAs sur le pathogène du blé *F. graminearum* a été caractérisé *in situ* grâce à différentes démarches expérimentales complémentaires. Cette étude a été menée de manière cinétique tout au long du cycle de vie des agents pathogènes dans les agrosystèmes que ce soit au stade de colonisation des épis ou lors des phases de survie dans les résidus de culture, ici des pailles de blé séchées. La capacité des BCAs à s'établir sur les matrices végétales et à inhiber l'agent pathogène ou ses toxines a été observée. En effet, la colonisation externe des épillets détachés à l'aide d'une échelle de notation visuelle a été complétée par une analyse de biomarqueurs par ddPCR pour avoir une vision plus juste de la colonisation par *F. graminearum*. Une méthode fiable a été mise au point pour doser la quantité de mycotoxines dans ces échantillons de faibles volumes. Grâce à cette analyse complète, les BCAs ont tous montré des capacités de réduction efficaces des traits pathogènes tels que la croissance (-80 % en moyenne), la production de mycotoxines (-99 % en moyenne) pendant la forme végétative, ou la production de périthèces (-95 % en moyenne) pendant la forme de survie. Les mécanismes complexes impliqués dans ces différentes interactions entre *F. graminearum* et les BCAs sur les matrices végétales sont examinés. Ce suivi des interactions contribuera à optimiser l'utilisation et l'efficacité de ces antagonistes mais aussi à évaluer de manière plus fiable les candidats potentiels.

Mot-clés : microbe interactions, mycotoxins, spikelet, ddPCR, plant protection.



Résumé graphique



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How Agents disturb life cycle interconnections of mycotoxigenic *Fusarium graminearum* and wheat ?

Lucile Pellan¹, Cheikh Ahmeth Tidiane Dieye¹, Noël Durand^{1,2}, Sabine Schorr-Galindo¹ and Caroline Strub^{1,*}

¹Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ d'Avignon, Univ de La Réunion, Université de Montpellier-Campus Triolet

F-34095 Montpellier Cedex 5

Montpellier, France;

Pellan.lucile@umontpellier.fr; noel.durand@cirad.fr ; sabine.galindo@umontpellier.fr ;

caroline.strub@umontpellier.fr

² CIRAD, UMR Qualisud, Maison de la Technologie, 73, avenue Jean-François Breton. F-34398

Montpellier, France

* Correspondence: Caroline STRUB: caroline.strub@umontpellier.fr

Abstract

Agricultural production is subject to a scissor effect with, on one hand, very evolutive climatic conditions that promote microbial development and enhance toxic mycotoxins production, on the other hand, the political determination to reduce the use of phyto-chemical products also toxic. The aim of this study is to propose effective and sustainable alternative solutions to control these two major environmental pollutions: the massive use of pesticides and the production of mycotoxins by plant pathogens. For this purpose, devices have been developed to evaluate the interactions between wheat spikelets, a mycotoxigenic pathogen *Fusarium graminearum* and several contrasting Biocontrol agents. This study was conducted throughout the life cycle of pathogens in agrosystems (colonization and survival). The ability of BCAs to establish and inhibit the pathogen or its toxins was observed. Thanks to this comprehensive analysis, the BCAs all showed effective reduction capacities of pathogenic traits such as growth (-80% on average), mycotoxins production (-99% on average) during vegetative form, or perithecia production (-95% on average) during survival form. The complex mechanisms involved in these different interactions between *F. graminearum* and BCAs on plant matrices are discussed. This interaction tracking will contribute to optimize the use and efficacy of

these antagonists and consequently to limit the use of phyto-pharmaceuticals, while maintaining healthy production and low mycotoxin levels.

Key-word: microbe interactions, mycotoxins, spikelet, ddPCR, plant protection.

1. Introduction

Nowadays, the sustainability of agricultural systems is widely questioned because they are at the crossroads of multiple health, environmental and economic issues. Firstly, global warming strongly disrupts crop development, but also the distribution of pathogens and the intensity of epidemics by creating conditions favorable to the proliferation of microorganisms (Paterson and Lima, 2010). Moreover, some of these pathogens, such as certain *Fusariums* strains, produce toxins that are particularly harmful to human and animal health, which are also modulated by environmental factors such as temperature. In large quantities, these mycotoxins, such as trichothecenes, can cause many health problems in humans and animals, ranging from food poisoning and acute intoxication leading to vomiting (emesis); to immunotoxic effects or reproductive disorder (Ferrigo et al., 2016). In 2019 in Central Europe, almost 70% of the samples analyzed contained mycotoxins (Gruber-Dorninger et al., 2019) and these mycotoxins are difficult to degrade and very stable during transformation process. Finally, fighting against these pathogens is mainly based on the pre-harvest massive use of pesticides, as few prevision programs or resistant varieties exist. These synthetic products can cause acute or chronic toxic effects, posing particular risk to children, and are recognized as an issue of global concern but also could present danger to environmental ecosystems (Kim et al., 2017). For these reasons, growing concerns about the impact of chemical pesticides on health and the environment have stimulated researchers and industry to explore alternative and more environmentally friendly solutions. Biocontrol and in particular the use of antagonistic microorganisms (BioControl Agents) may be a more integrative and health-friendly method for environment and consumer. These BCAs will colonize and prevent the progression of the pathogen in crops by various mechanisms including parasitism, competition or production of antifungal compounds (Torres et al., 2019). This complementary control method is particularly supported by political directive in the context of integrated crop protection (van Lenteren et al., 2018), as “Ecophyto II +” plan in France.

To develop this approach, one of the most devastating diseases of wheat Fusarium head blight (FHB) caused mainly by *Fusarium graminearum*, a mycotoxigenic fungus, was used as a model. *F. graminearum* is the dominant species of the FHB complex and provokes multifaced damage. This fungus leads to yield and quality losses of cereal production due to sterility of the florets and formation of wilt and discolored kernels, but also mycotoxins of trichothecene family. Indeed, cereals and cereal based-product are the first factor of consumers exposure to these mycotoxins (Goswami and Kistler, 2004), especially deoxynivalenol (DON). Structurally, DON is a polar organic compound, belonging to the type B trichothecenes. Its synthesis is influenced by many environmental parameters (Llorens

et al., 2004). Once ingested by humans or animals, it causes acute temporary nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and fever (Sobrova et al., 2010).

At the beginning of field epidemy and when environmental conditions are favorable, *F. graminearum* infects wheat plants at the flowering of the ears, during the formation of anthers. After spore germination the mycelium can penetrate the tissue via wounds or natural openings like stomata or empty space between the palea and the lemma of spikelet, near the floret. This colonization continues asymptotically for 20 to 70 hours after inoculation. After this first phase, described as biotrophic, the fungus triggers tissue necrosis by accumulation of mycotoxins and intracellular growth, allowing it to progress rapidly to invade other close spikelets. The production of mycotoxins is essential for the successful colonization of wheat ears by *F. graminearum*, and its synthesis is stimulated by the plant's defense compounds, such as H₂O₂ or polyamines known to be accumulated in plants attacked by pathogens (Boedi et al., 2016) Finally, the classical FHB symptom occurs with chlorosis of the whole head characterizing the necrotrophic phase of the pathogen activity. The life cycle of the pathogen then continues after the wheat harvest in the crop debris with saprophytic activity until the formation of the conservation structure, the perithecia. Indeed, the main source of inoculum comes from infected crop residues and the pathogen can survive in this form under unfavorable conditions for a long period of time (Bujold et al., 2001).

In order to fight this plant pathogen, only few biocontrol solutions are available in Europe: *Pseudomonas chlororaphis* are available as commercial products in Cerall® (Belchim Crop Protection) and *Pythium oligandrum* in Polyversum® (Biopreparáty/De Sangosse). However, this alternative to conventional method can enrich the set of tools to control FHB with more efficiency

From a technical point of view, the analysis of interactions between pathogens and biocontrol agents is mostly studied on synthetic media and it is clear that the complex tripartite interaction between the pathogen, the BCAs and the plant cannot most of the time be extrapolated from dual confrontation assays (Whitaker and Bakker, 2016). To better understand the potential capabilities of BCAs, realistic interaction easily performed than field test such as the use of detached organs (Comby et al., 2017) are needed. In addition, mechanisms of *F. graminearum* interaction with other microorganisms and their influence on DON biosynthesis in the plant-associated microbiome are currently poorly understood and require further research (Chen et al., 2018).

In this study, key stages of interaction between life cycle of wheat and *F. graminearum* were focused (establishment, colonization and survival) to analyzed the impact of three contrasted commercial BCAs on this patho-system at different levels from plant to gene. During the vegetative stage, the implantation of different microorganisms, pathogen or benefic, and the effect of BCAs on colonization or mycotoxin production by pathogen on detached spikelets was investigated with complementary techniques including microbiology, biochemistry and molecular biology . At survival stage, the antagonistic activities of BCAs on perithecia of *F. graminearum* on wheat straw was characterized. This research allowed to depict behavior of contrasted BCAs in interaction with mycotoxigenic

phytopathogen. It provides a method for identifying potential pathogenicity, growth capacity or antagonistic activities of microorganism on targeted plant and thus could reduce the diffusion or apparition two major environmental pollutions, pesticides and mycotoxins.

2. Material and Methods

2.1. Micro-organisms and culture condition

F. graminearum isolate BRFM 1967 was chosen for its isolation origin (wheat plant) and its strong ability to produce mycotoxins (CIRM, University of Aix-Marseille, Marseille, France) with a deoxynivalenol (DON/15-ADON) chemotype. Fungus were maintained under paraffin oil at 4°C and actively grown on PDA (PDA; BD Difco, Sparks, MA, USA) at 25°C for 7 days for spore production. Spore suspension was prepared from solid medium in sterile water-Tween (wT, 0,01%) and filtered with carded cotton.

Three commercial biological control agents were selected because of their contrasting type and use: Mycostop® (Lallemand Plant Care®), Xedavir® (Xeda International®), and Polyversum® (DeSangosse®). Explanations on how BCAs were selected and more information about their characteristics and their effects on growth and mycotoxinogenesis of *F. graminearum* in dual culture assays are available in previous work (Pellan et al., 2020c). All BCAs were isolated from their commercial formulation with a classical microbial isolation protocol and conserved in commercial product aliquots (4 °C) or in glycerol solution (15%/ V:V / -80 °C). Pure cultures were maintained on ISP4 (agar 18 g; starch 10 g; K₂HPO₄·3H₂O 1 g; MgSO₄·7H₂O 1 g; (NH₄)₂SO₄ 1 g; CaCO₃ 1 g; FeSO₄·7H₂O 0.001 g; MgCl₂ 0.001 g; ZnSO₄·7H₂O 0.001 g; per liter of distilled water), PDA, and V8 juice agar (V8 juice 200 ml (Campbell's, Camden, NJ, USA); agar 15 g; CaCO₃ 3 g; per 800 ml of distilled water), respectively, for Mycostop, Xedavir, and Polyversum at 25 °C for 7 days for production of spore suspensions or filaments for Poly. Spores suspension were prepared as described for *F. graminearum*. For the rest of the study, the strain isolated from commercial product were referred using the following abbreviations: M or Myco for Mycostop/*S. griseoviridis* (Lallemand Plant Care®), X or Xeda for Xedavir/*T. asperellum* (Xeda International®), and P or Poly for Polyversum/*P. oligandrum* (DeSangosse®).

