UNIVERSITÉ DU QUÉBEC À MONTRÉAL

IMPACT DES CULTURES DE COUVERTURES SUR LA COURGE (*CUCURBITA PEPO*) ET SON PATHOGÈNE *PSEUDOMONAS SYTINGAE* PV. *LACHRYMANS*: UNE ÉTUDE DU MICROBIOME DE LA PHYLLOSPHÈRE

THÈSE

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PRÉFACE

Parler de prédestination, c'est se protéger du chaos de l'aléatoire

AVANT-PROPOS

Faisant suite à des études en biologie moléculaire des plantes en France (universités de Bordeaux et Strasbourg), et notamment après une expérience de recherche dans un contexte d'agriculture (vigne), j'ai décidé de me spécialiser dans la génomique et les technologies de séquençage pour des plantes de grande culture avec une Maitrise à l'université Laval (Québec). Durant ce nouvel apprentissage, j'ai pu côtoyer le monde des microorganismes et ai été séduit par la métagénomique. J'ai donc naturellement choisi de poursuivre mes études académiques dans ce domaine. Dans l'idée de lier le monde de l'agriculture et les techniques scientifiques avancées pour contribuer à l'amélioration des connaissances sur des pratiques durables, j'ai réalisé ce doctorat, impliquant culture de couverture, contrôle de pathogène et métagénomique. Ce projet est une collaboration entre l'UQAM et AAC.

Cette thèse comporte trois articles scientifiques écrits en Anglais. J'ai été responsable de la planification scientifique, de l'échantillonnage, du travail de laboratoire, des séquençages, du traitement bio-informatique des séquences, des tests de compétition *in vitro* et essaies en serre, des analyses statistiques et de la rédaction des articles dont je suis le premier auteur. De nombreuses personnes m'ont assisté à la réalisation de ces travaux tel qu'indiqué ici :

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TABLE DES MATIÈRES

REMERCIEMENTS ii

PRÉFACE iii

AVANT-PROPOS iv

TABLE DES MATIÈRES v

LISTE DES FIGURES ix

LISTE DES TABLEAUX xi

LISTE DES ABRÉVIATIONS, DES SIGLES ET DES ACRONYMES xii

RÉSUMÉ xiii

ABSTRACT xv

INTRODUCTION 1

0.1 Revue de littérature 1

0.2 Microorganismes : considérations générales 1

0.2.1 Plantes : multiples habitats pour microorganismes. 2
0.2.2 Le sol : un environnement de recherche de prédilection pour l'études des microorganismes 3
0.2.3 La phyllosphère 4
0.2.3.1 Un système de recherche émergent 4
0.2.3.2 Environnement extrême 4
0.2.3.3 Stratégies de colonisation des bactéries 6
0.2.3.4 Bénéfice des interactions plante-bactéries 6
0.2.3.4.1 Augmentation de la biomasse des plantes 7
0.2.3.4.2 Augmentation de la production de métabolite 7
0.2.3.4.3 Tolérance au stress biotique : la résistance aux pathogènes 8
0.2.3.4.4 Qualité et cycles de vie de l'hôte 9
0.2.4 Étude de l'écologie des communautés 10
0.2.4.1 Enjeu pour appréhender des interactions complexes en agriculture 10
0.2.4.2 Des outils classiques dépendants des cultures aux outils de dernière génération, culture-indépendants. 11
0.2.5 La « révolution verte » : un système de culture intensif à bout de souffle 12
0.2.6 Culture de couverture 13
0.2.6.1 Considérations générales 13

0.3 Plan de thèse 14

0.3.1 Objectif et hypothèse générale 14

0.4 Chapitre 1 : Winter rye cover cropping changes squash (*Cucurbita pepo*) phyllosphere microbiota and reduces *Pseudomonas syringae* symptoms 14

0.4.1 Contexte....14
0.4.1.1 Du contrôle de pathogènes....14
0.4.1.2 Le cas de *Pseudomonas syringae*....15
0.4.1.3 Couvre-sol : modulation du microbiome de l'hôte et contrôle de *Psl*....15
0.4.2 Hypothèse et objectifs....16

0.5 Chapitre 2 : Functional characterization of bacterial communities on rye cover crop grown squash (*Cucurbita pepo*)....16

0.5.1 Contexte....16
0.5.1.1 Diversité fonctionnelle du microbiome de la phyllosphère....16
0.5.1.2 Les méthodes de séquençages : ciblées ou non?....17
0.5.2 Hypothèse et objectifs....18

0.6 Chapitre 3 : Winter rye cover crops shelter competent squash phyllosphere bacteria to reduce *Pseudomonas syringae* pv. *lachrymans* growth and angular leaf spot symptoms....18

0.6.1 Contexte....18
0.6.1.1 Associations plante-microorganismes....18
0.6.1.2 Les PGPB : cadre théorique....19
0.6.1.3 Les PGPB : cadre pratique et appliqué à l'agriculture....19
0.6.2 Hypothèse et objectifs....20

CHAPITRE 1 Winter rye cover cropping changes squash (*Cucurbita pepo*) phyllosphere microbiota and reduces *Pseudomonas syringae* symptoms....22

1.1 Abstract....23

1.2 Introduction....24

1.3 Material & Methods....25

1.3.1 Experimental design and field treatment....25
1.3.2 Microbial collection, DNA extraction and sequencing....26
1.3.3 Sequence analysis....27
1.3.4 Data analysis....28
1.3.4.1 *P. syringae* abundance analysis on squash leaves....28
1.3.4.2 Squash fruit health and marketability at harvest....28
1.3.4.3 Effect of cover cropping treatments on bacterial community diversity....29
1.3.4.4 Differential abundance analysis of ASVs....30

1.4 Results....30

1.4.1 Cover cropping reduced *P. syringae* abundance on squash leaves and improved fruit health and marketability.....30
1.4.2 Phyllosphere microbial communities differed between sampling dates and treatments....33
1.4.3 Cover cropping treatments influence squash phyllosphere community diversity and composition....33
1.4.4 *Sphingomonas* and *Methylobacterium* were more abundant with cover crops treatments....37

1.5 Discussion....39

1.6 Availability of data and materials....41

1.7 Acknowledgements....42

CHAPITRE 2 Functional characterization of bacterial communities on rye cover crop grown squash (*Cucurbita pepo*) 43
2.1 Abstract 44
2.2 Introduction 45
2.3 Material & Methods 47
2.3.1 Experimental design and field treatment 47
2.3.2 Microbial DNA collection 47
2.3.3 DNA extraction and Illumina sequencing 47
2.3.4 Sequence processing & annotation 48
2.3.5 Data analysis 49
2.3.5.1 Effect of cover cropping treatments on bacterial taxonomic and functional diversity 49
2.3.5.2 Effect of sequencing methods on taxonomic diversity 49
2.3.5.3 Differential abundance analysis of metabolic pathways 50
2.4 Results 51
2.4.1 Cover cropping treatments influence squash phyllosphere taxonomic diversity and composition 51
2.4.2 Sequencing methods do not influence overall taxonomic composition of squash phyllosphere 53
2.4.3 Cover cropping treatments influence squash phyllosphere metabolic pathways diversity 55
2.5 Discussion 59
2.5.1 Cover crop effects on microbial taxonomic composition and diversity 59
2.5.2 Cover crops influence phyllosphere microbial metabolic pathways 59
2.5.3 Comparison of 16S and metagenomic *shotgun* sequencing taxonomic annotations 61
2.6 Availability of data and materials 62
2.7 Acknowledgements 62
CHAPITRE 3 Winter rye cover crops shelter competent squash phyllosphere bacteria to reduce *Pseudomonas syringae* pv. *lachrymans* growth and angular leaf spot symptoms 63
3.1 Abstract 64
3.2 Introduction 65
3.3 Material & Methods 68
3.3.1 Experimental design and microbial collection 68
3.3.2 Microbial culture and isolation 68
3.3.3 Microbial DNA extraction and sequencing 69
3.3.4 Sequence processing and analysis 69
3.3.5 Data analysis 70
3.3.5.1 Phylogenetic analysis 70
3.3.5.2 Risk group classifications for bacterial candidate selection 70
3.3.6 *In vitro*: pre-selection and competition assay on Petri dish 72
3.3.7 *In vivo* pre-selection assay in greenhouse 72
3.3.8 *In vivo* symptoms control assay in greenhouse 73
3.4 Results 76

3.4.1 Bacterial isolation and sequencing identification....76
3.4.2 Screening for effective inhibitors of pathogen growth....79
3.4.3 Biological control effect confirmed with an *In vivo* greenhouse disease assay....82
3.4.4 Biological control of ALS disease symptoms was a function of leaf age....84
3.5 Discussion....86
3.6 Conclusion....89
3.7 Availability of data and material. 90
3.8 Acknowledgements....90
CONCLUSION....91
4.1 Résumé....91
4.2 Chapitre 1 : Les cultures de couvertures améliorent la qualité marchande des courges et façonne le microbiome de la phyllosphère de courge....92
4.3 Chapitre 2 : Des voies métaboliques bactériennes au service de la santé des plantes préférentiellement sélectionnées avec les cultures de couverture....93
4.4 Chapitre 3 - Des bactéries isolées en contexte de culture de couverture pour combattre *P. syringae* 94
4.5 Limites....96
4.6 Future recherche....98
4.6.1 Caractérisation complète des bactéries antagonistes sélectionnées par les couverts végétaux98
4.6.2 Explorer l'impact des cultures de couverture sur les fonctions de la plante hôte et du microbiome de la phyllosphère....98
4.6.3 Quantifier les changements de migration bactérienne en contexte de cultures de couverture 99
4.6.4 Améliorer les méthodes métagénomique avec implantation de procédures protocolaires rigoureuses....100
4.6.5 Approche holistique et systématique....100
ANNEXE A Matériel supplémentaire du Chapitre 1....101
ANNEXE B Matériel supplémentaire du Chapitre 2....118
ANNEXE C Matériel supplémentaire du Chapitre 3....127
BIBLIOGRAPHIE....138

LISTE DES FIGURES

Figure 0.1 : représentation schématique des différents compartiments d'une plante de courge (*Cucurbita pepo*) propre à la colonisation bactérienne……3

Figure 0.2 : représentation schématique de l'anatomie d'une feuille……5

Figure 0.3 : Différentes approches de séquençage, indépendantes de culture, pour l'étude des structures des communautés de microorganismes……12

Figure 1.1 : *P. syringae* populations on squash leaves for different cover cropping practices during 2016 and 2017. 32

Figure 1.2 : Alpha diversity (Shannon index) of phyllosphere bacterial communities for each treatment and each sampling date during the growing season of years 2016 and 2017. 34

Figure 1.3 : Non-metric multidimensional scaling (NMDS) ordination of bacterial community composition in squash phyllosphere samples from different cover cropping treatments in 2016 and 2017. 36

Figure 1.4: Log_2-fold change (LFC) heatmap of most differentially abundant ASV from DeSeq2 analysis for each sampling date of the 2017 samples. 38

Figure 2.1: Impact of cover crop treatments on bacterial taxonomic community diversity in the phyllosphere of squash. 52

Figure 2.2: Comparison of bacterial community relative abundances at the taxonomic phylum rank in different treatments between 16S rRNA gene amplicon sequencing and metagenomic *shotgun* sequencing. 54

Figure 2.3: Impact of cover crop treatments on bacterial metabolic pathways in the phyllosphere of squash. 56

Figure 2.4: Differentially abundant phyllosphere microbe metabolic pathways between cover crop and non-cover crop treatments. 57

Figure 3.1: Workflow from bacterial isolation to disease suppression validation. 71

Figure 3.2: Phylogenetic tree of bacterial isolates from winter rye cover crops and squash leaves collected during growing season 2016 and 2017..... 77

Figure 3.3: Relative number of the 584 isolates belonging to different taxonomic classes. 78

Figure 3.4: Inhibition of *P. syringae* **pv.** *lachrymans* growth by antagonist candidates over time..... 80

Figure 3.6: ALS lesion counts on squash leaves caused by *P. syringae* pv. *lachrymans* as a function of antagonist treatment..... 83

Figure 3.7 : ALS lesion counts as a function of leaf age for each antagonist treatment in the *In vivo* symptoms control assay. 85

LISTE DES TABLEAUX

Table 1.1: Proportion of squash fruit (mean +- standard deviation) with no *P. syringae* symptoms and marketable fruits with no damage for the two-growing seasons 2016 and 2017. 31

Table 3.1: Taxonomic identification of *P. syringae* pv. *lachrymans* antagonist candidate bacterial isolates selected for follow-up study in pathogen growth inhibition and disease control assays. 82