Antagonistic activities of BCAs against *F. graminearum* was assessed at keys stages of interaction between *F. graminearum* and wheat life cycle as presented in Figure X: 1) Inoculation of microorganisms at the flowering stage of wheat in relation to the natural attack phases of *F. graminearum*, 2) monitoring of external growth via a visual scoring scale and internal assessment via specific DNA quantification of the microorganisms on spikelet, 3) analysis of the production of DON on spikelets during interaction with BCAs and finally 4) the effect of BCAs on the survival structures of the pathogen, the perithecia.

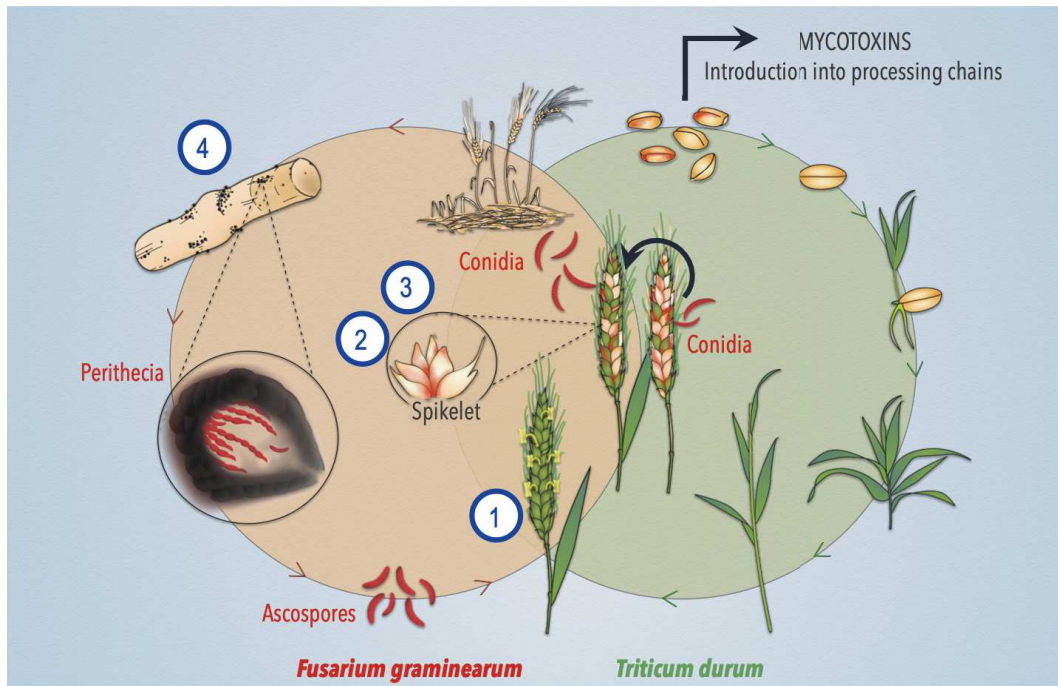


Figure 6: *Fusarium graminearum* and *Triticum durum* life cycles with a focus on their interconnection stages, and position of different assays carried out in this study. 1. Inoculation and establishment of microorganisms at the flowering stage of spikelets in accordance to the natural attack phases of *F. graminearum*, 2. Monitoring of external growth of *F. graminearum* via a visual scoring scale and internal assessment via DNA quantification of microorganisms on the spikelet, 3. Analysis of DON production by *F. graminearum* on spikelets and 4. Monitoring of BCAs effect on the survival structures of the pathogen, the perithecia.

2.2. Antagonistic activity of BCAs on wheat spikelets

2.2.1. Inoculation and visual growth assessment

Dual culture assay with *F. graminearum* and the three BCAs were conducted on detached wheat spikelet as described by (Comby et al., 2017). *Triticum durum* wheat, cultivar Miradoux® (Florimond Desprez®) were grown from untreated seeds on culture room (20 °C, photoperiod 16 h day/ 8 h night with 400W sodium lamp (Phillips, Amsterdam, Netherlands) up to the BCCH 55-60 stage (beginning of ear emergence and flowering). Spikes of homogenized stage were harvested and spikelets were detached and used immediately for confrontation.

Detached spikelets were dipped in spore suspension of BCAs or water-Tween (wT) control (1×10^5 spores.mL⁻¹) and placed in Petri dishes containing agar and water medium (3g.L⁻¹) with rachilla inserted in medium to conserve spikelets. After four days, spikelets was rapidly dipped in *F. graminearum* spore suspension (1×10^4 spores.mL⁻¹) or wT control and repositioned to the minimum medium. All spikelets were incubated at 20 °C (photoperiod 16 h day/8 h night). Every two days, a visual evaluation of the external colonization of *F. graminearum* was realized for each spikelet, using a visual scale modified from (Comby et al., 2017), during 12 days. Level 0 was assigned to no

symptom of disease, level 1 to spikelet tissue degeneration, level 2 to beginning of necrosis apparition, level 3 to visible mycelium on all spikelet tissue, level 4 to mycelium and necrosis and level 5 to spikelets strongly infected with mycelium, necrosis and possible presence of sporodochia. For each condition and sampling days, 15 spikelets were considered. Samples inoculated with the pathogens or BCAs were dissected and intern tissues was examined using a binocular loupe and a Zeiss PrimoStar optical microscope with Zeiss Axiocam ERc5s camera (Carl Zeiss Microscopy, Thornwood, NY, USA) to verify the colonization capacities of microorganisms.

2.2.2. Quantification of BCAs-Pathogens interaction in wheat spikelet tissues by ddPCR.

Three spikelets of each confrontation conditions were collected 8 days after inoculation, and stored at -80°C for further analysis. For the DNA extraction, samples were grinded using GenoGrinder (SpexSamplePrep, NJ, USA) with steel grinding balls. Total DNA was extracted using NuclioSpin DNA extraction kit (Macherey-Nagel, GmbH & Co. KG, Germany) according to manufacturer's instructions. Absolute quantification of *F. graminearum* in all conditions were realized by IAGE company, France. As Xeda presented an interesting inhibition profile (intermediate on growth and total inhibition of mycotoxins), it was selected to be detected in interaction with the pathogen. Detection and quantification of these two micro-organisms was performed with DropletDigitalPCR (ddPCR) technique. Biomarkers primers used in this study were detailed in Table X. They were chosen for the specific detection and quantification of *F. graminearum* and Xeda (*T. asperellum*). DNA was detected and quantified using the QX200™ Droplet Digital™ PCR system (Bio-Rad, Pleasanton, CA, USA). The ddPCR reaction mixture contained 2 µL of DNA template (22 µL total reaction volume); 450 nM of each EvaGreen primer and sterile water. Samples were partitioned with 70 µL of BioRad droplet oil in a BioRad QX200 droplet generator. Water-in-oil emulsions were transferred to a 96-well plate and amplified in a PCR thermocycler using the following cycling protocol: hold at 95°C for 5 min, 40 cycles of 95°C for 30 s, 62°C for 1 min, and a final enzyme deactivation step at 98°C for 10 min followed by cooling at 10°C. After the thermal cycling, the plate was then analyzed on droplet reader. Wells containing all reagents and RNA/DNA- free water were used as negative control. The software package provided with the ddPCR system (QuantaSoft 1.7, Bio-Rad) was used for data acquisition.

Table 2: Primer pairs characteristics used for absolute quantification by ddPCR.

Gene	Sequence	Amplicon Size	Targeted organisms	Source
<i>Tri5</i>	F GATCTGATGACTACCCTCAATTCCTT	71	<i>F. graminearum</i>	(Marín et al., 2010)
	R GCCATAGAGAAGCCCCAACAC			
RNA PolyB subII (<i>rpb2</i>)	F GGAGGTCGTTGAGGAGTACGAA	142	Xeda (<i>T. asperellum</i>)	(Gerin et al., 2018)
	R TTGCAGATAGGATTTACGACGAGT			

2.2.3. DON extraction and quantification on wheat spikelet

For mycotoxin analysis, each of twelve remaining spikelets by conditions was grinded with liquid nitrogen and 5 mL of acetonitrile/water/acetic acid (79:20:1, v/v/v) was added. Samples were homogenized by mechanical agitation for 20 min. Samples were preliminarily diluted 1:50 in water/acetic acid (99.5:0.5; mobile phase of analyzer) and filtered with a CA filter (0.45 µm, Carl Roth GmbH, Karlsruhe, Germany) before injection. Mycotoxin detection and quantification were achieved using an Ultra High-Performance Liquid Chromatography (UHPLC, Shimadzu, Tokyo, Japan) coupled with a mass spectrometer (8040, Shimadzu, Tokyo, Japan) as described in (Pellan et al., 2020c). During the development of this method, various parameters were tested to ensure the reliability of the analysis. At the extraction level, the quantity of spikelet (one or three), grinding (liquid nitrogen or fresh grinding), ratios of solvent (5ml or 15ml) and dilution in the mobile phase (1:50, 1:10 or 1:2) have been tested and the best parameters were selected. The following points were also checked: the absence of molecules in healthy spikelet having the same transitions as the mycotoxins, the high extraction yield (84.5%) using labelled mycotoxin standards added to healthy spikelets during extraction, repeatability of the analyses carried out on varied contaminated samples by inoculation of spores, and detection even of small quantities of mycotoxins (with DON limits of detection or quantification of 4 ng.mL⁻¹ and 14 ng.mL⁻¹, respectively).

2.3. Antagonistic activity on wheat straw and *F. graminearum* perithecia production

Dried wheat straws were collected at the end of the cycle of wheat plants used previously, to assess the antagonistic activity of BCAs against *F. graminearum* and monitoring the *F. graminearum* perithecia production (survival form) (Manstretta and Rossi, 2016). Three centimeters long straws were immersed in distilled water for 12 h, removed from the water, and autoclaved twice (121°C for 15 min). The sterilized straws were then dried in sterile filter paper, dipped in *F. graminearum* wT spore suspension (1x 10⁴ spores.mL⁻¹) for 2 min and were placed in center of Petri dish with small water-soaked sterile filter discs on the periphery. After two days, BCAs spore suspensions or Control were distributed along each straw pieces. Plates were incubated in culture room at 22°C in dark with 80% of relative humidity for 3 weeks (Bioclimatic chamber ICH750 C, Memmert, Schwabach, Germany) to allow the interaction of microorganisms on residue. Eighteen replicates of each condition were made and perithecia from each condition were counted, then gently crushed between slide and lamella in order to observe their contents at microscopic scale, using a binocular loupe and a Zeiss PrimoStar optical microscope with Zeiss AxioCam ERc5s camera (Carl Zeiss Microscopy, Thornwood, NY, USA). Another method consisting to incubate wheat straws in sterile wet soil was tested to produce *F. graminearum* perithecia and gave similar results to the selected method but the counting of perithecia was difficult because of similar soil color.

2.4. Data treatment and statistical analysis

With the aim of considering complete growth and mycotoxin production kinetics, and not only a final point, the area under disease progression and mycotoxin production curves were calculated. Statistical data analysis was performed with R Software (3.4.4, R Foundation for Statistical Computing, Vienna, Austria, 2017). Normality and homogeneity of variances were verified and the effect of BCA treatments or difference between conditions was tested with a one-way ANOVA and multiple comparisons of means were done with Tukey's test ($\alpha= 0.05$).

3. Results

Results of antagonistic activities of BCAs against *F. graminearum* was presented following keys stages of interaction between *F. graminearum* and wheat life cycle.

3.1. Wheat spikelet colonization by microorganisms

First of all, detached spikelets were inoculated with each microorganism separately. After 8 days of incubation, spikelets were dissected and internal tissues were observed to verify the endophytes capacities of pathogen and BCAs. In Figure 2, optical microscope images shown the different constituent parts of wheat spikelet (Awn, lemma, palea, anther, ovary) and colonization of microorganisms *in situ*. *F. graminearum* (**G**), rapidly colonize the palea/lemma and was able to penetrate the tissue via stomata and to produce large quantities of conidia. All three BCAs were able to colonize the internal tissues of the spikelet. Myco (**M**) colonized the epidermis of the tissues and produced numerous small pellet colonies. A characteristic white spore layer was produced on the surface. Xeda (**X**), invaded the tissues with aerial mycelium. It used damaged areas at the edge of palea tissues, or more fragile tissues such as the ovary inside the spikelet to produce sporulating colonies. Poly (**P**) was also able to settle and colonize the palea or base of the lemma awn and quickly produced a large quantity of characteristic oogonia containing oospores of sexual reproduction. None of the BCAs inoculated alone caused any apparent symptoms on the spikelets (no loss of chlorophyll, necrosis or desiccation) as compared to those inoculated with *F. graminearum* alone.

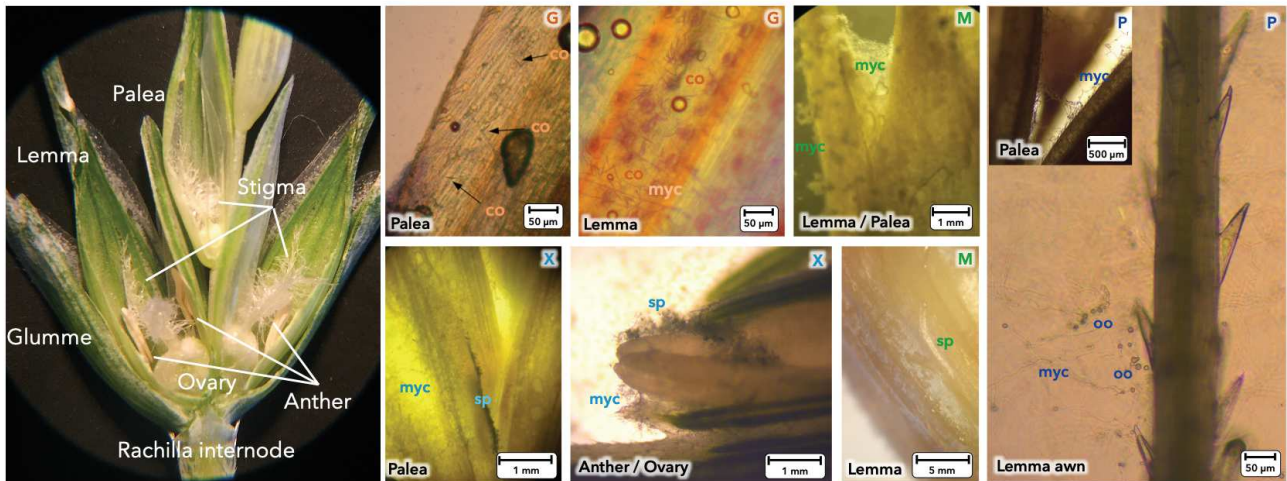


Figure 7 : Establishment of the different microorganisms on intern tissues of wheat spikelet. Bold letters: **G**, *F. graminearum* (orange); **M**: Mycostop® (green); **X**: Xedavir® (turquoise blue); **P**: Polyversum® (dark blue). Lower case letters indicate the different part of microorganisms: myc: mycelium, sp: sporulation, co: *F. graminearum* conidia, oo: Poly oogonia. Optical microscope images were taken on spikelets with only the pathogen or BCAs after 8 days of growth on spikelet at 20°C with photoperiod.

3.2. Inhibition of *F. graminearum* external colonization during antagonist assays in spikelet

Antagonist bioassays between *F. graminearum* and the BCAs were performed on detached spikelets and the external colonization of spikelets by *F. graminearum* was assessed using a scoring scale (from 0 to 5). Two clearly separate profiles of *F. graminearum* growth were observed between positive control with the pathogen alone and during BCAs treatment (Figure 3). *F. graminearum* alone clearly invaded spikelets tissues and produced high quantity of mycelium, pigment and spores. BCAs treatment provoked two significant important levels of inhibition with 77% and 93% of reduction of *F. graminearum* growth by Poly and Myco respectively. Xeda provided intermediate protection with 82% of *F. graminearum* growth inhibition. Over 12 days, the wT negative control spikelets started to dry out, making it impossible to conduct the experiment on alive spikelets any longer.

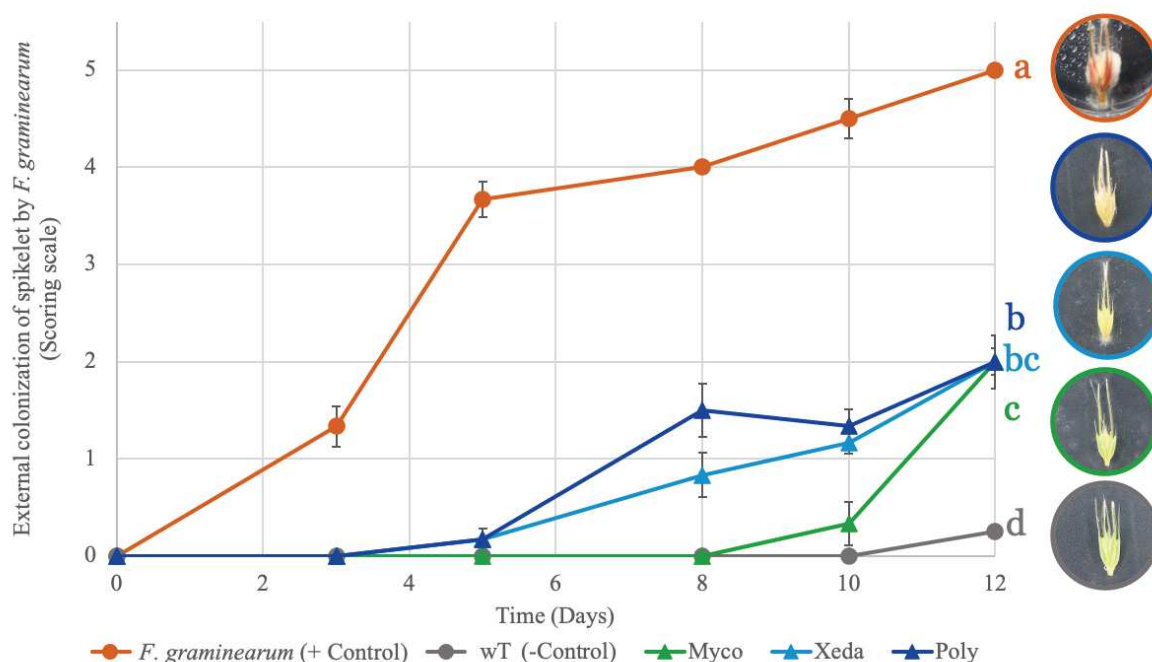


Figure 8 : Comparative effects of BCA treatments on the external colonization of spikelets by *F. graminearum* during antagonist bioassay. Colors of curve and photography outline correspond to different treatment: Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum® in confrontation with *F. graminearum*. The pictures illustrate the level of contamination after 10 days of growth of pathogen and BCAs. Antagonist bioassays were performed on detached spikelet for 12 days at 20°C with photoperiod. External colonization was expressed using scoring scale (from level 0, assigned to no symptom of disease to level 5 for spikelets strongly infected with mycelium, necrosis and possible presence of sporodochia), described in Experimental section (modified from Comby et al). ANOVA test were conducted on area under growth curves, p-value < 0.05.

3.3. Quantification of *F. graminearum* inhibition and Xeda-pathogen interaction in internal wheat spikelet tissues.

To complete the external assessment of *F. graminearum* on detached spikelets, absolute quantification of this fungi was made in all confrontation conditions via ddPCR. After 8 days of growth, the quantity of *F. graminearum* biomarker copies (*Tri5*) was assessed, and important inhibition caused by BCAs treatment could be observed (Figure 4). All BCAs triggered a quasi-complete disappearance of pathogen, similar to wT control, with 99.96%, 99.78% and 94.81% of DNA copy detected respectively for Myco, Xeda and Poly treatment. Xeda presented an interesting inhibition profile : intermediate on visual growth scale, to detect the pathogen also and total inhibition of mycotoxins, which is a definite advantage in the potential valorization of the cereal production. A focus was thus realized on *F. graminearum*-Xeda interaction. Quantity of biomarker copies of Xeda (*rpb2*) was analyzed on detached spikelets inoculated only with one of each microorganism and in *F. graminearum*-Xeda interaction. In the control conditions no non-specific amplification was detected

with the two biomarkers used. This BCA can colonize the spikelet up to 20% of the colonization of the pathogen alone in comparison, without completely invading it. Xeda biomarker and *F. graminearum* biomarkers were present in statistically equal amounts in spikelets treated only with Xeda and in spikelets inoculated with Xeda and *F. graminearum*, because the amount of pathogen in confrontation is so low compared to the control with only pathogen (99.78% of growth inhibition), illustrating the effectiveness of colonization and inhibition of this BCA.