LISTE DES ABRÉVIATIONS, DES SIGLES ET DES ACRONYMES

AAC	Agriculture et Agroalimentaires Canada
ACC	1-aminocyclopropane-1-carboxylate
acdS	ACC deaminase synthase
ANCOM	Analysis of Composition of Microbiomes
ANOVA	Analysis Of Variance
ALS	Angular Leaf Spot | tache angulaire des feuilles
ASV	Amplicon Sequence Variants
DNA/ADN	Deoxyribonucleic acid | Acide désoxyribonucléique
Bdg	Mutant BODYGUARD
BS	Bare Soil | Sol nu
CFU	Colony Forming Units | Unité Formant Colonies
clr	Centred Log Ratio
CT-RCC	Chemically Terminated Rye Cover Crop | Culture de couverture de seigle chimiquement exterminé
ISR	Induced Systemic Resistance | Résistance Systémique Induite
KB	King's B medium | milieu *King's B*
LFC	Log2-fold change
MAG	Metagenome Assembled Genome
MAMPs	Microbe-Associated Molecular Patterns
NMDS	Nonmetric Multidimensional Scaling
OD	Optical Density |Densité Optique
PC	Plastic Cover | Paillis plastique
PCA	Principal Component Analysis | Analyse en Composante Principale
PCR	Polymerase Chain Reaction | Réaction en chaîne par polymérase
PERMANOVA	Permutational Analysis Of Variance
PGPB	Plant Growth Promoting Bacteria | Bactéries favorisant la croissance des plantes
Psl	*Pseudomonas syringae* pv *lachrymans*
RCC	Rye Cover Crop | Culture de couverture de seigle
TSA	Tryptic Soy Agar
TukeyHSD	Tukey's honest significance test

RÉSUMÉ

Résumé

La "révolution verte" en agriculture, caractérisée par l'intensification des pratiques agricoles et l'utilisation d'intrants de synthèse après la deuxième moitié du XX^{e} siècle, a entraîné des conséquences environnementales et sanitaires. La résistance croissante aux produits chimiques a accentué l'utilisation de phytoprotecteurs synthétiques, amplifiant ainsi les problèmes écologiques et de santé. L'agriculture du début de XXI^{e} siècle fait face à la contraction énergétique globale, un manque de surfaces cultivable accentué par le réchauffement climatique, dont elle-même contribue à son augmentation par d'importantes émissions de gaz à effet de serre. Ces enjeux mettent en péril la sécurité alimentaire mondiale. À ceci, vient s'ajouter des pertes de productions causées par les mauvaises herbes, les animaux nuisibles et les maladies. Face à ces défis, il est capital d'implanter des pratiques résilientes et durables. En ce sens, l'adoption croissante des cultures de couverture comme une pratique agricole alternative s'impose. Alors que leurs avantages en matière de lutte contre les mauvaises herbes et d'amélioration de la santé des sols sont de plus en plus reconnus, leur impact sur la phyllosphère reste à explorer. La phyllosphère, composée par les organes des plantes situés au-dessus de la surface du sol, est le lieu d'habitat de nombreux microorganismes, dont les pathogènes. Il est connu que les interactions plante-microorganismes pourraient être à l'origine du contrôle de certaines maladies. Étudier la dynamique des communautés du microbiome en contexte de culture de couverture semble capital pour mieux appréhender les bénéfices apportés par cette pratique agriculturale. Cependant, aucune recherche n'a encore exploré leur potentiel de contrôle de maladies bactériennes, en général, et de la maladie de la tâche angulaire des courges causée par *Pseudomonas syringae* pv *lachrymans* (*Psl*).

Le principal objectif de cette thèse était d'approfondir notre compréhension de l'impact des pratiques de cultures de couverture sur le microbiome de la phyllosphère de la courge, ainsi que leur potentiel de biocontrôle contre le pathogène *Psl*. Dans cette optique, le premier chapitre visait à décrire la diversité taxonomique des communautés bactériennes associées à la phyllosphère de la courge en conditions de culture de couverture, à évaluer la réduction potentielle de l'abondance de *Psl* à la surface des feuilles de courge, et à examiner des paramètres agronomiques tels que la santé des plantes et la qualité marchande des fruits à la récolte. Ce premier chapitre a ainsi fourni un profil inédit des communautés bactériennes

de la phyllosphère de la courge en contexte de culture de couverture, jetant les bases pour des applications futures.

Afin d'approfondir notre compréhension de l'impact des cultures de couvertures sur le phytobiome, le deuxième chapitre a exploré les voies métaboliques des communautés de la phyllosphère de la courge grâce à un séquençage métagénomique de type *shotgun*. Cette approche a permis de préciser nos connaissances fondamentales sur l'effet potentiellement combiné de biocontrôle du pathogène et d'élicitation des défenses de la plante lors de la mise en place de cultures de couverture.

Enfin, le troisième chapitre avait pour objectif d'isoler par culture des bactéries de la phyllosphère de la courge en contexte de culture de couverture, puis d'évaluer leur potentiel de biocontrôle de *Psl* à travers des tests de compétition *In vitro* et *In vivo*. Cette contribution significative au domaine agronomique ouvre des perspectives pour des applications futures dans des stratégies de gestion en lutte intégrée.

Les travaux présentés ici constituent une exploration originale des cultures de couverture dans le contexte de la contamination par *Psl*. Cette approche combine l'étude des dynamiques des communautés de la phyllosphère de la courge, des méthodes dépendantes de la culture, des tests de compétition subséquents et des cultures indépendantes, en utilisant des techniques de séquençage de nouvelle génération. Nos expérimentations sur le terrain ont été conçues en tenant compte de diverses pratiques agricoles, telles que la culture de couverture de seigle d'hiver (*Secale cereale)*, la culture de couverture de seigle d'hiver préalablement chimiquement détruite, le paillis de plastique noir et le sol nu, afin d'ancrer nos travaux dans le contexte concret de la production de courge.

Mots clés :

Culture de couverture, microbiome, phyllosphère, *Pseudomonas syringae*, biocontrôle, agriculture durable, métagénomique

ABSTRACT

Abstract

The "Green Revolution" in agriculture, characterized by the intensification of farming practices and the use of synthetic inputs after the second half of the 20th century, has led to environmental and health consequences. The increasing resistance to chemicals has amplified the use of synthetic pesticides, exacerbating ecological and health issues. Agriculture in the early 21st century faces global energy constraints, a lack of cultivable land intensified by climate change, contributing to its increase through significant greenhouse gas emissions. These challenges jeopardize global food security. Additionally, there are losses in production caused by weeds, pests, and diseases. In the face of these challenges, it is crucial to implement resilient and sustainable practices. In this regard, the growing adoption of cover crops as an alternative agricultural practice is essential. While their benefits in weed control and soil health are increasingly recognized, their impact on the phyllosphere remains to be explored. The phyllosphere, composed of plant organs above the soil surface, is the habitat for numerous microorganisms, including pathogens. It is known that plant-microorganism interactions play a determining role in controlling certain diseases. Studying the dynamics of microbiome communities in the context of cover crop cultivation seems crucial to better understand the benefits of this agricultural practice. However, no research has yet explored their potential for controlling bacterial diseases in general, and the angular leaf spot disease of squash caused by *Pseudomonas syringae* pv *lachrymans* (*Psl*).

The main objective of this thesis was to deepen our understanding of the impacts of cover crop practices on the phyllosphere microbiome of squash and their biocontrol potential against the *Psl* pathogen. In this context, the first chapter aimed to describe the taxonomic diversity of bacterial communities associated with the phyllosphere of squash under cover crop conditions, evaluate the potential reduction of *Psl* abundance on squash leaves, and examine agronomic parameters such as plant health and marketable fruit quality at harvest. This first chapter provided a novel profile of bacterial communities in the phyllosphere of squash under cover crop cultivation, laying the groundwork for future applications.

To further our understanding of the impact of cover crops on the phytobiome, the second chapter explored the metabolic pathways of squash phyllosphere communities through shotgun metagenomic

sequencing. This approach helped refine our fundamental knowledge of the potentially combined effects of pathogen biocontrol and plant defence elicitation when implementing cover crops.

Finally, the third chapter aimed to isolate, through culture, phyllosphere bacteria of squash under cover crop cultivation and evaluate their biocontrol potential against *Psl* through *In vitro* and *In vivo* competition tests. This significant contribution to the agronomic field opens perspectives for future applications in integrated pest management strategies.

The work presented here constitutes an original exploration of cover crops in the context of *Psl* contamination. This approach combines the study of phyllosphere community dynamics, culture-dependent methods, subsequent competition tests, and independent cultures, using next-generation sequencing techniques. Our field experiments were designed, taking into account various agricultural practices such as winter rye (*Secale cereale*) cover cropping, chemically terminated winter rye cover cropping, black plastic mulch, and bare soil, anchoring our work in the concrete context of squash production.

Keywords :

Cover crops, Microbiome, Phyllosphere, *Pseudomonas syringae*, biocontrol, Sustainable agriculture, Metagenomics

INTRODUCTION

La question centrale posée par cette thèse concerne l'impact des pratiques de cultures de couverture sur le microbiome de la phyllosphère de la courge (*Cucurbita pepo*) et leur potentiel de biocontrôle sur le pathogène *Pseudomonas syringae* pv *lachrymans* (*Psl*). Alors que les avantages de cette technique agricole, tels que le contrôle des mauvaises herbes et l'amélioration de santé des sols, ont déjà été démontrés, la compréhension du contrôle des maladies bactériennes était un terrain vierge à explorer. À travers les différents chapitres, nous évaluons l'hypothèse selon laquelle les cultures de couverture augmentent la diversité des communautés bactériennes, réduisent les populations de *Psl* et améliorent finalement la santé des plantes et la qualité marchande des fruits. Compte tenu de la nature multifactorielle de l'assemblage des communautés de la phyllosphère, nous explorons aussi l'influence des cultures de couverture sur la diversité fonctionnelle du phytolliome des courges. Finalement, pour explorer ces hypothèses, cette thèse intègre des technologies de séquençage de pointe avec des techniques microbiologiques traditionnelles, mariant ainsi des approches indépendantes et dépendantes de cultures bactériennes, pour fournir un examen complet du microbiome de la phyllosphère, à la fois dans des expériences contrôlées et des conditions réelles de culture.

0.1 Revue de littérature

0.2 Microorganismes : considérations générales

Les microorganismes forment la plus grande diversité de vie, incluant notamment les bactéries, les archées, les fungi, les algues, et les virus (Pace, 1997). Ils vivent dans de nombreux habitats tant au niveau de la biosphère (Wommack et Ravel, 2013) que de l'atmosphère (Chen *et al.*, 2020) en passant par les niches aquatiques (Rodriguez-Brito *et al.*, 2010). Les microorganismes peuvent être trouvés dans des habitats très divers, incluant même les environnements extrêmes, comme les surfaces marines profondes ou les sources géothermales (Pedersen, 1993). Ces microorganismes contribuent à la vie terrestre, tant animale que végétale. Ils façonnent les dynamiques environnementales et peuvent être un atout considérable dans la gestion des pratiques agricoles : ils sont déjà utilisés dans des stratégies de remédiation (Xu *et al.*, 2010) et de contrôle de stress biotiques, comme des pathogènes (Maksimov *et al.*, 2015) et abiotiques (Yang *et al.*, 2009). D'autre part, en science expérimentale, les modèles microbiens représentent un atout majeur. En effet, ceux-ci sont de petite taille, facilement manipulables, et présentent une très courte période de génération, avec une moyenne estimée entre 30 minutes à une heure, sur un intervalle de 20 minutes

(*Escherichia coli*) à plus de 15 heures (*Mycobacterium tuberculosis*). Les bactéries sont donc parmi les organismes vivant avec les temps de génération les plus courts, juste après les virus. Ceci permet aux scientifiques de produire des réplicas à travers une large gamme d'échelle et de temps. De plus, des informations génétiques et physiologiques sont déjà disponibles pour de nombreux microorganismes. Finalement, leur forte implication dans divers processus dynamiques contribue à amener le modèle microbien en haut de l'échelle de potentiel de recherche dans le domaine de la théorie de l'écologie (Jessup *et al.*, 2004).

0.2.1 Plantes : multiples habitats pour microorganismes.

La majorité des tissus végétaux peuvent héberger divers microorganismes (figure 0.1); tant la rhizosphère, la zone au pourtour des racines, que la phyllosphère (leurs surfaces aériennes) et l'endosphère (leurs tissus internes), peuvent être colonisés par des microorganismes. Des compartiments plus spécifiques peuvent leur servir d'habitat : l'anthosphère, habitat associé aux fleurs (Aleklett *et al.*, 2014), la carposphère, habitat associé aux fruits (Leff et Fierer, 2013), la spermosphère, la section de sol sous influence des graines en germination (Schiltz *et al.*, 2015), et finalement les graines elles-mêmes (Shade *et al.*, 2017). La plupart de ces microorganismes sont donc étroitement associés à leur plante hôte et influent sur ses fonctions et son métabolisme. Les voies hormonales sont souvent citées comme processus cible (Dodd *et al.*, 2010 ; Glick, 2012), mais l'impact des communautés de microorganismes s'étend bien au-delà. En effet, elles peuvent, par exemple, fournir des capacités nutritionnelles et biosynthétiques vitales pour la plante, comme la fixation d'azote, la production d'hormones de croissance et la défense contre les pathogènes (Friesen *et al.*, 2011).