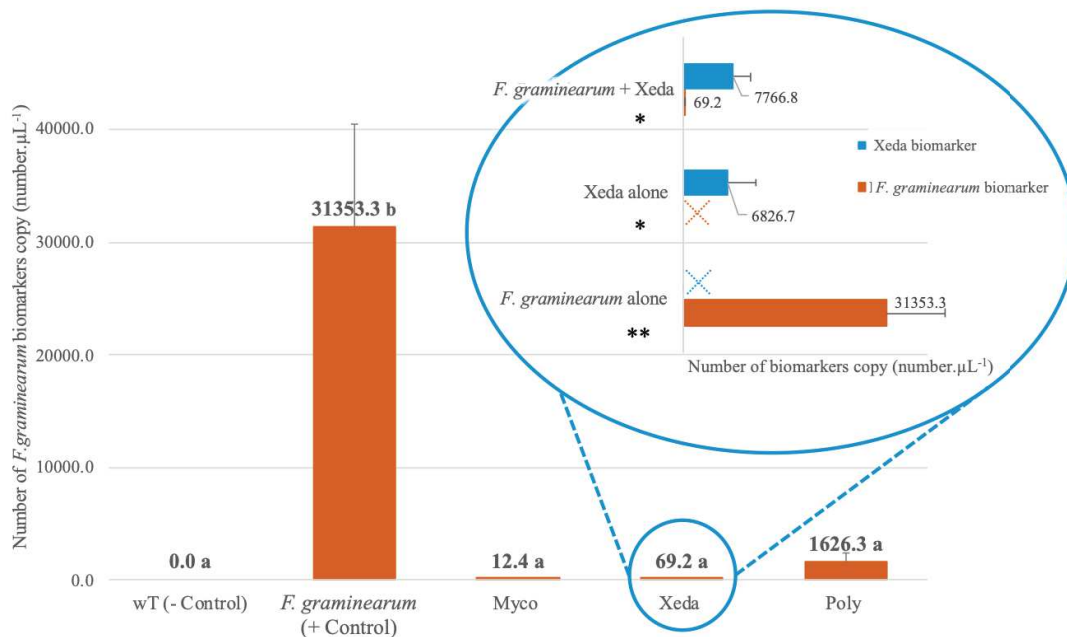


Figure 9 : Comparative quantification of *F. graminearum* and Xeda biomarkers on detached spikelet. Antagonist bioassays were performed on detached spikelet at 20°C with photoperiod, and analyzed after 8 days of co-culture. Main graphic presented quantity of *F. graminearum* biomarkers in number of copy.µL⁻¹ in presence of different BCAs treatment. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum®. ANOVA test, p-value < 0.01.

A focus was realized on *F. graminearum*-Xeda interaction in blue circle: quantity of *F. graminearum* biomarkers copy and Xeda biomarkers in number of copy.µL⁻¹ was compared alone or in confrontation on detached spikelet.

3.4. Inhibition of DON production by *F. graminearum* during antagonist assay in spikelet

A method for extraction and quantification of DON produced by *F. graminearum* in unitary wheat spikelets samples were optimized and used to monitor the evolution of mycotoxins production during the antagonist bioassays with BCAs. Results presented in Figure 5 shows a bell-like evolution with a detection of DON as early as 3 days on infection and peak of mycotoxin production between 5 and 8 days on *F. graminearum* positive control samples. This peak production is completely avoided in samples treated separately by the three types of BCAs (p-value<0,01), which showed total inhibition

of DON production until day 8. Xeda, in particular, maintains this inhibition throughout the observed kinetics.

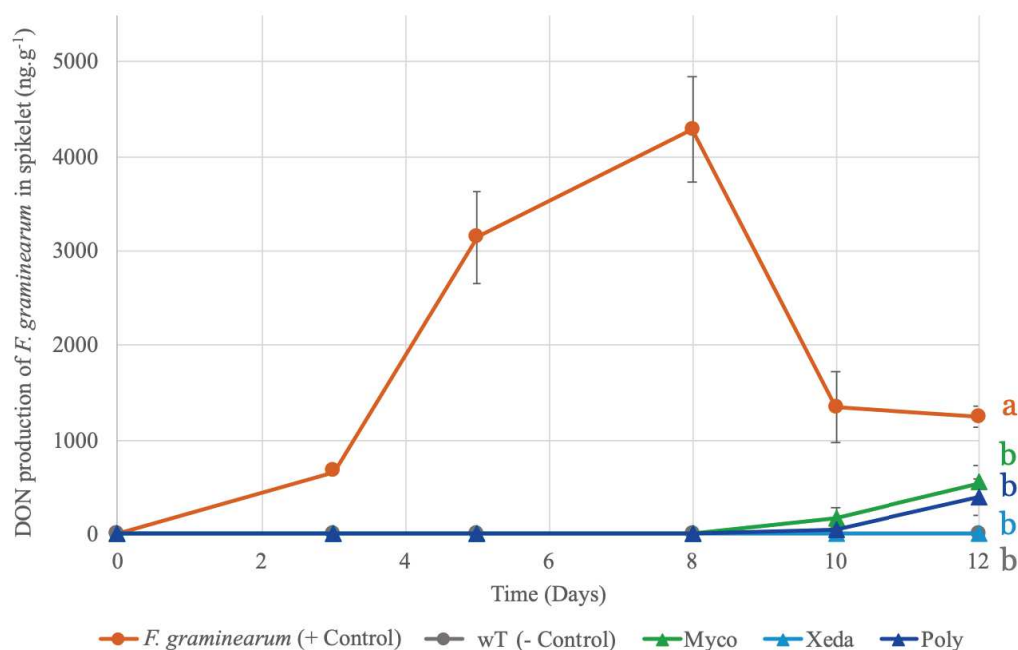


Figure 10 : Comparative effects of BCA treatments on DON production of *F. graminearum* in spikelet during antagonistic bioassay. Colors of curve correspond to different treatment: Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum® in confrontation with *F. graminearum*. Antagonistic bioassays were performed on detached spikelet for 12 days at 20°C with photoperiod. DON production was expressed in ng.g^{-1} of spikelet. ANOVA test were conducted on area under mycotoxin production curves, p -value < 0.01.

3.5. BCAs impact on perithecia synthesis and quantity on wheat straw.

A selected method used for antagonist assays on wheat straws permits to decipher the impact of BCAs on production of perithecia, the survival form of *F. graminearum* during inter-season. After three weeks of incubation with high humidity, *F. graminearum* formed many dark perithecia (ten per wheat straw on average, but which can be up to 30 perithecia per wheat straw) as it can be seen in Figure 6. On the contrary, the pieces treated with BCAs presented a very strong inhibition of perithecia formation by the pathogen. Xeda caused the strongest effect with almost 99% reduction, and Myco and Poly resulted in an average 90% reduction in the quantity of perithecia. In spite of this strong inhibition, some samples treated with Poly could nevertheless allow the development of these conservation structures with up to 14 perithecia at most.

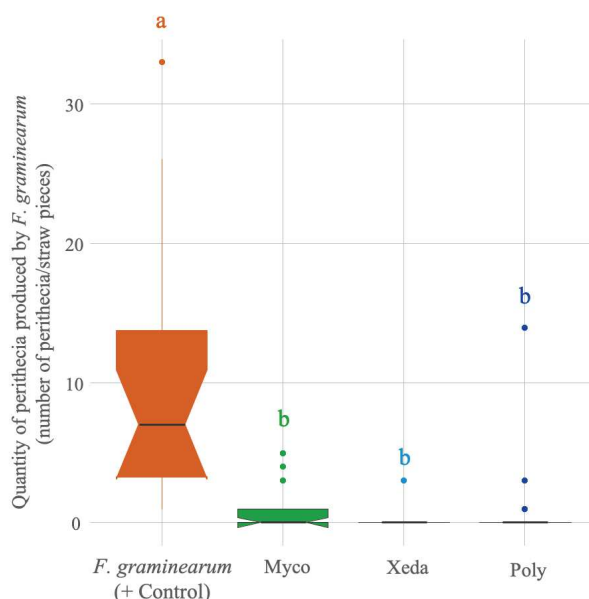


Figure 11 : Comparative effect of BCAs on perithecia production by *F. graminearum* during antagonistic bioassay on wheat straw. Myco: Mycostop®, Xeda: Xedavir®, Poly: Polyversum® in confrontation with *F. graminearum*. Antagonistic bioassays were performed on wheat straw for 3 weeks at 20°C in dark. Boxplot represents the distribution of the 18 replicates per conditions, ANOVA test, p-value < 0.05.

Once the perithecia were counted, the wheat straw was observed under the microscope at different scale and photographed (*in situ* or on microscope slide), in order to highlight particular modes of action of the BCAs when interacting with the pathogen perithecia (Figure 7). At first scale (3-1 cm), perithecia appeared on all positive control samples with only *F. graminearum*. Important quantity of characteristic colonies of Myco and Xeda were spread along the pieces of wheat. Poly presented very little visible mycelial growth and only few perithecia produced by *F. graminearum* compared to control. Increasing the magnification to the scale of the perithecia (1-0.5 cm), the control samples showed both developing and mature perithecia. In confrontation with Myco, the BCA colonies developed in contact with emerging perithecia and the few mature perithecia was reduced in size. In dual bioassay against Xeda, perithecia in formation are covered with Xeda sporulating mycelium. Some mature perithecia were directly parasitized by BCA colonies. The perithecia formed during the interaction between *F. graminearum* and Poly all showed abnormalities such as early excretion of ascospores in cirrus, external wall deformations and droplet exudations. The perithecia of the control samples contained large quantities of ascospores with a particular star-shaped organization where each branch was composed of imbricate ascospores. This spore cluster conformation could not be observed on any perithecia that had been treated with BCAs. Myco and Xeda prevented rigidification of parts of the perithecia wall (lighter areas), and very few ascospores of the pathogen could be observed when the perithecia were burst. Colonies of these BCAs could be observed on the perithecia fragments, confirming the presence of BCAs inside perithecia. Samples treated with Myco also

showed the presence of conidia of *F. graminearum*. Xeda produced many visible spores when observed close to the perithecia residues. In a completely different way, the perithecia deformed by the action of Poly produced very numerous disorganized ascospores in a mucilage inside the perithecia. These ascospores were very small compared to those produced in the controls (respectively 15 and 25 μm on average).