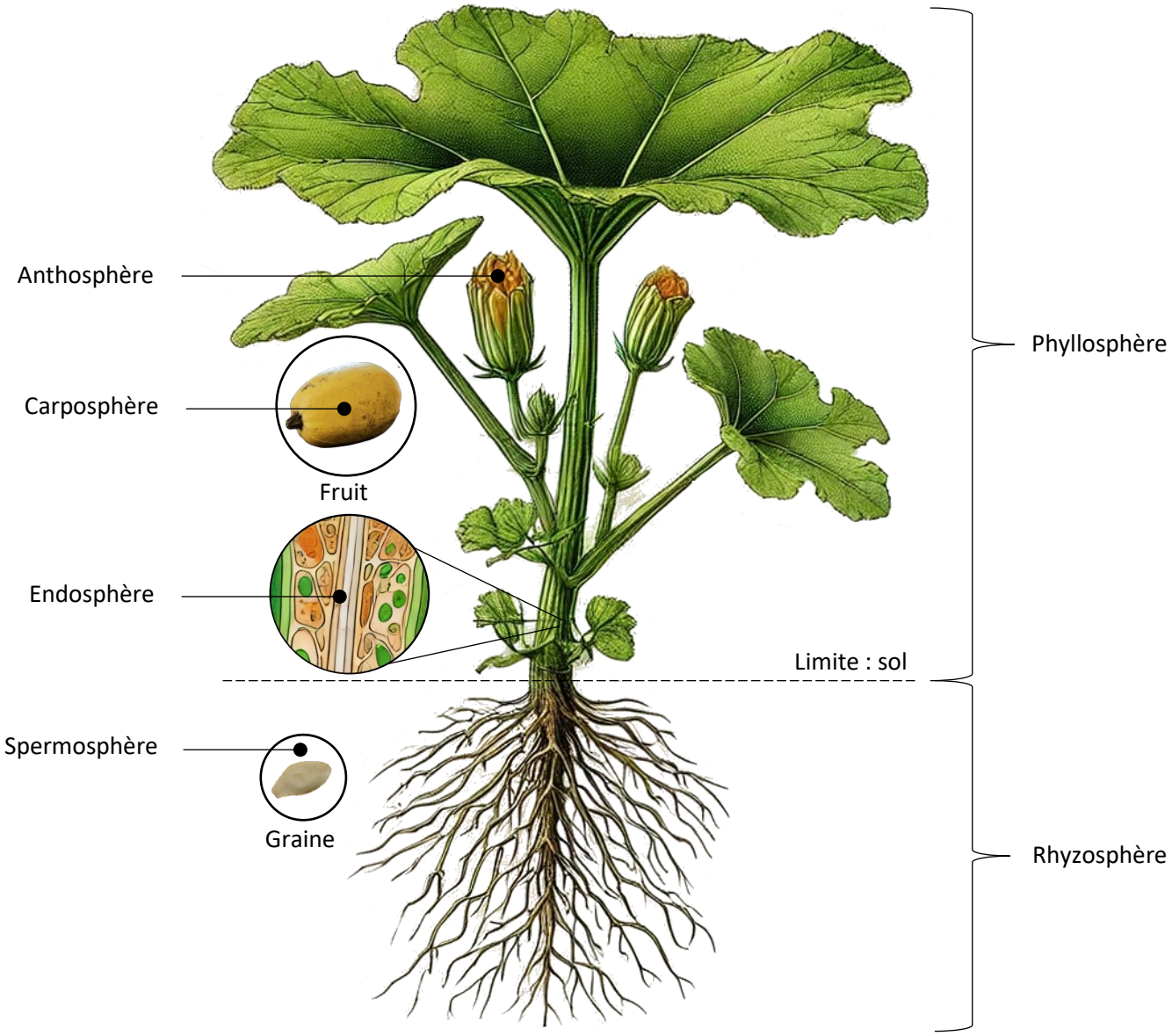

Figure 0.1 : représentation schématique des différents compartiments d'une plante de courge (*Cucurbita pepo*) propre à la colonisation bactérienne.

0.2.2 Le sol : un environnement de recherche de prédilection pour l'études des microorganismes

La plupart des attentions de recherches sur le microbiome des plantes se sont portées sur l'habitat des sols (Hartmann *et al.*, 2008), certainement dû au large spectre de fonctionnalités d'intérêt agronomique apportés par des associations hôte-mycorhize. On retrouvera parmi ces avantages l'absorption de nutriments (Van Der Heijden *et al.*, 2008), des contributions au niveau du cycle du carbone (Zhu et Miller, 2003) et de l'azote (Kowalchuk et Stephen, 2001 ; Tiedje, 1988), dans la formation des sols (Rillig et

Mummey, 2006) et leur respiration (Högberg *et al.*, 2001), de l'aide dans le système d'immunité des plantes (Van Wees *et al.*, 2008 ; Van der Ent *et al.*, 2009 ; Zamioudis et Pieterse, 2011), et aussi pour la protection contre les pathogènes (Doornbos *et al.*, 2012 ; Loon, 2007 ; Lugtenberg et Kamilova, 2009). Ainsi, le sol représente un terrain fertile pour les scientifiques produisant bon nombre de publications sur les dynamiques de la communauté de microorganismes associés à cet habitat et leur impact sur la plante; cependant, durant la dernière dizaine d'années, la phyllosphère jouit d'un intérêt croissant (Vorholt, 2012a).

0.2.3 La phyllosphère

0.2.3.1 Un système de recherche émergent

La phyllosphère, dont le terme a été publié pour la première fois en 1961 (Ruinen, 1961), représente la surface de la plante au-dessus du sol, composée de ses feuilles et tiges. Elle correspond à un habitat de vaste surface pour des microorganismes vivants, dont sa globalité a été estimée à 4×10^8 km^2 (Morris et Kinkel, 2002). La population de bactéries de la phyllosphère pourrait représenter 1×10^{26} cellules (Vorholt, 2012a). Chaque plante pouvant produire des feuilles permettra de générer un habitat potentiel pour des microorganismes, et ce à la fois au niveau de la surface des feuilles, pour les microorganismes épiphytes, et des tissus internes, pour les endophytes (Hallmann *et al.*, 1997). De plus, les microorganismes sont capables de coloniser non seulement la phyllosphère des plantes terrestres, mais aussi aquatiques (Goulder et Baker, 1991). Ainsi, l'offre d'habitat fourni par la phyllosphère étant très large, il permet d'être colonisé par une grande gamme de microorganismes, parmi lesquels on retrouvera bactéries, champignons, oomycètes, nématodes et archaea (Lindow et Brandl, 2003 ; Vorholt, 2012a). D'un point de vue anatomique, une feuille présente trois types de compartiments pouvant être colonisés : l'épiderme, le mésophile et les faisceaux vasculaires. Les microorganismes peuvent être trouvés à la surface de l'épiderme, dans les espaces intercellulaires du mésophile, mais pas dans les cellules de la plante (Hirano et Upper, 2000a).

0.2.3.2 Environnement extrême

D'un point de vue d'un organisme, la surface d'une feuille fait figure d'environnement extrême. En effet, c'est un milieu soumis à de forts stress biotiques et abiotiques. Hirano et Upper (2000) ont même suggéré que la colonisation bactérienne pouvait être soumise à des conditions stressantes durant la période d'un seul jour. Cette surface est aussi le lieu d'une forte contrainte de survie : les bactéries seront sujets aux

radiations, aux ultra-violets (Paul *et al.*, 1997 ; Jacobs et Sundin, 2001) et pressions climatiques, comme le vent et la pluie, entrainant de fortes variations d'humidité (Ercolani, 1991). D'autre part, la phyllosphère présente une teneur très faible en nutriments. La disponibilité des nutriments est un des facteurs principaux nécessaires pour la colonisation bactérienne et leurs succès de croissance (Ruppel *et al.*, 2008). Pourtant, les sources essentielles de carbone et de sulfure sont limitées sur une feuille (Mercier et Lindow, 2000). D'autre part, l'habitat foliaire présente un gradient de nutriments sur sa surface : en suivant la consommation bactérienne de sucre avec *Erwinia herbicola* possédant une construction plasmide-GFP réagissant au fructose et au sucrose, Leveau and Lindow (2001) ont pu estimer par observation *in situ* (méthode FISH) une abondance hétérogène en nutriments disponible pour *E. herbicola*, après son inoculation dans la phyllosphère de haricot (*Phaseolus vulgaris*). Finalement, à l'échelle d'une communauté bactérienne, la surface foliaire a une topographie qui présentes de fortes variations (figure 0.2). En effet, la cuticule, protégeant et imperméabilisant l'épiderme, la cutine et les cires associées, permettant une faible perméabilité, les stomates et les trichomes, toutes ces structures foliaires représentent à l'échelle d'une bactérie de véritables sommets, vallées, grottes et plaines à surmonter (Hirano et Upper, 2000a).

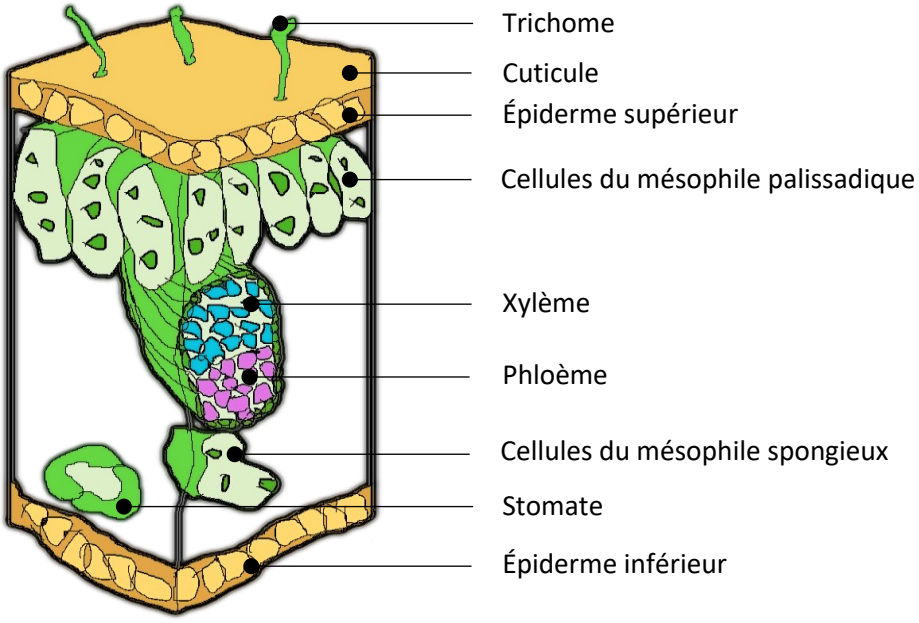

Figure 0.2 : représentation schématique de l'anatomie d'une feuille

0.2.3.3 Stratégies de colonisation des bactéries

Pourtant, les bactéries ont réussi à développer des stratégies pour coloniser la phyllosphère. La colonisation bactérienne dans le contexte de la phyllosphère fait référence à l'établissement et à la prolifération de communautés bactériennes sur les surfaces foliaires, régulées par les effets *top-down* (prédateurs, pathogènes Díaz-García *et al.*, 2021) et *bottom-up* (ressources, conditions abiotiques Remus-Emsermann *et al.*, 2012), ainsi que par le concept de l'effet de priorité, où les premiers colonisateurs peuvent fortement influencer la composition des communautés ultérieures (Carlström *et al.*, 2019). Les niches, réservoirs source de colonisation bactérienne de la phyllosphère, sont multiples : on y trouve le sol, l'eau, l'air, les plantes avoisinantes et les graines (Lindow et Brandl, 2003 ; Vorholt, 2012a). Certaines communautés ont réussi à s'adapter à cet environnement stressant en collaborant pour former des biofilms, une enveloppe protectrice (Danhorn et Fuqua, 2007). Brièvement, le biofilm est un ensemble de bactéries enfermées dans une matrice extracellulaire sécrétée par cette même communauté (Hall-Stoodley *et al.*, 2004). D'autre part, les bactéries, en produisant un large spectre de métabolites, possèdent une flexibilité fonctionnelle d'adaptations aux conditions de vie dans la phyllosphère. On citera l'exemple des bactéries *Proteobacteria*, *Chlorobi*, *Chloroflexi*, *Firmicutes* and *Acidobacteria* (Bryant *et al.*, 2007 ; Xiong et Bauer, 2002) capable d'utiliser la lumière par photosynthèse anoxygénique (Atamna-Ismaeel *et al.*, 2012a). De plus, une étude du microbiome de la phyllosphère de différentes espèces de plantes, incluant le riz et le soja, à même montré la présence de rhodopsine d'origine microbienne, une protéine sensible à la lumière impliquée dans le système de pompe à protons, mais dont le spectre d'absorption est différent de celui des feuilles suggérant un métabolisme microbien lié à la lumière et indépendant de celui de la plante hôte pouvant permettre une survie en milieu pauvre tel que la surface d'une feuille (Atamna-Ismaeel *et al.*, 2012b). Cependant, le métabolisme et les fonctions associés aux communautés microbiennes de la phyllosphère restent à être explorés.

0.2.3.4 Bénéfice des interactions plante-bactéries

Avec différentes stratégies de colonisation, de multiples taxons microbiens sont associés aux fonctions de la plante. En effet, de nombreux microbes sont reconnus pour avoir un effet bénéfique sur la croissance des plantes et sont, en ce sens, nommés PGPB, de l'anglais Plant Growth Promoting Bacteria (PGPB) (Kloepper *et al.*, 1989). De nombreuses études se sont penchées sur le cas des PGPB (Begum *et al.*, 2018 ; Dhawi *et al.*, 2015 ; Esitken *et al.*, 2010 ; Pardo-Díaz *et al.*, 2021). Ces bactéries influencent de nombreuses fonctions de leur hôte, de la production de métabolite à l'accumulation de biomasse, en passant par la

tolérance à certains stress biotique, et abiotiques. De plus, la composition des communautés microbiennes façonnerait la qualité des semences, leur capacité de germination, et le cycle de vie de l'hôte.

0.2.3.4.1 Augmentation de la biomasse des plantes

Il a été démontré que le microbiome associé aux plantes pourraient induire une augmentation de la biomasse de l'hôte. Par exemple, Panke-Buisse *et al.*, (2015) ont montré comment la sélection de différentes composition de microbiomes du sol peut influencer de manière reproductible la fonction des plantes, et augmenter significativement la biomasse des plantes hôtes : les microbiomes associés aux sols des plantes à floraison tardive ont augmenté de deux à cinq fois la biomasse totale de *Brassica rapa* et la biomasse des inflorescences de trois génotypes *d'Arabidopsis thaliana*. Cette augmentation de biomasse a aussi été observée dans la phyllosphère. Dans le but de comprendre l'effet de la déaminase 1-aminocyclopropane-1-carboxylate (ACC), une hormone qui améliore la croissance des plantes en réduisant les taux d'éthylène, sur la phyllosphère, Herpell et collaborateurs (2023) ont comparé la croissance de plants de tomate inoculés avec la souche sauvage de *Paraburkholderia dioscoreae* Msb3 à ceux inoculés avec une souche mutante dépourvue du gène ACC deaminase synthase (acdS). Ils ont ainsi démontré que la souche Msb3 colonise avec succès la phyllosphère de la tomate et favorise la croissance des plantes grâce à la production de la déaminase ACC, codée par le gène acdS. Les chercheurs ont aussi pu explorer le potentiel de manipulation du microbiote foliaire avec l'inoculation par pulvérisation de bactéries en tant que stratégie pour augmenter le rendement à la récolte. Je clorai ce paragraphe en notant que ces manipulations de microbiome font parties d'un grand ensemble de recherche : l'ingénierie des microbiome (Mueller et Sachs, 2015).

0.2.3.4.2 Augmentation de la production de métabolite

Le microbiote des plantes peut moduler la chimie de l'hôte de manière générale, et des métabolites de manière spécifique (Badri et al. 2013, voir aussi la revue de littérature Etalo et al. 2018). Cette dépense énergétique par la bactérie se justifie notamment pour son propre fonctionnement et pour la survie de son hôte (voir la revue de littérature de Chaudhry *et al.*, (2021)) Le phyllobiome, microbiome de la phyllosphère, peut augmenter la production de métabolites de l'hôte grâce à la production de substance organique secondaires tels que les flavonoïdes, les coumarines et les terpénoïdes (revue de littérature par Hussain et al. 2022). Plus spécifiquement, il a été rapporté que les PGPB augmentent la production de terpènes, impliqués dans la tolérance à la sécheresse (Schweiger *et al.*, 2014), mais aussi de composés

phénoliques (voir la revue de littérature in Saleem 2021), tels que l'acide gallique qui confère une résistance aux plantes de pois (*Pisum sativum*) à *Sclerotinia sclerotiorum* (Jain *et al.*, 2015), ou de l'acide salicylique qui favorise la croissance des plants de tomate (Wu *et al.*, 2018), et des coumestrols, qui ont été rapportés par Cho and Harper (1991) comme permettant d' augmenter la nodulation des racines du soja. Certains PGPB permettent aussi de stimuler la production de composés alcaloïdes tels que la tomatidine qui améliore la tolérance au sel des plants de tomate (Rivero *et al.*, 2018), ou de monocrotaline et de séneçonine qui fournissent une défense contre les herbivores (Hill *et al.*, 2018 ; Irmer *et al.*, 2015). Cette liste d'améliorations non exhaustives permet tout de même d'apprécier que le microbiote des plantes puisse stimuler la production d'une gamme diversifiée de métabolites chez les plantes, améliorant potentiellement leur capacité à faire face au stress et aux ravageurs. Cependant, deux récentes études invitent à nous attarder sur le sujet. Tout d'abord, Howe et al. (2023) ont étudié le microbiome des feuilles des cultures de millet vivace (*Panicum virgatum*) et de *Miscanthus*, identifiant des membres écologiquement importants du microbiome. Ils ont en effet constaté que les populations bactériennes associées aux feuilles sont assemblé selon une dynamique saisonnière et une réponse aux signaux de l'hôte, avec l'expression de voies associées au stress et l'activation de voies de biosynthèse pour les terpènes et les peptides non ribosomiques. Ensuite Kusstatscher et al. (2020) ont analysé les communautés microbiennes présentes dans la phyllosphère et les trichomes (voir figure 0.2 pour cette structure foliaire) de deux génotypes de tomates et ont constaté que les trichomes abritent un microbiote distinct et diversifiée par rapport aux autres échantillons de feuilles. La présence de familles bactériennes spécifiques, telles que les *Sphingomonadacées*, dans les trichomes suggère que les métabolites produits par les trichomes peuvent façonner une communauté microbienne unique et enrichir potentiellement les taxons bénéfiques. Cette étude a surtout mis en lumière l'importance des micro-niches, comme les trichomes, pour façonner les communautés bactériennes de la phyllosphère et suggère que l'analyse des bactéries associées aux trichomes pourrait conduire à la découverte de nouveaux composés bioactifs. Ces deux études nous invitent à entrevoir que la majorité des métabolites modelés par les PGPB restent inexplorés, d'une part, mais aussi que les dynamiques qui façonnent les profils métaboliques dépassent le seul cadre de l'interaction avec un ou plusieurs organismes, d'autre part.

0.2.3.4.3 Tolérance au stress biotique : la résistance aux pathogènes

Les interactions plante-bactéries contribuent donc à façonner le métabolome - l'ensemble des métabolites - de l'hôte. Ces modulations physiologiques apportent de nombreux bénéfices comme la résistance à certains pathogènes. On notera à cet effet la contribution de Hernández-León *et al.*, (2015) qui ont

caractérisé les effets antifongiques des composés organiques volatils produits par des souches de *Pseudomonas fluorescens* : lorsqu'inoculé à *Medicago truncatula* ces souches augmentent le contrôle de *Botrytis cinerea* et la biomasse de la plante hôte. Une autre étude majeure dans ce domaine est celle de Ritpitakphong et al. (2016). Les chercheurs ont exploré le rôle du microbiome de la phyllosphère d'*Arabidopsis thaliana* dans la résistance des plantes au pathogène fongique *Botrytis cinerea*. En restaurant la résistance des mutants bdg (BODYGUARD) sensibles par des inoculations composées du microbiome de la phyllosphère d'*A. thaliana*, ils ont démontré que le microbiome foliaire contribue à la résistance des plantes *d'Arabidopsis* contre *B. cinerea*. De plus, ils ont également découvert qu'une espèce appartenant au genre *Pseudomonas* isolée du microbiome des mutants bdg conférait une résistance contre *B. cinerea* sur *A. thaliana* et des pommes.

0.2.3.4.4 Qualité et cycles de vie de l'hôte

Il est bien sûr facile d'appréhender les bénéfices économiques qu'un contrôle du microbiome pourrait avoir sur la gestion des pathogènes en contexte agricole. Cependant, son implication a aussi des liens plus subtils avec le grand cycle de vie de son hôte, allant même jusqu'à des interactions avec les graines. Links et al. (2014) ont cherché à caractériser le microbiote bactérien et fongique associée aux surfaces des graines de *Triticum* et de *Brassica* et à examiner les interactions de ces microbiotes. Leurs résultats ont montré qu'il existerait un microbiome commun entre les graines de divers genres de plantes, mais aussi des souches bactériennes spécifiques (*Pantoea agglomerans*) à chaque graine qui ont présenté des propriétés antagonistes envers un isolat fongique (*Alternaria sp.*) sur les graines de *Triticum* et *Brassica*. Selon les auteurs, l'étude d'un microbiome commun des graines pourrait avoir un potentiel d'application dans la production et le stockage de graines saines et de haute qualité. De plus, en comparant les communautés bactériennes des graines de mauvaises herbes et du sol, Overbeek et al. (2011) ont montré que le microbiome des graines des mauvaises herbes dans les sols cultivables peut augmenter la diversité microbienne du sol, ce qui pourrait potentiellement influencer la qualité des graines et leur germination. Les interactions graine-microorganismes semblent donc décisives dans la qualité des semences. De plus, avec un nouveau dispositif de culture développé pour faire pousser des plants de chêne dans des conditions dépourvues de microbes tout en maintenant les parties de la plante situées en dessous et au-dessus du sol séparées, Abdelfattah et al. (2021) ont prouvé l'existence d'une transmission directe du microbiome de la graine à la phyllosphère. En effet, le microbiote de la graine semble plutôt localiser au niveau de structures comme le péricarpe et l'embryon ce qui expliquerait la transmission aux organes foliaires lors de la germination. Ces résultats renforcent l'implication du microbiome de la graine dans

l'apport potentiel de microbiomes bénéfiques à la phyllosphère. Mais les interactions hôte-microbiome dépassent cette structure reproductive garant de la pérennité de l'hôte. En effet, elles auraient aussi des implications dans le cycle de vie même de l'hôte, de la dormance à la floraison. En ce sens, Goggin et al. (2015) ont étudié le rôle des cytokinines d'origine végétale et bactérienne dans la médiation de l'état de dormance et de la réponse au fluridone dans les graines de *Lolium rigidum*. Les auteurs ont montré que les graines dépourvues de bactéries étaient incapables de perdre leur dormance dans l'obscurité à moins d'être alimentées en gibbérelline exogène ou en fluridone, suggérant que la microflore résidente des graines de *L. rigidum* contribue à leur état de dormance.

Les bénéfices apportés par les interactions plante-microorganismes sont donc indéniable pour l'agriculture. Dans les contraintes actuelles de limitations de surfaces cultivables, à la contraction énergétique globale et des pressions climatiques, il apparait essentiel d'étudier non seulement les interactions des communautés microbiennes avec leur environnement, mais aussi leur dynamique et diversité en contexte d'optimisation et de transition de production agricole. C'est ce que l'on nomme l'écologie des communautés.

0.2.4 Étude de l'écologie des communautés

0.2.4.1 Enjeu pour appréhender des interactions complexes en agriculture

L'amélioration de traits fonctionnels associés aux performances de la plante dépend de la composition et de l'abondance des communautés de bactéries de la phyllosphère (Saleem *et al.*, 2017). On peut donc trouver un intérêt certain à étudier les dynamiques écologiques qui façonnent les communautés microbiennes de la phyllosphère. L'étude de ces processus écologiques constitue ce que l'on nomme écologie des communautés. Les approches d'étude de ces dynamiques, en contexte écologique, se basent généralement sur des différences de diversité. La diversité d'une communauté est représentée par les espèces, taxons, ou fonctions qui la compose et leurs abondances (Vellend and Agrawal, 2010). Différentes mesures de diversités existent permettant de mettre en lumière les relations présentes entre les communautés. Ainsi, on retrouve l'α-diversité, diversité au sein d'un échantillon, la β-diversité, ayant attrait à la quantité de variation de composition entre les échantillons d'une collection, et la γ-diversité, diversité regroupant l'ensemble des diversités α de multiples échantillons, nommée aussi « diversité régionale » (Whittaker, 1972 ; McCune *et al.*, 2002). La β-diversité peut-être aussi considérée comme une

mesure de différence de diversité faisant référence à un changement entre des populations locales (Nemergut *et al.*, 2013).