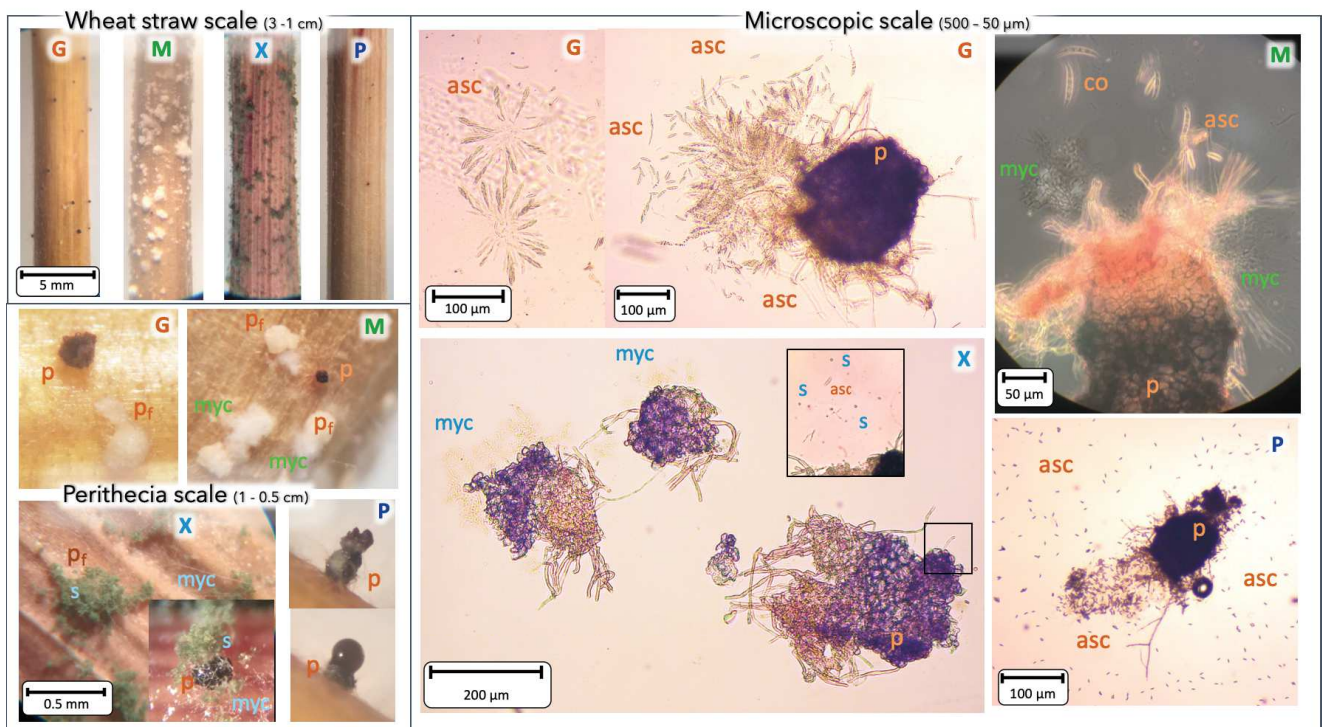


Figure 12: Macroscopic and microscopic BCAs impact on perithecia produced by *F. graminearum*. Pictures show the interaction between *F. graminearum* perithecia and the three BCAs at different level *in situ* or on microscope slide. Bold letters: **G**, *F. graminearum* (orange); **M**: Mycostop® (green); **X**: Xedavir® (turquoise blue); **P**: Polyversum® (dark blue). Lower case letters indicate the different part of microorganisms: myc: mycelium, sp: sporulation, asc: *F. graminearum* ascospores, co: *F. graminearum* conidia, pf: perithecia in formation, p: perithecia. Optical microscope images were taken after 3 weeks of incubation on wheat straw at 20°C.

4. Discussion

Experiments characterizing the impact of BCAs on *F. graminearum* have been carried out through the phases of the natural interaction between the life cycles of wheat and the pathogen (establishment, colonization and survival of pathogen). This chronological continuity allows to propose ways to improve the use of BCAs and to identify the best application stages during field epidemic (Beck et al., 2014) to make them more competitive than conventional agrochemicals. In most screenings for new BCAs, few differences can be observed between potential candidates of the same

species, hence the interest of studying BCAs of different types to observe a maximum of phenotype (Luongo et al., 2005; Schisler et al., 2006). The antagonistic bioassays on detached spikelets were analyzed at different levels (visual notation, DNA quantification and mycotoxin production) which provided a more accurate vision of the events occurring during the *in-situ* interaction, while optimizing the cost, time and space required. The use of spikelets compared to the whole plant allows to test a large quantity of biotic and abiotic conditions from a small area of wheat growing, while approaching the natural matrices on which the interaction takes place in the field. This step can help identify the most promising BCA candidates prior to the whole plant study. Indeed, during the study of the screening of different BCAs against *F. graminearum* on detached spikelet, a divergence was observed between the results obtained during the duals culture tests on synthetic medium and thus obtained on spikelets (Comby et al., 2017) while correlations could be observed between tests on detached spikelet and greenhouse assays on whole plants in other study (Rojas et al., 2020).

These kinds of results on effects of BCAs in dual culture assay *in vitro* or *in situ*, can also be compared in our study. Indeed, the BCAs studied were analyzed in terms of growth reduction and mycotoxin production in dual culture assays on synthetic media during a previous study (Pellan et al., 2020c) and significant differences were observed with the results obtained *in situ* on detached spikelets. Concerning the pathogen, the global mycotoxin maximum level was reduced by one third in plant tissues and the chemotype has completely switched from 15-ADON produced mainly *in vitro* with a small amount of DON, to exclusively DON produced on spikelets. Usually in field studies, DON is the most common (Chen et al., 2014; Siou et al., 2014), which underlines the importance of identifying appropriate tests, closer to natural infection conditions for a reliable interpretation of the mode of interaction. Regarding the antagonistic activities of BCAs on *F. graminearum*, they have all been underestimated on *in vitro* dual assays to varying degrees. Myco which had a very low level of inhibition of *F. graminearum in vitro* (23% and 15% of growth and mycotoxin inhibition respectively) showed a strong inhibition in detached spikelet (93 and 98% of visual growth and mycotoxin inhibition respectively). With intermediate profile, Xeda allowed 82% and 100% inhibition of visual growth and mycotoxin inhibition respectively *in situ* versus 48% and 72% of visual growth and mycotoxin inhibition respectively in dual culture assays. Finally, *F. graminearum* inhibition caused by Poly on detached spikelet was best estimated by analysis in classic dual culture assays with 77% and 99% of visual growth and mycotoxin inhibition in detached spikelet against 79% and 93%). This difference between the two types of tests were similar that which already highlighted in a study of (Comby et al., 2017). Special attention has been paid to keep the spikelet healthy to replicate the natural conditions as closely as possible. In the first days of colonization of the spikelets by the pathogen, limited mycelium was visible but discoloration of the spikelet tissues began to appear, suggesting that the pathogen was in the biotrophic phase and was colonizing the tissues without apparent classical symptoms. The exponential mycotoxin production at the beginning of analysis (<5 days) tended to decrease as the pathogen entered the saprophytic phase, while the mycelium developed abundantly. These results

are consistent with a comparative study of the colonization of *F. graminearum* on live and dead wheat ears (Boedi et al., 2016). One of the conclusions of the same study indicates that despite only a limited number of in planta- expressed genes of the pathogen require the living plant for induction but the majority uses simply the plant tissue as signal. Therefore, even if senescence processes occur after detachment of the spikelet from the ear, confrontation on plant tissue already allows to get closer to real interaction than *in vitro* duals culture assays.

Concerning the test of microorganism establishment and growth on spikelets, good tissue colonization was observed and no BCAs caused symptoms when inoculated alone on the spikelets. Xeda mainly accumulates on already weakened areas, suggesting symbiotic or saprophytic activity (Vargas et al., 2009). Several *Trichoderma spp.* have already shown features including opportunistic and avirulent plant symbionts that cause substantial changes in host metabolism, favoring growth and disease resistance (Hermosa et al., 2012). Nevertheless, this observation method (tissue dissection) allow to suspect but not to guarantee the endophytic character of the BCAs tested and would require further disinfection of the spikelets before microscopic evaluation.

The assessment of *F. graminearum* colonization in plant tissues is frequently characterized using a scoring scale (Bekalu et al., 2020; Li et al., 2011; Váňová et al., 2001). Correlation was found between reduced symptom expression and reduced biomass of *F. graminearum* (Rojas et al., 2020). In our study, all BCAs showed a significant reduction in the development of *F. graminearum* infection, assessed using visual scoring scale. Xeda and Myco that had particularly developed on spikelets, could be good competitor against pathogens. Indeed, these two BCAs compared to *F. graminearum* alone showed good nutritional competition capacity on synthetic medium, and Xeda only, good space competition capacity in previous study (Pellan et al., 2020a). It is therefore relevant to point out that Myco is also spatially competitive on spikelets. Nevertheless, visual distinction between BCA colonization and pathogen may be difficult depending on the BCA considered. Moreover, describing the infection only by extern visual scoring of the spikelets does not allow to know precisely the internal development of the pathogen, even by adapting the scoring scale by taking into account the signs of early colonization by this pathogen (level 1: discoloration of wheat tissues). To overcome this problem, an innovative method for the detection and quantification of microorganisms, via ddPCR, has been developed. This technique has already been successfully used to analyze the presence of other pathogens in plant tissues (Gutiérrez-Aguirre et al., 2015) and has the advantage to be a highly precise method for sensitive DNA detection and absolute quantification. Moreover, ddPCR is less susceptible than qPCR to substances that inhibit PCR reactions and favorize reproducibility and specificity of assays (Khurana and Gaur, 2019). This method can therefore allow a specific and early detection of the pathogen, confirming a definite advantage in the case of pathogens with similar symptoms or which appear late in the colonization process, such as *F. graminearum*. To our knowledge, this is the first report of *F. graminearum* quantification on wheat via this method. By

comparing the results of visual scores and quantification, similar trends can be observed (same order of effectiveness of the three BCAs: Control > Poly > Xeda > Myco). However, external observation alone overestimated the relative presence of *F. graminearum* in the samples treated with BCAs compared to the control (84% versus 98% inhibition on average recorded during visual scoring or absolute quantification of *F. graminearum*). In previous tests, Xeda caught our attention because it showed strong inhibition of pathogen growth (but not complete allowing potential pathogen detection), and total inhibition of mycotoxin production. Xeda biomarker was also detected. This is why a focus was made on the interaction between this BCA and the pathogen, and this assay demonstrated that Xeda was able to colonize spikelet but also to drastically reduce the presence of *F. graminearum*. Surprisingly, its growth capacity does not seem to be affected by the presence of *F. graminearum* during interaction, which contributes to its efficiency during field epidemics (Rojas et al., 2020). This innovative method of detection (ddPCR coupled with specific primers) permits a precise quantification of pathogen and BCAs co-inoculated on wheat tissues, and highlights competitive modes of action. It opens an interesting way to future studies on BCAs colonization and biocontrol competition against phytopathogens. Biomarkers specific to other BCAs could be used to confirm these conclusions.