0.2.4.2 Des outils classiques dépendants des cultures aux outils de dernière génération, culture-indépendants.

Afin d'estimer la diversité d'une communauté, différentes méthodes expérimentales sont à disposition. Originellement, on retrouve les approches dépendant de cultures de microorganismes. Mais, limitées dans leur pouvoir de détection, un intérêt croissant est porté aux approches, culture-indépendantes, par séquençage (Xu, 2006). En effet, la puissance et la précision des techniques de séquençage de nouvelle génération permettent de capturer l'ensemble des séquences présentes dans un environnement donné, et ceci associé à une forte diminution du coût par bases séquencées ces dix dernières années (Nowrousian, 2010). Généralement pour les bactéries, les régions variables des gènes des sous-unités ribosomiques 16S sont isolées par amplifications PCR et permettent de déterminer la composition taxonomique et l'abondance relative d'une communauté par séquençage des amplicons obtenus. Puisqu'il est possible d'étiqueter ces séquences, la quantification d'une communauté peut donc se faire pour chaque échantillon. D'autres techniques de séquençage existent, notamment les approches de métagénomique où l'ADN total d'un échantillon environnemental est fragmenté et séquencé (*shotgun sequencing*). Ceci permet d'obtenir en plus de la composition taxonomique une composition fonctionnelle, mais aussi une estimation plus précise de l'abondance des transcrits (de Vries *et al.*, 2023 ; Khachatryan *et al.*, 2020); **Error! Reference source not found.**3). Couplé à cette technique, il est possible de réaliser de la méta-transcriptomique avec un échantillon d'ARN ou de la méta-protéomique avec les protéines. Considérées ensemble, ces approches permettent de déterminer efficacement et précisément la composition et l'abondance (quantité de transcrits et/ou de protéines) fonctionnelles d'une communauté. Actuellement, faire le lien entre les dynamiques écologiques qui façonnent la structure d'une communauté et les fonctions associées à leur écosystème apparaît comme capital. Comprendre quelles activités les communautés bactériennes entretiennent dans leur habitat pourrait mener à mieux cerner les enjeux agronomiques soulevés par les changements climatiques et nos pratiques agricoles contemporaines.

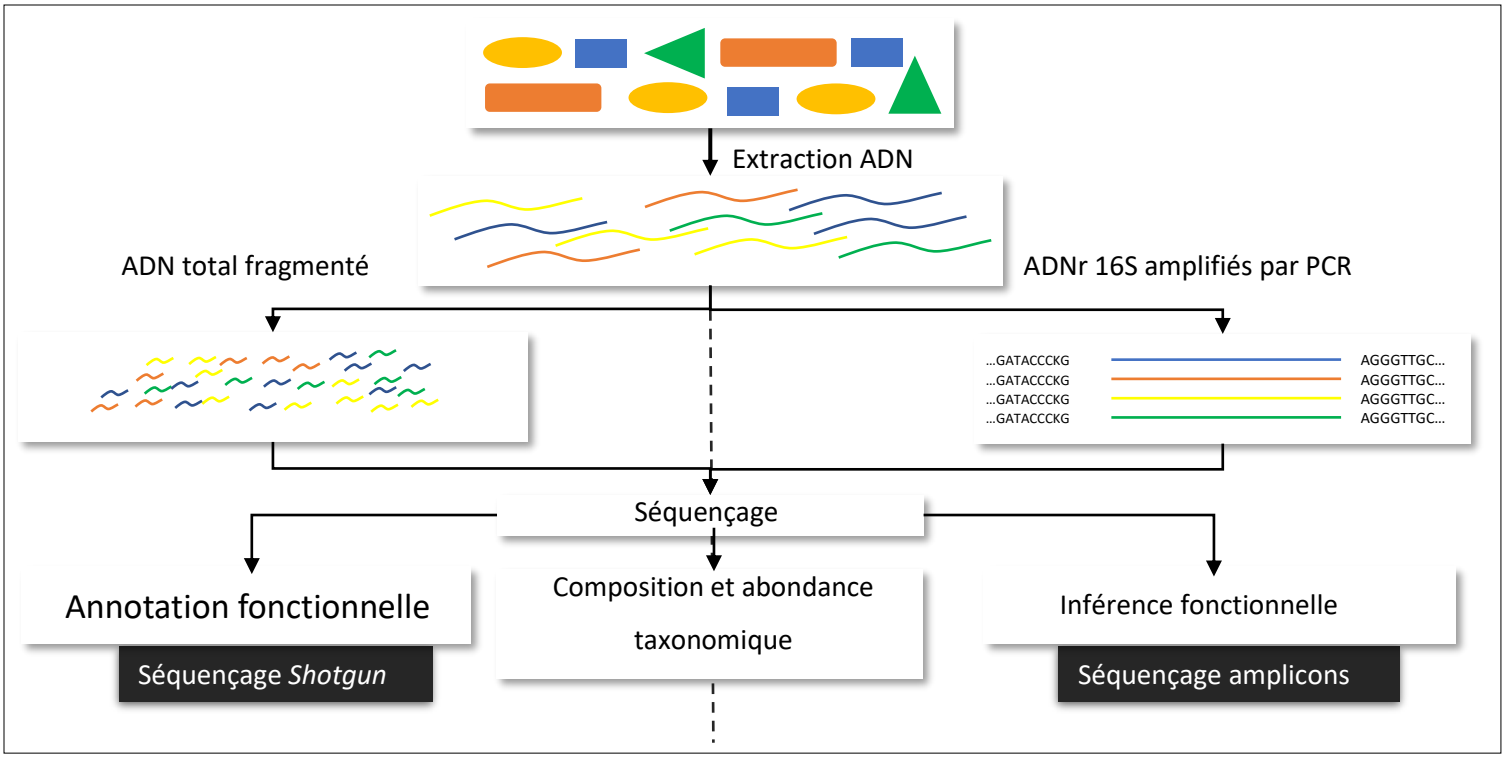

Figure 0.3 : Différentes approches de séquençage, indépendantes de culture, pour l'étude des structures des communautés de microorganismes. Contrairement à l'analyse des génomes complets, l'approche par séquençage d'amplicons ne permet de réaliser qu'une inférence de la composition fonctionnelle.

0.2.5 La « révolution verte » : un système de culture intensif à bout de souffle

La «révolution verte» est le résultat de l'intensification des pratiques agricoles notamment grâce à l'utilisation de cultivar à haut potentiel de rendement (Evenson et Gollin, 2003 ; Hedden, 2003), l'amélioration des technologies de mécanisation et d'irrigation, et l'augmentation massive de l'utilisation de produit chimique (pesticides et engrais). Les pratiques agricoles intensives génèrent une altération de l'environnement, et ce à travers divers processus : érosion des sols (Pimentel *et al.*, 1995), destruction des ressources naturelles (Repetto et Holmes, 1983), persistance des produits chimiques agricoles (Bowman, 1989). En plus de l'impact négatif sur l'environnement, ces pratiques pourraient aussi avoir des conséquences sur la santé humaine puisque les produits chimiques persistants peuvent être retrouvés dans la nourriture, l'air et l'eau (Fawell et Nieuwenhuijsen, 2003). D'autre part, l'intensification de l'agriculture est entravée par les mauvaises herbes, les animaux nuisibles et les maladies qui seraient responsables d'une perte de la production mondiale de 34%, 18% et 16% respectivement (Oerke, 2006). Par contre, l'inquiétude publique relative aux impacts des additifs chimiques de synthèse sur la santé et l'environnement ont eu pour effet de restreindre l'utilisation de pesticides efficaces (Carvalho, 2006). De

plus on observe une constante augmentation de la résistance aux antibiotiques, herbicides, insecticides et fongicides (Huesing *et al.*, 2016). Tout ceci a pour effet d'induire, dans les objectifs d'amélioration de rendements, une augmentation de l'utilisation, et donc de coût, de phytoprotecteurs de synthèse, venant nourrir par conséquent la spirale d'impacts environnementaux et de santé humaine susmentionnés. Ainsi, il est donc nécessaire d'identifier des approches innovantes afin de permettre une amélioration de la productivité tout en garantissant un faible impact sur les écosystèmes.

0.2.6 Culture de couverture

0.2.6.1 Considérations générales

Parmi les approches adaptées aux pratiques agricoles occidentales, on retrouve le système des cultures en rotation, par alternance du genre cultivé. Un point fort des cultures en rotation est de dynamiser la diversité du phytobiome associé, incluant les microorganismes, notamment du sol (Maeder *et al.*, 2002). D'autre part, la culture sur différents matériaux de couverture serrait une composante importante des systèmes de rotation en culture sans labour (Chen *et al.*, 2017 ; Teasdale, 1996). Ces couvertures peuvent être de type plastique (Nachimuthu *et al.*, 2017), mais présentent des défis pour leur retrait et des risques pour la santé des sols (Steinmetz *et al.*, 2016). Une alternative biologique existe, avec l'utilisation de paillis végétaux, composés généralement de seigle (Eckert, 1988). La culture de couverture, ou culture sur paillis vivant, représente l'implantation d'un couvert végétal qui a été roulé sur le sol : les tiges ne sont pas coupées mais cassées lors du roulage, permettant le maintien du paillis tout au long de la saison de production. À rendement équivalent, ces derniers pourraient donc constituer un fort potentiel de développement durable (LU et al., 2000) puisque les couvres sols offre un contrôle des pathogènes et des mauvaises herbes (Teasdale, 1996). Une étude de culture de laitue en couverture a même montré que cette technique pouvait améliorer les rendements (Stirzaker et White, 1995). D'autre part, un intérêt agronomique certain concerne la lutte contre les pathogènes et il semblerait que l'utilisation d'herbacé, du genre *Tagetes*, en couverture apporterait des résistances aux nématodes pour de nombreuses cultures (Hooks et al., 2010). De plus, si la santé du phytobiome associé s'en trouvait améliorée l'utilisation de paillis végétaux constituerait une alternative solide aux techniques traditionnelles. Pourtant, aucune littérature ne traite actuellement de cette problématique.

0.3 Plan de thèse

0.3.1 Objectif et hypothèse générale

Ce projet de doctorat est axé principalement autour de la thématique des cultures de couverture en paillis végétal et de leurs implications sur la diversité du microbiome de la phyllosphère de courge associée. En effet, même si des bénéfices directs de ces approches peuvent être observés en champ (augmentation de rendement, lutte contre des pathogènes), aucune étude n'a encore clairement démontré leur raison. Avec les différents chapitres, nous évaluerons l'hypothèse selon laquelle cette approche agriculturale permet une amélioration de la santé des plantes par l'entremise de son phyllobiome. Pour soutenir cette hypothèse, l'objectif général de cette thèse sera l'utilisation des nouvelles technologies de séquençage, pour l'étude des structures de communautés, combinées avec une approche d'étude microbiologique culture-dépendante. Le tout sera intégré avec des essais en culture *In vivo*, en serre. Durant ce projet, la courge (*Cucurbita pepo*), plante d'intérêt économique dans la province de Québec, a été utilisée comme hôte modèle. Cette thèse s'articule autour de trois chapitres, qui sont des articles publiés ou en processus de publication.

0.4 Chapitre 1 : Winter rye cover cropping changes squash (*Cucurbita pepo*) phyllosphere microbiota and reduces *Pseudomonas syringae* symptoms

0.4.1 Contexte

0.4.1.1 Du contrôle de pathogènes

Dans un monde à la recherche d'une agriculture durable, les approches de cultures de couvertures en paillis végétal ont montré de forts potentiels d'amélioration de la santé des plantes cultivées et de leur rendement. Des études pratiques en champ ont montré des augmentations de rendements pour le blé (et rendements équivalents pour le soja) avec différents types de paillis organiques; seigle, radis et trèfle rouge (Fawcett *et al.*, 2015, 2017). Les espèces composant le paillis peuvent être adaptées selon les conditions géographiques et les besoins : quantité de biomasse du paillis, lutte contre des pathogènes et apport azoté (Snapp *et al.*, 2005). D'autre part, en plus de l'amélioration de la santé des plantes, les approches de culture en couverture permettent d'améliorer la qualité des sols. Elles favorisent la réduction de l'érosion des sols (Dabney *et al.*, 2007), même dans le cas de sols non labourés (Stirzaker et White, 1995). De plus, les paillis végétaux contribuent à augmenter les apports de matière organique

limitant donc l'utilisation d'intrants azotés (Sainju et al., 2002). Finalement, un élément essentiel des cultures de couvertures réside dans leur contribution à la lutte contre certains pathogènes : Motisi et al. (2009) ont montré une réduction de l'infection de *Rhizoctonia solani*x chez la betterave à sucre. Leur utilisation peut aussi s'avérer utile dans le cas de contrôle des nématodes (Abawi et Widmer, 2000 ; Collins *et al.*, 2006);. De plus, des suppressions de *Phytium* spp. ont même été observées dans des cultures de couvertures de *Brassica* (Lazzeri et Manici, 2001). Finalement, Lazarus et White (1984) ont rapporté que les paillis de seigle permettaient de diminuer l'utilisation de pesticide chez de nombreuses plantes cultivées.

0.4.1.2 Le cas de *Pseudomonas syringae*

Des observations préalables à ce doctorat, issues de travaux réalisés dans le laboratoire du Dr V. Toussaint, ont montré que les cultures de couvertures sur paillis de seigle pouvaient contribuer à diminuer la pathogénicité de *Psl* chez la courge (*Cucurbita pepo*). Ce pathogène, de type gram-négatif, peut coloniser un nombre multiple d'environnements. Très étudié dans le domaine agricole pour sa pathogénicité, il peut être retrouvé hors de ce domaine, associé à des biofilms, dans des rivières, des nuages, pouvant même induire des cristallisations d'eau en glace et serait donc impliqué dans le cycle de l'eau (Morris *et al.*, 2008). Plus spécifiquement, de toutes les bactéries pouvant coloniser la phyllosphère, *Psl* est le plus étudié. En effet, dans sa phase primaire de développement, il colonise la surface des feuilles et s'y établit en épiphyte (Hirano et Upper, 2000a). Mais les symptômes associés à *Psl* génèrent des pertes conséquentes. Le contrôle de ce pathogène en système agricole est donc un enjeu capital.