This pathogen growth analysis can be compared to mycotoxin production results to characterize an overall protective effect of BCAs. The method developed for the precise quantification of mycotoxins allows to observe significant differences between the evolution of visual and/or absolute growth (ddPCR) and mycotoxin production. This toxin is mainly produced during the primary phase of the biotrophic mode of pathogen (Brown et al., 2017), which is coherent with our results. All the BCAs studied were very effective in the reduction of mycotoxins extracted in detached spikelets in this first phase (<5 days) and even beyond (<10 days). In particular Xeda which induced a complete inhibition of the DON produced by *F. graminearum*. This reduction corresponded to an inactivation of pathogen system attack. It may be due to many mechanisms such as inhibition of pathogen growth, biotransformation of the mycotoxins produced (Venkatesh and Keller, 2019) or epigenetic changes caused by BCAs that prevent pathogenic Fusarium pathogens by inhibiting the expression of genes of mycotoxin biosynthesis pathways (Chen et al., 2018), but these activities remain complex. The small increase of mycotoxins at the end of the kinetics can be explained by a lower activity of BCAs during the saprophytic phase of the pathogen, but the presence of BCAs has greatly reduced the amount of toxins detected below the limit recommended by the European Union for DON in unprocessed durum wheat: 1750 $\mu\text{g.kg}^{-1}$ (Commission Regulation (EC) No 1881/2006, (Kyprianou, 2006)).

During this vegetative phase, all BCAs demonstrate potentialities for the reduction of colonization and mycotoxins production of *F. graminearum*. As suggested by the analyses on the modes of action of these BCAs *in vitro*, competition, mycophagy or mycotoxin bio-transformation phenomena could be responsible for the success of these BCAs. The mechanisms implemented by these BCAs during *in*

vitro tests (Pellan et al., 2020b, 2020a) could be investigated more precisely in order to prove their deployment in natural conditions.

Turning now to the conservation phase of the pathogen, it is well described that conservation structures, such as perithecia, preserved in culture debris are the primary source of inoculum for future epidemics (Gilbert and Fernando, 2004). The activity of BCAs against perithecia of *F. graminearum* was evaluated. These results are consistent with those obtained *in vitro* on Carrot agar in another study (80% inhibition on average, (Pellan et al., 2020a), not published). All BCAs showed a substantial reduction in the number of mature perithecia. However, the mechanisms involved in this reduction seem to vary according to the BCA considered. Indeed, Myco and Xeda seemed to enter directly into spatial competition with *F. graminearum* and parasitize the perithecia, causing a weakening of the structure of the perithecia and a strong reduction in the quantity of ascospores. This effect may be due to the direct synthesis of antimicrobial compounds that will interact with the formation of these structures (Inch et al., 2011). On the contrary, Poly will allow more perithecia to reach maturity, and will stimulate the production of ascospores with atrophied structure.

5. Conclusion and perspectives

Based on this integrative analysis, all the commercial BCAs showed effective reduction capacities in both phases of the *F. graminearum* cycle (vegetative and survival stages), whereas their initial use is limited to aerial parts (Poly) or for general soil treatments (Myco and Xeda). Future efforts to improve BCAs disease control on wheat spikes and in the phyllosphere of various plants should focus on application timing, which could be extended throughout the pathogen cycle in case of commercial BCAs or tested on this different stage for new potential BCAs.

BCAs are able to act at different levels of the pathogen's life cycle but also through different mechanisms such as the reduction of toxins, nutritional and/or spatial competition, or production of anti-microbial compounds. They are therefore an effective means of limiting the use of pesticides through integrated management strategies, while maintaining a level of food safety of production. The proposed model on spikelets allows to have quickly and easily answers on the mode of interaction of microorganisms closer to real situations thanks to this complex matrix, and ddPCR method is a rapid and reliable solution to quantify the pathogen and BCAs development. The developed protocols can be used to observe other patho-systems, only with the pathogen for the assessment of resistant varieties or abiotic factors, or in biocontrol studies with pathogen and other agents or natural substances.

Author contributions: L.P., C.S. and S.S.-G. designed the study. C.A.T.D. participated in development of detached spikelet experiments. N.D. performed mycotoxin analysis via HPLC MS-MS. L.P. carried out the entire range of manipulations and experimental design approaches (except

ddPCR) and analyzed the data. L.P., C.S. and S.S.-G. interpreted the data. L.P. wrote the original draft. C.S. and S.S.-G. carefully supervised and reviewed the paper. All authors have read and agreed to the published version of the manuscript.

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Discussion générale et Conclusion

4. DISCUSSION GENERALE ET CONCLUSION

Après avoir abordé le contexte de ces travaux lors de la synthèse bibliographique, il apparaissait que la gestion des mycotoxines et de leurs producteurs de manière intégrée dès leur apparition en pré-récolte, grâce notamment au biocontrôle, était un enjeu important pour maintenir la sécurité alimentaire et sanitaire des populations tout en préservant les agrosystèmes. En effet, ces micro-organismes bénéfiques non-pathogènes sont capables d'avoir une activité antagoniste afin de limiter ou arrêter la progression du pathogène, et ces inhibitions sont provoquées par l'activation de tout un arsenal de défenses qu'ont à leur disposition certains BCAs, comme la production de composés antimicrobiens, le parasitisme, la compétition ou la détoxification. Malgré une abondante littérature sur le screening, l'activité antagoniste et les potentiels modes d'action des BCAs, peu de produits sont commercialisés en France et de nombreuses incertitudes persistent quant à leurs efficacités contre les pathogène du genre *Fusarium* et leurs impacts sur la production de mycotoxines mais également quant à certains de leurs mécanismes d'action connus.

Les mécanismes biologiques à l'origine du biocontrôle sont complexes : directs ou indirects ; combinés ou seuls ; caractéristiques d'un BCA ou spécifiques d'une espèce pathogène ou d'un stade d'évolution. Pour pouvoir mettre en place des interactions entre micro-organismes bénéfiques et pathogènes mycotoxinogènes efficaces lors d'application au champ, il est essentiel d'évaluer leurs impacts sur la production de mycotoxines par les *Fusarium*, et de mieux comprendre quels sont les mécanismes d'action déployés par les agents de biocontrôle (BCAs) pour inhiber les traits de virulence de ces pathogènes.

C'est donc avec ces informations en tête que les objectifs de la thèse ont été définis :

- (i) Évaluer les effets d'Agents de Biocontrôle sélectionnés sur la croissance et la production de mycotoxines des espèces pathogène du genre *Fusarium* en conditions contrôlées.
- (ii) Décrypter les mécanismes d'actions potentiels des BCAs à l'origine des inhibitions observées, grâce à la mise au point et la réalisation de tests spécifiques, à l'identification et la quantification de certains modes d'action, mais aussi par leur chronologie dans la cinétique d'interaction BCA-pathogènes.
- (iii) Transposer les confrontations *in situ* et mettre en relation les effets *in vitro* et *in situ* des BCAs sur *F. graminearum*

Pour répondre à ces objectifs, des souches de *Fusariums*, fortement productrices de mycotoxines *in vitro* et *in situ*, et trois BCAs commercialisés, de type et d'usage variés, ont été sélectionnés comme micro-organismes modèles pour optimiser l'observation et la caractérisation de phénomènes variés et représentatifs des interactions de biocontrôle potentielles contre les *Fusaria* mycotoxinogènes.

Dans un premier temps, des milieux de culture adaptés ont été soigneusement sélectionnés pour optimiser les conditions de confrontation des BCAs et pathogènes. C'est un point important qui est rarement pris en compte dans les études sur la gestion des pathogènes producteurs de mycotoxines, et qui a permis de sélectionner des milieux de culture qui favorisent la production des mycotoxines de chaque pathogène, afin d'identifier les BCAs qui ont un impact considérable même en cas de forte production. De plus, la toxigenèse des pathogènes est un phénomène complexe et instable, ce qui souligne l'importance d'une conservation optimale des pathogènes et la nécessité d'avoir identifié, calibré et uniformisé ou adapté au maximum l'ensemble des paramètres communs aux différentes expérimentations (production, concentration et quantité des inocula, quantité de milieux de culture, condition d'incubation, condition d'extraction et d'analyses des mycotoxines, etc.)

Les premières analyses réalisées sur les organismes seuls ont également permis de mettre en évidence d'importantes différences entre les comportements des deux *Fusarium*, qui vont être confirmées tout au long de l'étude, et qui nous ont poussé à considérer les analyses de données séparément. En effet, ils proviennent de différents environnements (*F. graminearum* a été isolé d'épis de blé tandis que *F. verticillioides* a été isolé de grains de maïs) ; ils produisent des types distincts de mycotoxines et leurs niveaux de production *in vitro* sont également différents. Grâce à l'étude des cinétiques de mycotoxinogénèse sur différents milieux de cultures, *F. graminearum* produit des trichothécènes (DON et 15-ADON) à hauteur de 15 000 $\mu\text{g.kg}^{-1}$ de milieux en moyenne, et *F. verticillioides* produit des fumonisines (FB1+FB2) à hauteur de 5 000 $\mu\text{g.kg}^{-1}$ de milieux en moyenne. Leurs stratégies de colonisation sont également différentes : *F. graminearum* a une croissance rapide mais produit lentement et peu de spores, contrairement à *F. verticillioides* avec une expansion plus lente, mais compensé par une production rapide et importante de spores. Ces éléments peuvent aussi expliquer le caractère spécifique de certains BCAs ou mode d'action contre l'un ou l'autre des pathogènes.

D'un point de vue méthodologique, la caractérisation initiale des confrontations *in vitro* directes des pathogènes et BCAs sur différents milieux de cultures a permis d'identifier des effets d'inhibition contrastées sur la croissance et la production de mycotoxines des souches de *Fusarium* en fonction des agents de biocontrôle. Cette approche a permis de réaliser des macro et micro-observations des zones de contact entre les deux types de micro-organismes en confrontation, mais aussi d'émettre des hypothèses quant au type de mécanismes mis en jeu lors des interactions.

La deuxième partie de l'étude s'est donc concentrée sur la confirmation des premières hypothèses et l'élucidation des mécanismes mis en jeu lors des inhibitions observées *in vitro*, mais aussi sur les aspects de spécialisation et la potentielle chronologie des événements durant les premières phases puis les phases plus tardives de l'interaction. Les mécanismes ont donc été recherchés pendant toute la durée du cycle de vie des pathogènes (de la germination des spores à la survie), et permet de mieux localiser spatialement et temporellement les moments clés de la relation antagoniste. Les modes d'actions suivants ont été recherché chez les BCAs : (1) inhibition de la germination des

spores pathogènes, (2) mycophagie, (3) synthèse de chitinase, (4) émission de composés volatiles antimicrobiens, (5) inhibition de la synthèse des mycotoxines, (6) biotransformation des mycotoxines produites, (7) compétition nutritionnelle et (8) spatiale, (9) impact sur la production des périthèces. Cette étude nous a permis à la fois de rassembler ou designer des protocoles d'expérimentations fiables et pertinents pour identifier les modes d'action des BCAs contre les champignons mycotoxinogènes, mais aussi de s'en servir pour mettre en évidence ces comportements divergents chez les BCAs sélectionnés.

D'autre part, afin de comparer les effets et mécanismes des BCAs identifiés *in vitro* et ceux qui apparaissent lors du contact des micro-organismes sur la plante, des expériences ont été menées au niveau des différentes phases de l'interaction naturelle entre les cycles de vie du blé et de l'agent pathogène *F. graminearum* (établissement, colonisation et survie de l'agent pathogène) sur des organes détachés *in situ*. Des essais d'antagonismes sur les épis détachés ont été analysés à différents niveaux (notation visuelle, quantification d'ADN par ddPCR et teneur en mycotoxines), ce qui a permis d'obtenir une vision plus précise des événements se produisant lors de l'interaction *in situ*, tout en optimisant le coût, le temps et l'espace requis. Des essais ont également été réalisés sur paille de blé séché pour mesurer l'impact des BCAs sur le nombre et le contenu des périthèces formés pendant la phase de conservation du pathogène.

Le premier BCA, *S. griseoviridis* ou Myco a d'abord montré de faible capacité d'inhibition des deux pathogènes lors des confrontations *in vitro* avec seulement 24% de réduction de croissance en moyenne et un faible potentiel sur la réduction de mycotoxines avec 10 à 20% de réduction des fumonisines de *F. verticillioides* et quasiment aucune réduction voire une stimulation des trichothécènes (DON et 15-ADON) pour *F. graminearum*, qui pouvait être encouragée par l'observation de vésicules à l'extrémité des hyphes du pathogène à proximité de ce BCA. Néanmoins la présence de halos d'inhibition de croissance avant contact et l'observation d'arrêt brutal de la progression ou de la germination de *F. verticillioides* suggérait la diffusion de composés antimicrobiens.

En effet, lors de son passage au travers de la toolbox d'identification des mécanismes, un des premiers mécanismes mis en place par Myco est la diminution de la germination des spores des pathogènes, notamment *F. verticillioides*, que ce soit sous forme de spores mais surtout sous forme d'extrait de culture (39% et 78% de réduction du pourcentage de germination respectivement). Le fait que cet extrait de culture sans pathogène actif ait un effet souligne la présence de composés antifongiques excrétés par le BCAs. *F. verticillioides* va également être très sensible à l'activité mycophage de Myco et ce dès le début de l'interaction dans le dispositif privant le BCA d'autres ressources. Lors de ce test, il a également stimulé la production de mycotoxines de *F. graminearum* ce qui conforte les premières inquiétudes soulevées lors des tests de confrontation initiaux et souligne l'importance de mesurer l'impact des BCAs sur la production de mycotoxines. Il a tout de même

montré d'autres capacités intéressantes qui restent dans le domaine de l'antibiose durant les premières phases de l'interaction. Même si son taux de croissance reste plus faible que les autres BCA, Myco produit rapidement différentes enzymes lytiques comme des endochitinases, et des exochitinases, qui avec différents modes d'action lui permettent une efficacité de dégradation maximale des parois qui est stimulée par la présence des pathogènes. Les composés antimicrobiens produits par Myco peuvent également être volatils, et les COVs produits par Myco ont eu un effet assez spécifique contre les mycotoxines produites par *F. verticillioides* avec 56% d'inhibition de la production spécifique des fumonisines. Myco fait également partie des BCAs capables de concurrencer les agents pathogènes en termes de nutriments, en particulier pour les sucres solubles rejetés par les plantes et qui sont également consommés par les agents pathogènes, suggérant un bon établissement dans le milieu naturel. Lors du test de compétition spatiale, Myco induit une nouvelle fois une inhibition de la croissance de *F. verticillioides* avant contact, ce qui confirme encore l'hypothèse de la diffusion de composés antimicrobiens abordée précédemment. A la fin du cycle de *F. graminearum*, Myco a également fortement réduit la formation de périthèces.

Comme indiqué précédemment le screening en boîte de Petri reste un outil accessible et intéressant dans les premières phases de caractérisation des BCAs mais on peut passer à côté d'organismes bénéfiques qui ne sont pas adaptés à la croissance sur milieu synthétique. Myco illustre bien cette problématique. Avec un très faible niveau d'inhibition de *F. graminearum in vitro*, il devient le BCA le plus efficace sur épis détachés *in situ* avec respectivement 93 et 98% d'inhibition de la croissance visuelle et de l'inhibition des mycotoxines du pathogène. Il présente de forte capacité de colonisation sur épis, ce qui, associé aux hypothèses émises lors du test *in vitro* de compétition nutritionnelle, indique qu'il pourrait entrer en compétition avec la moisissure mycotoxinogène pour des composés issus du blé. De manière plus surprenante, il est aussi compétitif en termes d'espace sur les matrices végétale. Il peut aussi réduire fortement la formation de périthèces sur pailles et en altérer le contenu en ascospores.

Le second BCA étudié, *Trichoderma asperellum* ou Xeda a lui aussi été d'abord évalué en confrontation directe avec les pathogènes et ses capacités d'inhibition semblaient déjà prometteuses. Il a pu réduire de 75% en moyenne la croissance et la production de mycotoxine globale de *F. verticillioides* mais a aussi été efficace contre *F. graminearum* avec 48 et 72 % d'inhibition de la croissance et de la production des mycotoxines. Cette importante réduction des mycotoxines, qui n'était pas expliquée uniquement par la réduction de croissance, pouvait suggérer que Xeda est capable de bloquer la synthèse ou de dégrader les trichothécènes, DON et 15-ADON. Avec de bonnes capacités de colonisation, il semble pouvoir rivaliser avec le développement des agents pathogènes. Lors du contact, il peut former une barrière mécanique qui bloque physiquement le développement des champignons pathogènes et il semble synthétiser des enzymes dégradant le mycélium pathogène. Ces hypothèses de parasitisme ont pu être explorées lors de l'analyse des

mécanismes mis en place par Xeda pour provoquer ces effets antagonistes, et de nombreux mode d'action ont pu être mis en évidence en présence de ce BCA. Xeda a provoqué une inhibition importante de la germination des spores pathogènes et notamment de *F. graminearum* (48 et 83% d'inhibition de germination pathogène en présence de spores ou d'extrait de culture du BCA respectivement). Bien que parfois la germination puisse avoir lieu, les tubes germinatifs restent atrophiés empêchant le développement du pathogène. *F. graminearum* est également plus sensible à une autre activité précoce du BCA, la mycophagie, qui n'a pas pu être détectée au contact de *F. verticillioïdes*. Durant ce test en conditions nutritionnelles très limitées, Xeda a pu maintenir l'inhibition des mycotoxines, suggérant qu'il possède des mécanismes dédiés à leur contrôle. L'hypothèse de synthèse de composés permettant une dégradation des parois des pathogènes abordée précédemment a pu être confirmée par une forte détection des activités exochitinasés de ce BCAs stimulées par la perception des pathogènes. Peu d'études ont discutées l'efficacité différentielle au sein de la famille des exochitinasés mais il semblerait que Xeda soit capable d'inverser fortement les rapports entre les deux exochitinasés étudiées au fil du temps. Il est également capable de produire des composés volatiles ayant des effets antifongiques (contre *F. verticillioïdes*) ou antimycotoxinogènes (contre les deux pathogènes). De plus, d'importantes décolorations du mycélium ont pu être observées soulignant l'impact de ces COVs sur la physiologie des pathogènes. L'analyse de cette piste prometteuse pourrait être complétée par l'identification des composés extraits et analysés par GC-MS, comme celle abordée dans la partie de résultats complémentaires. Aux vues des fortes capacités de réduction des mycotoxines par Xeda dans les précédents tests, la capacité de bio-transformation des mycotoxines au cours du temps semble être une piste intéressante, avec près de 55% de dégradation de la DON isolée.

De plus, ce mécanisme semble être à l'origine d'une proportion importante des inhibitions globales observées lors des confrontations directes, mais ce n'est pas le seul. Xeda est aussi capable d'agir directement sur les voies de biosynthèse des mycotoxines avec une réduction de près de 56% et 39% de l'expression des gènes de biosynthèse des trichothécènes et fumonisines respectivement. Concernant ces capacités de compétition, il montre des activités intéressantes de compétition pour les composés du blé ou du maïs, que ce soit en termes de types de composés assimilables par les deux types de micro-organismes ou de demande forte pour certains composés (comme le saccharose). De plus, il maintient un bon ratio de croissance en milieu non limitant, ce qui confirme son potentiel comme compétiteur spatial. Enfin, Xeda limite fortement le développement de périthèce pendant la phase de survie de *F. graminearum*, ce qui peut également être lié aux phénomènes de compétition abordés précédemment.

Lors des essais *in situ*, Xeda a augmenté ses capacités antagonistes avec 82% et 100% d'inhibition de la croissance et de la production de mycotoxines de *F. graminearum*. Il s'est particulièrement développé sur les épillets, et pourrait être un bon compétiteur contre les agents pathogènes, comme abordé *in vitro* au travers de ces aptitudes de consommation des nutriments et de colonisation

spatiale. De manière surprenante, sa capacité de croissance ne semble pas être affectée par la présence de *F. graminearum*, qu'il réduit drastiquement lors de l'interaction, ce qui peut contribuer à son efficacité lors des épidémies au champ. Xeda permet donc aussi une inhibition complète de la DON produite par *F. graminearum*. Cette réduction peut correspondre à la synergie de trois phénomènes identifiés chez Xeda : sa capacité à réduire la croissance du pathogène, à inhiber l'expression des gènes des voies de biosynthèse des mycotoxines et enfin à transformer ou détoxifier les mycotoxines produites. Au niveau de la conservation du pathogène, Xeda limite fortement le développement des périthèces de *F. graminearum* et est capable de parasiter et fragiliser la structure des périthèces formés.