0.4.1.3 Couvre-sol : modulation du microbiome de l'hôte et contrôle de *Psl*

Certains pathogènes peuvent être contrôlés par la diversité de la communauté bactérienne à laquelle ils sont associés (Kim *et al.*, 2011). Ainsi, réussir à capturer la composition et l'abondance de la communauté de la phyllosphère de courge contribuerait à appréhender les dynamiques de pathogénicité de *Psl* en culture de couverture. Ceci permettrait d'évaluer l'efficacité de techniques durables que sont les cultures de couverture en paillis végétaux sur le contrôle de *Psl*. D'autre part, nous avons vu précédemment que la constitution des plantes dépendait en partie des microorganismes qui lui sont associés. Plus spécifiquement, les communautés de bactéries associées à la phyllosphère influeraient positivement sur la croissance de la plante et sa tolérance à des pathogènes (Abanda-Nkpwatt *et al.*, 2006 ; Vorholt, 2012a). En effet, après avoir observé par métaprotéogénomique (analyse des génomes et des protéomes associés

à une communauté) que les espèces *Methylobacterium* et *Sphingomonas* étaient très représentées dans la phyllosphère de *Arabidospsis thaliana* (Delmotte *et al.*, 2009), mais aussi du trèfle et du soja, l'équipe du Dr J.Vorholt ont montré que certaines souches de *Sphingomonas* pouvaient conférer une suppression de symptômes et une diminution de la croissance de *Psl* chez *A. thaliana*.

0.4.2 Hypothèse et objectifs

Pour ce premier chapitre, nous avons émis l'hypothèse que le mécanisme d'un effet protecteur des cultures de couverture de seigle contre la tache bactérienne des feuilles de courge pourrait être modulé par les effets de la culture de couverture sur le microbiote de la plante, qui à son tour pourrait contrôler *Psl*.

Les objectifs du premier chapitre de cette thèse visent donc à décrire, par approche de séquençage d'amplicons, la diversité taxonomique des communautés bactériennes associées à la phyllosphère de courge en condition de culture de couverture. Nous avons évalué en premier si les cultures de couverture pouvaient contribuer à réduire les populations de *Psl* à la surface des feuilles de courge en mesurant directement l'abondance du pathogène sur les feuilles. Nous avons également pris en compte la santé et la commercialité des fruits à la récolte dans de telles pratiques culturales. Ensuite, nous avons estimé les effets de différentes méthodes de cultures de couverture sur les communautés bactériennes de la phyllosphère en quantifiant la diversité et la composition du microbiome foliaire à l'aide d'une approche de séquençage par métabarcoding bactérien 16S. Enfin, nous avons identifié les taxa bactériens les plus fortement influencés par les pratiques de cultures de couverture.

0.5 Chapitre 2 : Functional characterization of bacterial communities on rye cover crop grown squash (*Cucurbita pepo*)

0.5.1 Contexte

0.5.1.1 Diversité fonctionnelle du microbiome de la phyllosphère

En plus d'une étude de structure taxonomique des communautés bactériennes, il apparaît essentiel d'en étudier le profil fonctionnel, d'en évaluer les processus métaboliques, dans le but de déterminer leur rôle dans la physiologie de la plante (Vorholt, 2012a). La contribution fonctionnelle des bactéries de la phyllosphère peut s'observer avec une amélioration de la santé des plantes, avec par exemple la fixation

d'azote (Martínez *et al.*, 2003), l'augmentation de l'absorption minérale (Malinowski *et al.*, 2000) et production facteur de croissance (Kang *et al.*, 2007). En plus de l'amélioration de la croissance, la santé des plantes bénéficie d'un apport de fonctions bactériennes contribuant au contrôle de pathogène avec notamment le renforcement des mécanismes de défense (Bakker *et al.*, 2007), la diminution de la sévérité de certaine maladie (Kloepper *et al.*, 2004), la production de composé antimicrobien (Berg *et al.*, 2005 ; Großkinsky *et al.*, 2016 ; Mazinani *et al.*, 2017) et antifongique (Valverde *et al.*, 2017). Plus particulièrement, l'expression de gènes bactériens impliqués dans la synthèse de cytokinine apporterait un contrôle de *Psl* chez *Arabidopsis* (Großkinsky *et al.*, 2016, p. 20-18). Dans un cadre de culture de couverture, il serait donc pertinent d'évaluer l'effet de cette technique sur les fonctions bactériennes de la phyllosphère. Les analyses fonctionnelles contribueraient à identifier les bénéfices apportés lors des associations plante-microorganismes (Berg *et al.*, 2014). Ainsi, dans le cadre des cultures de couvertures, de telles approches favoriseraient la compréhension du rôle du microbiome de la phyllosphère de courge.

0.5.1.2 Les méthodes de séquençages : ciblées ou non?

Comme nous l'avons vu précédemment, les méthodes de séquençage jouent actuellement un rôle prédominant dans la caractérisation des communautés bactériennes. Elles se déclinent en deux approches principales : le séquençage ciblé, qui amplifie spécifiquement le gène de l'ARN ribosomal 16S avant le séquençage, et le séquençage métagénomique *shotgun*, une approche non ciblée qui séquence tout le matériel génétique présent dans un échantillon, comprenant à la fois l'ADN microbien et l'ADN hôte. Le séquençage 16S, malgré son coût moindre et son adaptabilité à de grands échantillonnages, présente des limites en termes de résolution taxonomique et de capacité à fournir une phylogénie précise au rang de l'espèce. De plus, l'inférence des fonctions à partir des séquences 16S est complexe (Aßhauer *et al.*, 2015 ; Douglas *et al.*, 2020), particulièrement dans les écosystèmes à base de plantes (Lajoie *et al.*, 2020). Le séquençage métagénomique *shotgun*, en revanche, offre une vue exhaustive de la composition et de la fonction des communautés microbiennes, ainsi que la possibilité d'assembler *de novo* des gènes (Methe *et al.*, 2020) et des génomes métagénomiques (Su *et al.*, 2022). Cette méthode a montré son efficacité dans la caractérisation fine des communautés microbiennes de plantes et la détection des fonctions métaboliques clés. Elle possède cependant certaines limites : un besoin de couverture plus important pour capturer certains groupes rares par exemple, ce qui entraine une augmentation du coût de séquençage (Tremblay *et al.*, 2022) et les bases de données manquent de références pour les domaines autres que les bactéries. Bien que le séquençage 16S puisse être amélioré par des marqueurs et des corrections de biais, le séquençage métagénomique *shotgun* reste l'approche privilégiée pour une compréhension globale et

approfondie des interactions entre les microbiomes de plantes et leur environnement, ouvrant la voie à de nouvelles stratégies de gestion agricole durable et de biocontrôle des pathogènes.

0.5.2 Hypothèse et objectifs

L'hypothèse choisie pour ce deuxième chapitre est que l'utilisation des cultures de couverture de seigle aurait un impact sur la composition taxonomique et fonctionnelle des communautés microbiennes de la phyllosphère. De plus, nous avons aussi formulées l'hypothèse selon laquelle certaines fonctions préférentiellement sélectionnées par ces cultures de couverture seraient associées avec une diminution des populations et des symptômes de *Psl*.

Pour tester cette hypothèse, nous avons mené une analyse de séquençage métagénomique *shotgun* afin de comparer les communautés bactériennes de la phyllosphère de la courge (*Cucurbita pepo*) cultivée avec différentes pratiques agricoles : des cultures de couverture de seigle (terminées ou non par un herbicide), au sol nu, en passant par les couverts en plastique noir (pratiques de gestion les plus courantes au Québec et dans l'est de l'Amérique du Nord). Comme pour le premier chapitre, nous avons d'abord évalué l'effet de ces pratiques culturales sur le microbiome de la courge en quantifiant la diversité taxonomique et la composition de la communauté bactérienne de sa phyllosphère. Nous avons ensuite testé si les annotations taxonomiques obtenues par le séquençage métagénomique *shotgun* étaient similaires à celles obtenues à partir du séquençage d'amplicons du gène 16S généré dans le premier chapitre. Enfin, nous avons quantifié l'impact des cultures de couverture sur la composition fonctionnelle et la diversité des voies métaboliques dans la phyllosphère.

0.6 Chapitre 3 : Winter rye cover crops shelter competent squash phyllosphere bacteria to reduce *Pseudomonas syringae* pv. *lachrymans* growth and angular leaf spot symptoms

0.6.1 Contexte

0.6.1.1 Associations plante-microorganismes

Les associations plante-microorganismes peuvent être de plusieurs types. Mutualisme, qui est un bénéfice direct et réciproque entre les deux partenaires, commensalisme, le microorganisme peut tirer bénéfice de son hôte avec une approche non parasitaire, et pathogène où le parasite tire un bénéfice au détriment de la plante. L'ensemble des espèces assemblées dans un espace d'association forme une unité écologique

que l'on nomme holobionte (Vandenkoornhuyse *et al.*, 2015). C'est dans cet espace que la plante peut tirer bénéfice des associations avec ses communautés bactériennes. Nous avons vu que ces bénéfices pouvaient être de plusieurs types : métabolique, immunitaire, etc. De manière générale, certaines bactéries peuvent avoir un impact positif sur la santé et le développement de son hôte : c'est ce que l'on a décrit précédemment comme les PGPB.

0.6.1.2 Les PGPB : cadre théorique

L'utilisation du potentiel bénéfique des associations plante-microorganismes est déjà en cours d'investigation pour des applications dans un contexte d'agriculture avec des améliorations de rendements et de qualité pour de nombreuses cultures. Récemment de nombreux projets s'intéressent à la manipulation du microbiome dans le but d'améliorer les capacités de la communauté pour optimiser les fonctions d'intérêts des plantes cultivées (Quiza *et al.*, 2015a ; Ryan *et al.*, 2009). Ces explorations se font surtout au niveau des PGPR ou PGPF, pour Plant Growth Promoting Rhizobacteria et Fungi, respectivement. Les souches de PGPB appartiennent généralement aux genres *Acetobacter*, *Achromobacter*, *Anabaena*, *Arthrobacter*, *Azoarcos*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, *Enterobacter*, *Flavobacterium*, *Frankia*, *Hydrogenophagae*, *Kluyvera*, *Microcoleus*, *Phyllobacterium*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Streptomyces*, et *Vibrio* (Bashan et De-Bashan, 2005). L'action de ces PGPB n'est pas encore complètement décrite, mais implique :

1- La production d'hormones utile à la plante, comme les cytokines (García de Salamone *et al.*, 2001), les gibbérellines (Gutiérrez-Mañero *et al.*, 2001), les auxines (Egamberdiyeva, 2005 ; Patten et Glick, 2002),) et elles seraient même responsables d'une inhibition de la production d'éthylènes par la production d'AAC déaminase (Glick *et al.*, 1995 ; Jacobson *et al.*, 1994 ; Li *et al.*, 2000 ; Penrose et Glick, 2001).
2- La fixation d'azote (Dobbelaere *et al.*, 2002 ; Şahin *et al.*, 2004).
3- La solubilisation des phosphates inorganiques (Vessey, 2003).

0.6.1.3 Les PGPB : cadre pratique et appliqué à l'agriculture

Les PGPB présentent un potentiel important pour la lutte contre certains pathogènes par des approches de contrôle biologique durable. Le 1er produit de biocontrôle, *Agrobacterium radiobacter* isolat 84, a été commercialisé en 1972 (Kerr, 1972) et permettait de lutter efficacement contre la galle d'inoculum *A.*

tumefaciens. Depuis, les approches d'intégration des PGPB en lutte durable contre les pathogènes se sont multipliées (Weller, 1988). En 1998, Klopper et al., montraient encore que l'ajout de PGPB permettait de lutter contre *Rhizoctonia solani* avec des résultats plus efficaces qu'un traitement fongicide de synthèse. Le potentiel de ces PGPB étant indéniable, il est nécessaire de continuer les investigations afin d'identifier de futurs candidats. Dans le cas où des pratiques agricoles favoriseraient l'établissement d'une communauté bactérienne améliorant la santé de la plante, il semble essentiel d'en isoler et caractériser les individus la composant lors de validation biologique avec une approche par inoculation contrôlée. Cependant, la combinaison de différents PGPBs affecterait leur efficacité. Par exemple, Jetiyanon et Kloepper, (2002) ont rapporté que l'inoculation de PGPRs mélangées présentait plus de suppression de symptômes de différents pathogènes que la PGPR individuelle . Puis, Esitken et al.(2010) ont montré que des assemblages de *Pseudomonas* BA-8, *Bacillus* OSU-142 et *Bacillus* M-3 pouvaient induire une augmentation de plus de 30% de la production de fraises par plante. Cette étude est d'autant plus intéressante puisqu'elle a exploité les potentiels de PGPB au niveau de la phyllosphère. Récemment, Innerebner et al. (2011) ont montré par approche d'inoculation que plusieurs souches de *Sphingomonas* pouvaient lutter efficacement contre les développements de symptôme de plants d'*A. thaliana* contaminés avec *Pseudomonas syringae* pv. tomato DC3000. De plus, ils ont prouvé que les souches de *Sphingomonas* inoculées individuellement présentaient différents potentiels de protection, jusqu'à une efficacité nulle. Ainsi, dans les cadres des cultures de couvertures, il est donc impératif de réaliser une caractérisation complète des communautés bactériennes cultivables associées à des améliorations de la santé des cultures d'intérêts afin d'en isoler et d'en contrôler les PGPB pour de futures applications. Pourtant, aucune validation biologique par approche d'inoculation n'existe à ce jour pour déterminer l'impact des communautés de la phyllosphère de courge dans ce contexte agricole. Nous sommes en droit de nous demander si les communautés de bactéries de la phyllosphère de courge associée aux cultures de couverture sont responsables de la diminution de la pathogénicité de *Psl*.