Le troisième et dernier BCA considéré, *Pythium oligandrum* (Poly) a lui aussi permis d'inhiber la croissance et les mycotoxines des pathogènes avec une approche encore différente. Dans les tests préliminaires de confrontation, il a montré parmi les plus forts effets avec près de 53% et 86 % d'inhibitions de croissance et de production globale de mycotoxines en moyenne pour *F. verticillioides* et *F. graminearum* respectivement. L'observation macro et microscopique des interactions a permis de mettre en évidence une dépigmentation de *F. graminearum*, la capacité de Poly à coloniser rapidement les milieux de culture, voire les colonies pathogènes lors des confrontations, suggérant une interaction étroite dépendante du contact des pathogène et du BCA. Après le même temps d'incubation, par rapport à Xedavir, qui a une progression plus lente, le contact a eu lieu de manière assez subtile. Il semble que peu d'enzymes de dégradation des parois fongiques aient été synthétisées. De plus, une forte stimulation de la reproduction sexuée du BCA semble être liée à la présence des pathogènes.

Ces premières hypothèses ont pu être éclairées par les performances de Poly à travers les différents tests d'identification de ses mécanismes de biocontrôle. Bien qu'il n'ait montré aucune capacité contre la germination des spores pathogènes, sa grande capacité de colonisation lui a permis de détourner rapidement les nutriments des pathogènes lors du test de mycophagie. Il est capable d'interagir directement avec le mycélium vivant des pathogènes en l'absence d'autre ressource, ce qui rejoint les observations microscopiques de son interaction lors des confrontations sur lame avec *F. graminearum*. Poly a pu passer au travers du mycélium de l'agent pathogène et induire la formation de zones d'hyphes aux parois lysées. Elles pourraient être considérées comme des espaces privilégiés pour l'échantillonnage des nutriments par le BCA, ce qui suggère à nouveau une interaction de proximité comme le mycoparasitisme ou la prédation microbienne des deux pathogènes. Encore une fois la présence des pathogènes stimule fortement la reproduction de Poly, ce qui lui permet de maintenir l'avantage en termes de colonisation. Poly est aussi capable de produire toutes les chitinases testées, mais de manière constitutive à des niveaux relativement faibles, en présence ou non des pathogènes. Ces observations soutiennent les hypothèses émises lors de l'observation de connexions limitée entre Poly et les hyphes pathogènes dans un premier

temps. Il synthétise également des COVs qui peuvent réduire les concentrations de DON mais n'agissent pas sur la croissance. Ces éléments suggèrent que les composés synthétisés par Poly peuvent avoir une action inhibitrice directe sur la voie de biosynthèse des mycotoxines des agents pathogènes. L'analyse de l'expression des gènes liés à la production des mycotoxines chez les deux pathogènes a pu confirmer que Poly avait l'impact le plus fort sur l'expression des gènes, avec 85% d'inhibition en moyenne des gènes de biosynthèse des trichothécènes et de fumonisines respectivement. Cette piste apparaît comme le phénomène majeur de réduction de mycotoxines car les faibles quantités de mycotoxines biotransformées par le BCA ne peuvent être à l'origine que de faibles réductions. Dans le test de compétition spatiale, Poly ne permet pas l'inhibition des agents pathogènes avant le contact, ce qui soutient l'hypothèse de mécanismes directs préférentiels. Cependant, il a une très forte capacité de colonisation comparés aux pathogènes. Pour ce type de micro-organismes à croissance très rapide, le test de compétition spatiale pourrait être amélioré pour visualiser la colonisation des micro-organismes sur des matrices plus complexes en utilisant notamment des gènes rapporteurs (GUS, GFP, etc.) ou en quantifiant des biomarqueurs spécifiques par qPCR ou ddPCR par exemple. En intégrant l'impact du milieu de culture, Poly est le BCA le plus sensible au changement des ressources nutritionnelles. Néanmoins, il est intéressant de noter que la thréonine est un composé qui était consommé uniquement par Poly et ne favorise pas la croissance d'autres micro-organismes, y compris les deux pathogènes mycotoxinogènes. Ainsi, cet acide aminé pourrait être envisagé comme stimulateur de l'activité de ce BCA par des formules d'inoculation. Enfin, Poly limite fortement la formation de périthèces. C'est la première fois qu'il pourrait être envisagé pour le traitement des périthèces de *F. graminearum* dans le sol, ce qui ferait de lui un BCA adaptée à toutes les phases du cycle de vie de l'agent pathogène (bonne colonisation des parties aériennes et décontamination des résidus de culture).

Enfin, l'inhibition de *F. graminearum* causée par Poly sur organes détachés été la mieux estimée par les analyses de confrontation *in vitro*, avec 77% et 99% de réduction de croissance et de mycotoxines sur épillets détachés contre respectivement 79% et 93% d'inhibition lors des confrontation *in vitro*. Au contraire des autres BCAs, Poly permettra à davantage de périthèces d'atteindre la maturité, mais stimulera la production de grandes quantités d'ascospores atrophiées.

Les études *in vitro* réalisées semblent représenter des avancées importantes ainsi que des outils efficaces pour améliorer les stratégies de sélection et la compréhension des mécanismes d'action de divers BCAs. Les discussions à propos des effets des BCAs sélectionnés sur les agents pathogènes permettent de mettre en évidence diverses stratégies partiellement interconnectées, avec leurs avantages et leurs inconvénients : Le mycoparasitisme est plus spécifique, l'antibiose peut générer la production de composés nocifs pour les écosystèmes, et la compétition spatiale et nutritionnelle semble plus adaptée à l'implantation dans les milieux naturels. La dégradation peut être un mécanisme à double tranchant en fonction de la toxicité des sous-produits. Les meilleurs résultats

sont observés lorsqu'un seul BCA combine plusieurs modes d'action, et de nombreuses synergies ont pu être observées. La spécificité des BCAs sur les pathogènes est un aspect intéressant pour proposer des actions ciblées sans effet secondaire sur les communautés microbiennes déjà présentes dans les agrosystèmes.

D'autre part, le modèle d'étude proposé sur épis et pailles, permet d'avoir rapidement et facilement des réponses sur le mode d'interaction des micro-organismes, plus proches des situations réelles, et la méthode ddPCR est une solution rapide et précise pour quantifier le développement de l'agent pathogène et des BCAs. Tous les BCAs commerciaux ont montré des capacités de réduction efficaces dans les deux phases du cycle de *F. graminearum* (stades végétatifs et de survie). Comme le suggèrent les analyses et résultats sur les modes d'action de ces BCAs *in vitro*, les phénomènes de compétition, de mycophagie ou de biotransformation des mycotoxines pourraient être responsables du succès de ces BCAs en conditions naturelles. La continuité chronologique de l'étude tout au long du cycle pathogène permet aussi de proposer des moyens pour améliorer l'utilisation des BCAs et identifier les étapes d'application clés lors d'une épidémie au champ.

Perspectives

5. PERSPECTIVES DE POURSUITE DU PROJET

Cette étude permet également d'ouvrir de nombreuses perspectives quant aux voies d'amélioration possibles des interactions entre BCAs et souches mycotoxinogènes de *Fusarium*, mais aussi pour poursuivre les investigations sur les phénomènes d'antagonisme,

D'un point de vue fondamental, plusieurs perspectives peuvent être considérées, par exemple :

- Compléter ces approches avec des outils « omics » permettrait d'aller plus loin sur l'identification et la caractérisation des gènes et métabolites à l'origine des modes d'action des BCAs. Avec le large tableau de mécanismes spécifiques décrit expérimentalement, la mise en relation des familles de transcrits produits dans les différentes conditions avec les mécanismes identifiés pourrait permettre de distinguer des biomarqueurs fonctionnels, représentatifs de l'activation de modes d'action spécifiques.
- Analyser le comportement de collection de même type de micro-organismes, genre, espèces ou souches afin de comprendre à quel point les caractéristiques et modes d'action identifiés sont liés au type de BCAs.
- Étendre l'utilisation de telle méthodes de profilage à d'autres conditions d'incubation, d'autres pathogènes filamenteux et/ou d'autres variétés ou types de plante ; mais aussi améliorer la gamme de tests proposés en incluant des dispositifs permettant d'identifier des modes d'action impliquant la plante entière, et mieux comprendre les interactions entre plante et micro-organismes bénéfiques.

D'un point de vue plus appliqué plusieurs perspectives peuvent également être envisagées, par exemple :

- Optimiser l'utilisation : usage aérien/traitement de sol, en préventif/curatif, adaptation du BCA choisi à la menace (pas globalement *Fusarium* toutes espèces mais des cibles précises), mais aussi à l'environnement dans lequel il est implanté (climat, température, humidité, pH, etc.).
- Renforcer la formulation des BCAs par des nutriments (qu'il est le seul à consommer, comme la thréonine pour Poly), des éliciteurs de composés antimicrobiens (forme de « priming » des BCAs, comme l'utilisation de poudre de pathogènes inactifs stimulant la synthèse de chitinases), ou encore des composés protecteurs permettant d'améliorer la durée de vie des produits. La formulation de produits de traitement pourrait aussi être réalisée à partir d'un consortium de micro-organismes bénéfiques, qui pourrait produire des effets additifs ou synergiques en combinant les différents mécanismes des souches isolées. Ou au contraire,

isoler les composés responsables des effets inhibiteurs pour s'affranchir de l'aspect biologique et de survie des traitements (formulation de traitement à partir de composés d'antagonismes, bio-fumigation des sols par les COV...).

- Améliorer la sélection et la caractérisation de nouveaux candidats de biocontrôle à l'aide des dispositifs mis au point pour les deux approches complémentaires *in vitro* et *in situ*. Notamment, par l'évaluation de l'impact écologique de l'application massive de BCAs pour une utilisation sûre, il faut en effet porter une attention particulière à la stimulation de production des mycotoxines par ces méthodes, en dépit d'effets intéressants sur la croissance.

Cet ensemble d'études met aussi en avant les avantages à combiner des méthodes d'approches variées et complémentaires dans le cadre de l'étude du biocontrôle : *in vitro* en condition contrôlée pour cibler, identifier, et quantifier des modes d'action précis, et *in situ* sur la plante pour mesurer les effets de ces modes d'action en conditions naturelles.