0.6.2 Hypothèse et objectifs

Dans le troisième et dernier chapitre, nous avons émis deux hypothèses : tout d'abord que nous serions en mesure d'isoler des bactéries de la phyllosphère de la courge et du seigle utilisés comme traitement de culture de couverture, capable de contrôler *Psl*. Puis, qu'un isolat seul présente moins de potentiel antagoniste contre *Psl* qu'un assemblage de plusieurs bactéries cultivables.

Pour tester ces hypothèses, nous avons poursuivi l'objectif d'isoler et de constituer une banque de bactéries issues des différentes pratiques mises à l'expérience. Dans un deuxième temps, nous procéderons à une validation biologique du potentiel de biocontrôle de ces isolats sur *Psl* avec des tests *in vitro* sur Pétri et *in vivo* avec des inoculations de courges en serres. Pour les tests *in vivo*, nous désirons aussi déterminer le niveau de diversité de l'inoculum nécessaire permettant de reproduire les effets estimés par le test *in vitro*.

CHAPITRE 1

Winter rye cover cropping changes squash (*Cucurbita pepo*) phyllosphere microbiota and reduces *Pseudomonas syringae* symptoms

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Dans ce chapitre (Résumé).

L'implantation de cultures de couverture est une pratique de conservation des sols qui pourrait réduire les impacts du pathogène économiquement important *Pseudomonas syringae* pv *lachrymans* (*Psl*) sur des cultures telles que la courge (*Cucurbita pepo*). À ce jour, aucune étude n'a directement quantifié l'effet des cultures de couverture de seigle sur les populations de *Psl*, ni sur la communauté bactérienne des feuilles de courge. Dans ce travail, nous avons testé l'hypothèse selon laquelle les effets protecteurs des cultures de couverture sur la courge pourraient être médiés par les effets de ces cultures sur le microbiote de la plante, qui protège à son tour contre *Psl*. En utilisant des approches combinées de séquençage 16S et de culture, nous avons montré que les cultures de couverture de seigle protègent la courge contre *Psl*, en réduisant la taille de la population du pathogène sur les feuilles de courge et en améliorant la santé des fruits ainsi que leur commercialisation à la récolte. Nous avons également trouvé des preuves d'un fort effet des cultures de couverture de seigle sur les communautés bactériennes de la phyllosphère de la courge. Ces résultats étaient plus marqués en début de saison de production. Enfin, nous avons identifié de nombreuses bactéries de la phyllosphère appartenant aux genres *Sphingomonas*, *Methylobacterium* et *Pseudomonas* qui étaient favorisées par les cultures de couverture de seigle. Dans l'ensemble, nos résultats suggèrent que la culture de couverture est efficace pour la gestion durable de *Psl* sur la courge et peut fournir un réservoir potentiel d'agents de biocontrôle microbien colonisant la phyllosphère.

1.1 Abstract

Cover cropping is a soil conservation practice that may reduce the impacts of the economically important pathogen *Pseudomonas syringae* on crops including squash (*Cucurbita pepo*). To date, no studies have directly quantified the effect of rye cover crops on *P. syringae* populations, nor on the bacterial community of squash leaves. In this work, we tested the hypothesis that the protective effects of cover cropping on squash may be mediated by cover cropping effects on the plant's microbiota that in turn protects against *P. syringae*. Using combined 16S sequencing and culture-based approaches, we showed that rye cover cropping protects squash against *P. syringae*, by decreasing pathogen population size on squash leaves and increasing fruit health and marketability at harvest. We also found evidence of a strong effect of rye cover crops on bacterial communities of the squash phyllosphere. Those findings were more striking early in the growing season. Finally, we identified numerous phyllosphere bacteria belonging to the genera *Sphingomonas*, *Methylobacterium* and *Pseudomonas* that were promoted by rye cover crops. Overall, our findings suggest cover cropping is effective for the sustainable management of *P. syringae* on squash and may provide a reservoir of potential microbial biocontrol agents colonizing the phyllosphere.

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1.2 Introduction

Cover cropping, or the growth of a plant to cover the soil for environmental benefits rather than for its harvest, is an increasingly popular option available to farmers to address the environmental and human health challenges associated with agricultural intensification (Fawell et Nieuwenhuijsen, 2003). Cover cropping allows equivalent yield (LU *et al.*, 2000) or an increase in yield (Fawcett *et al.*, 2015, 2017 ; Stirzaker et White, 1995), weed control (Teasdale, 1996), nematode control (Hooks *et al.*, 2010), and reduces soil erosion (Dabney *et al.*, 2001 ; Stirzaker et White, 1995). Winter cover crops used in northern countries are a promising avenue to reduce soil erosion and depletion by covering the soil during the winter (Dabney *et al.*, 2001). Cover crops can also improve water quality by reducing herbicide runoffs (Hall *et al.*, 1984), and improve soil condition by reducing temperature variations and water loss (Teasdale et Mohler, 1993). Many studies have demonstrated the benefits of cover cropping, although there is controversy about potential negative effects of cover cropping such as the potential transfer of pathogens from cover crop to crop (Bakker *et al.*, 2016). Cover cropping is known to shape the soil microbiome (Hartman *et al.*, 2018), but to date no study has quantified cover crop effects on aboveground microbial communities.

The phyllosphere microbiome, the microbial communities of aboveground plant parts, particularly leaves, are composed of a broad range of microorganisms such as bacteria, viruses, fungi and archaea (Lindow et Brandl, 2003). The microbiota on the aboveground parts of plants can improve plant fitness and biomass, primarily by reducing pathogen symptoms thanks to direct competition or associated with plant volatile compounds (Abanda-Nkpwatt *et al.*, 2006 ; Ritpitakphong *et al.*, 2016). Microorganisms are also important pathogens of the phyllosphere: 20–30% of crop production losses worldwide are due to various pests and pathogens (Savary *et al.*, 2019), and microbial pathogens are estimated to account for 16% of potential losses (Oerke, 2006). *Pseudomonas syringae*, one of the most widely studied bacterial plant pathogens, can infect a wide range of host plants including many economically important crops; it begins life as a leaf epiphyte, colonizes the host apoplast through wounds and stomates, and then damage fruits (reviewed in Hirano and Upper, 2000; Xin et al., 2018). Long-term intensive and frequent monocropping favours emergence of local pathogenic *P. syringae* reservoirs (Lindemann *et al.*, 1984). Efforts have been made to biologically control this pathogen on leaves using microbial competition; Lindemann and Suslow (1987) used a competition population of *P. syringae* disarmed with an ice nucleation mutation to prevent pathogen-related frost damage on strawberry plants and Innerebner et al. (2011) used several *Sphingomonas* species on leaf surfaces to protect *Arabidopsis thaliana* plants from *P. syringae*. Moreover,

many studies of plant induced systemic resistance (ISR) have noted potential leaf control of *P. syringae* based on the microbe-associated molecular patterns (MAMPs) mechanism and, interestingly, via microbial competitors located in the root-associated microbiome (reviewed in Pieterse et al., 2014; Van Wees et al., 2008). Finally, prior colonization of the plant phyllosphere by the beneficial bacterium is a well-known strategy to pre-emptively prevent the growth of the pathogen (reviewed in Andrews, 1992).

Despite the potential for microbiota-based biological control of *P. syringae*, management of this pathogen is primarily done through copper application, which has led to the development of resistance (Bender et Cooksey, 1986). Past studies have suggested that cover crops may provide soil borne biological control of pathogens (Abawi et Widmer, 2000 ; Collins *et al.*, 2006), and we have found that rye cover crops helped to reduce *P. syringae* symptoms incidence on squash leaves (Toussaint et al., personal communication). The mechanism of this protective effect of rye cover crops against squash bacterial leaf spot is not known, but we hypothesize that it may be mediated by cover cropping effects on the plant's microbiota that in turn protects against *P. syringae*.

In this study we used sequence and culture-based approaches to quantify the effects of different cover cropping approaches on bacterial communities on squash leaves infected by *P. syringae*. We first evaluated if cover crops could help to reduce *P. syringae* populations on squash leaf surfaces by direct measurement of pathogen abundance on leaves. We also considered the fruit's health and marketability at harvest in such cropping practices. We then estimated the effects of different cover cropping methods on phyllosphere bacterial communities by quantifying leaf microbiome diversity and composition using a bacterial metabarcoding approach. Finally, we identified the bacterial taxa that were most strongly influenced by cover cropping practices.

1.3 Material & Methods

1.3.1 Experimental design and field treatment

All samples in this study were collected from the Agriculture and Agri-Food Canada L'Acadie Experimental Farm at Saint-Jean-sur-Richelieu, Quebec, Canada (45°17'48.7"N 73°20'14.8"W). The experiment comprised 6 replicates of 4 cover cropping treatments in a fully randomized block design, with a total of

24 plots containing 3 raised beds with a single line of squash each (see Supplemental Figure 1.1). The cover cropping treatments were Rye (Secale cereale) Cover Crop (RCC), Chemically Terminated Rye Cover Crop (CT-RCC), Plastic Cover (PC) and Bare Soil (BS). Specifically, for 2016 and 2017 growing season, the RCC treatment consisted of fall rye (cv Gauthier) seeded at a rate of 250kg/ha on September 14 2015 and September 16 2016 and rolled to the ground the following spring by crimping rye with a 3-sectional roller crimper (I&J Mfg., PA, USA) on June 13 2016 and 2017 (see Supplemental Figure 1.2 for crimping process); the CT-RCC treatment was the same as RCC except rye was killed with a herbicide (glyphosate (Roundup, WeatherMax^{MD}, Bayer, Canada) at a rate of 2.16 kg a.e. ha^{-1}) before the rye crimping (on June 10 2016 and June 8 2017); the PC treatment consisted of the application of an agricultural plastic mulch over each raised bed within plots; and the BS treatment was a bare soil mound lane that did not receive any cover cropping treatment. All the soil was covered by rye and most of the cover crop material remained on the soil during the growing season and, except for a change in color, no significant decay was observed (see Supplemental Figure 1.2). Squash seeding was performed on June 15 2016 and 2017; mechanically with a seeder for the bare soil and both rye treatments, or manually for the plastic cover (see Supplemental Figure 1.2). To monitor treatment effects on pathogen populations, we inoculated squash seeds with a rifampicin-resistant *P. syringae* strain prior to direct seeding into raised beds. While plants are frequently naturally inoculated by seedborne or soil pathogen reservoirs, we needed to ensure potential infection to be able to quantify the effects of the cover cropping treatments. Strain pathogenicity was confirmed by hypersensitive reaction (HR) testing in tobacco leaves and seed inoculation was validated by growing on-field negative control plants at the border of each treatment plot. Control plants were asymptomatic during the entire growing season.

1.3.2 Microbial collection, DNA extraction and sequencing

Microbial communities of the phyllosphere were collected from squash at three different times each growing season in 2016 (July 12, August 1, and September 1), and 2017 (June 12, July 31, and September 5), defined as Early, Mid, and Late season. Each sample consisted of a mix of young and old leaves harvested from the squash canopy by clipping an average of 16.8±8.3 g and 20.7±4.8 g of leaves for years 2016 and 2017 respectively (see Supplemental Table 1.1) from an individual plant into sterile sample bags (SCR-7012-ID, Innovation Diagnostics Inc., Blainville, Canada) with surface-sterilized shears. Replicate samples (3 per plot) were collected for a total of 72 samples per sampling date (3 samples x 4 treatments x 6 replicates). Microbial cells were then gathered by washing each leaf sample with 110 ml of saline buffer [0.85% NaCl] and using a homogeniser blender (Stomacher® 400, Seward, UK) for 30 sec at 250 rpm.

A volume of 1ml of wash solution from each sample was placed on King's B (KB) medium with cycloheximide (50mg/l) (C7698, Sigma Aldrich, Oakville, CA) and rifampicin (50mg/l) (R3501, Sigma-Aldrich, Oakville, CA), allowing us to estimate *P. syringae* population size by counting colony forming units (CFU) after 4 days of growth at 28°C.

The remaining 100 ml wash solution was divided into two 50ml Falcon tubes; one was centrifuged at 11,500 × g for 20 min and the other at 4,500 × g for 20 min. The aqueous phase was removed from both tubes and the pellet in the Falcon tube centrifuged at 4,500 × g was frozen at -80°C. The DNA of the remaining pellet was extracted using MoBio PowerSoil DNA extraction kits (CA-11011-418, VWR, Mont-Royal, CA) and stored at -20°C for future processing. Amplicon libraries were prepared for Illumina sequencing using PCR targeting the V5–V6 region of the bacterial 16S rRNA gene using cyanobacteria-excluding primers [16S primers 799F-1115R] (Chelius et Triplett, 2001 ; Redford *et al.*, 2010) to exclude chloroplast DNA (for a 16S amplicon structure overview, see Supplemental Figure 1.3). The 25 µL PCR reactions consisted of 5 µL 5x HF buffer (Thermo Scientific, Waltham, MA, USA), 0.75 µL DMSO, 0.5 µL dNTPs (10 mM each), 0.25 µL Phusion Hot Start II polymerase (Thermo Scientific), 1 µL each primer (5 µM), 1 µL of genomic DNA, and 15.5 µL molecular-grade water (IDT, Coralville, IA, USA). We included a negative control (1 µl of sterile water; IDT, Coralville, IA, USA) as well as a positive control (1 µl of *P. syringae* DNA) in each 96 well library plate. Libraries were checked on Agarose gels (2%), normalized with SequalPrep kit (A1051001, Life Technologies, Burlington, CA) on a Gilson robot (Middleton WI, USA) and sequenced on a MiSeq (Illumina, San Diego, CA, USA). For each year, samples were randomly assigned between two sequencing runs, representing a total of 4 runs.

1.3.3 Sequence analysis

Sequencing adaptors were removed with the bbduk tool from bbmap (38.86, https://sourceforge.net/projects/bbmap/), with the following parameters: ktrim=r k=23 mink=11 hdist=1 tpe tbo (Bushnell, 2014). Sequences were thereafter demultiplexed allowing one mismatch on the barcode sequence with deMulMe (https://github.com/RemiMaglione/ genomicScript/tree/master/deMulMe). Sequence barcodes were removed with cutadapt 2.10 in paired-end mode (Martin, 2011). In total we obtained 11,929,677 and 11,816,138 demultiplexed paired-end sequences, for 2016 and 2017 respectively. All subsequent data processing and computations were done with DADA2 1.12.1 (Callahan *et al.*, 2016) in R 3.6.0 (RC Team, 2013) and graphs were produced using the ggplot2 3.2.1 package (Wickham, 2016, p. 2). Sequences were trimmed and quality filtered with filterAndTrim with default parameters

except: trimLeft = c(19, 26), truncLen = c(230, 210), maxEE=c(2,3); trimleft was set to remove PCR primers used for library preparation, truncLen and maxEE were set to yield filtered sequences with a quality around a phred score of 30. Amplicon sequence variants (ASVs) were constructed from filtered sequences with the following set of built-in DADA2 functions and their default parameters except as mentioned: dada in pseudo-pooling mode, mergePairs with minOverlap = 30, collapseNoMismatch with minOverlap = 240, removeBimeraDenovo with method="pooled". ASVs were then taxonomically annotated by assignTaxonomy with the SILVA version 128 database (Quast *et al.*, 2013 ; Yilmaz *et al.*, 2014). For 2016 and 2017 data, the DADA2 pipeline yielded a mean of 10,484,056 filtered paired-end sequences used to identify 7,604 ASVs, which represent an average of 29,652 sequences and 165 ASVs per sample.

A preliminary evaluation of control samples was performed by comparing the composition of control and phyllosphere samples using a principal component analysis ordination of a distance matrix obtained with centred log ratio (clr) transformation of the original community matrix (Gloor, 2016). Since negative control samples would be lost by excluding samples with very few sequences, the clr transformation allowed us to keep all the samples while identifying outlier samples. Since the positive and negative control samples were distinct compositionally from the phyllosphere samples (see Supplemental Figure 1.4), they were removed from all further analyses. Positive control samples were examined and determined to be dominated by ASVs corresponding to the expected mock community species composition.

1.3.4 Data analysis

1.3.4.1 *P. syringae* abundance analysis on squash leaves

P. syringae count differences between treatments were evaluated with a linear mixed model of treatment effects on *P. syringae* abundance, for 2016 and 2017 data. Effects of experimental blocks were integrated as a random effect. Effect of treatment was estimated with a Tukey's honest significance (TukeyHSD) post-hoc test performed on the above-mentioned model.

1.3.4.2 Squash fruit health and marketability at harvest

We quantified squash fruit health and marketability by harvesting fruit within a 10m x 10m area within each plot. Fruit health and marketability was determined with 4 categories of *P. syringae* symptoms based on the visually estimated proportion of fruit affected by the considered symptoms: P.*syringae* symptoms outside of the fruits, P.*syringae* symptoms that penetrate the fruits, P.*syringae* symptoms that left a scar

at the surface of the fruits and P.*syringae* symptoms that generate squash rot. Marketability was assessed based on these categories of *P. syringae* symptoms, where more than 1% of fruit affected in at least one category prevents marketability. This cut-off was chosen to address the actual market plasticity, where squash fruits with low symptoms can still be sold. Healthy fruit was defined as a squash fruit with no *P. syringae* symptoms. Thus, marketability and fruit health were binomially distributed and their differences among treatments were evaluated with generalized mixed linear model, where blocking effect was integrated as random variable, for both years. Effects of treatment on fruit health and marketability was estimated with a TukeyHSD post-hoc test (using the glht function of multcomp R package) performed on the above-mentioned model.

1.3.4.3 Effect of cover cropping treatments on bacterial community diversity

Diversity analyses were performed using the R package phyloseq 1.30.0 (McMurdie et Holmes, 2013), picante 1.8.1 (Kembel *et al.*, 2010), and vegan 2.5-6 (Oksanen *et al.*, 2007). To evaluate the effect of treatments on community diversity on squash leaves, samples were randomly rarefied to 5000 sequences per sample: this threshold was chosen to preserve the maximum number of samples with a sufficient quantity of ASVs to capture the majority of the diversity in each sample (see Supplemental Figures 1.5 and 1.6). For all diversity analyses, rarefactions and their subsequent analyses were repeated 1000 times but no qualitative differences were observed between iterations, and so we report here the results of a single random rarefaction of the data. The uniformity of relative abundance distributions of ASVs (alpha diversity) was assessed with the Shannon index (Haegeman *et al.*, 2013). The effect of treatment on alpha diversity was evaluated with a post-hoc test (TukeyHSD) of a linear model (alpha diversity as a function of treatment). Variation in bacterial community structure among samples was quantified with the Bray-Curtis index (Bray et Curtis, 1957). Major gradients in community composition were evaluated with nonmetric multidimensional scaling (NMDS) ordination of weighted Bray-Curtis distances among samples. We partitioned the variance in phyllosphere bacterial community structure explained by sampling date and treatment using permutational analysis of the variance (PERMANOVA) in Bray-Curtis dissimilarities, and linear mixed models of sample scores on the NMDS ordination axes. We evaluated whether treatments were associated with compositionally distinct groups of samples with a least squares comparison (emmeans v1.4.8 R package; Lenth et al., 2018) of a linear mixed model with NMDS axis scores as a function of treatment (fixed effect) with experimental block as random effect.

1.3.4.4 Differential abundance analysis of ASVs

Differential abundance analysis of ASVs among treatments were performed with DeSeq2 3.11 (Love *et al.*, 2014). The ASV matrix was filtered using the CoDaSeq R package 0.99.4 (Gloor, 2016), with the codaSeq.filter function with the following parameters: min.reads=1000 (minimum reads per sample), min.prop=0.00001 (minimum proportional abundance of a read in any sample), min.occurrence=0.005 (minimum fraction of non-zero reads for each variable in all samples). Since DeSeq2 takes non-zero positive integers as input, we transformed the abundance matrix to pseudocounts by adding 1 to each cell in the matrix prior to analysis (Nearing *et al.*, 2021). DeSeq2 analysis was executed with parameters recommended for single-cell analysis that better fit data with a zero-inflated negative binomial distribution such as our community matrix. We tested for differential abundance by contrasting ASV abundances across all six possible treatment comparisons: Rye Cover Crop versus Chemically Terminated-Rye Cover Crop, Rye Cover Crop versus Plastic Cover, Rye Cover Crop versus Bare Soil, Chemically Terminated-Rye Cover Crop versus Plastic Cover, Chemically Terminated-Rye Cover Crop versus Bare Soil and Plastic Cover versus Bare Soil. We used the following model: design = ~ block + treatment and the blocking random variable was controlled through the reduced parameter. Only contrasts with adjusted P-value<=0.01 and $\log_2$-fold-change ≥ 1 were considered to be significantly differentially abundant. Taxonomic annotations were set at the genus level for the differential abundance analysis of ASVs. Phylogenetic trees have been built with Fastree2 2.1.3 (Price *et al.*, 2010) on ASVs sequences alignment with QiiME1 (PyNAST as default method ; Caporaso et al., 2010a, 2010b).

1.4 Results

1.4.1 Cover cropping reduced *P. syringae* abundance on squash leaves and improved fruit health and marketability.

We found that *P. syringae* was less abundant on the leaves of squash grown with rye cover crops (Figure 1.1) and harvested squash fruits were more marketable and healthier with rye cover crop treatments (Table 1.1). In 2016, *P. syringae* population size was significantly lower for the Rye Cover Crop treatment compared to Plastic Cover and Bare Soil treatments during the Early season (Tukey HSD post-hoc on linear model; Figure 1.1). There were no significant differences among treatments during Mid and Late season sampling in 2016. On the other hand, in 2017, *P. syringae* population size was significantly lower during the Early season for both rye cover crop treatments (Rye Cover Crop and Chemically-Terminated Rye Cover

Crop) compared to Plastic Cover and Bare Soil treatments (Tukey HSD post-hoc test on linear mixed model; Figure 1.1). No *P. syringae* colonies were retrieved at Mid season on the squash leaves grown with rye cover crops, and pathogen populations were lower for Chemically-Terminated Rye Cover Crop as compared to Plastic Cover and Bare Soil treatments. Finally, pathogen viable cells counts were significantly lower in Rye Cover Crop as compared with Bare Soil at Late season sampling. Taken together, *P. syringae* populations showed the greatest reduction in the rye cover crop treatments early in the growing season. This result was consistent during both years (see Supplemental Tables 1.2 and 1.3). Moreover, fruits health and marketability were significantly different across all years of harvest ($p<0.05$, Tukey HSD). Indeed, marketability with Rye Cover Crop was significantly different from Bare soil and Plastic Cover. Average marketability with Rye Cover Crops was 13% higher in 2016 and 6.2% higher in 2017 as compared to Bare Soil, and 8% higher in 2016 and 4.3% higher in 2017 as compared to Plastic Cover (Table 1-1). Fruit health increased 14% in 2016 with Rye Cover Crops treatment as compared to Bare Soil and 13% in 2017 with Chemically-Terminated Rye Cover Crop treatment as compared to Bare Soil (Table 1-1). No further significant differences among treatments were observed for fruit health and marketability in both years of harvest.

Table 1.1: Proportion of squash fruit (mean +- standard deviation) with no *P. syringae* symptoms and marketable fruits with no damage for the two-growing seasons 2016 and 2017. Differences among treatments were tested using TukeyHSD test, based on a generalized mixed model with General Linear Hypotheses provided by glht function of multcomp package. Within each year, treatments that do not share a letter were significantly different according to the Tukey HSD test ($p<0.05$).

Year	Plastic Cover	Rye Cover Crop	Chemically-Terminated Rye Cover Crop	Bare Soil
Proportion of squash fruit without *P. syringae* symptoms (%)				
2016	56.8 ± 6.7 a**b**	63.2 ± 7.7 **a**	59.7 ± 10.4 a**b**[1]	49.2 ± 9.3 **b**
2017	74.0 ± 13.0 a**b**	73.2 ± 7.8 a**b**	78.7 ± 12.1 **a**	65.7 ± 7.7 **b**
Proportion of marketable squash fruit with no damages (%)				
2016	79.5 ± 5.4 **a**	87.5 ± 5.8 **b**	81.3 ± 3.4 a**b**	74.5 ± 7.3 **a**
2017	91.2 ± 7.2 **b**	95.5 ± 3.4 **a**	94.2 ± 3.0 a**b**[2]	89.3 ± 3.0 **b**

[1]Difference between CT-RCC and Bare soil was marginally significant (p=0.057). [2]Difference between CT-RCC and Bare soil was marginally significant (p=0.064)

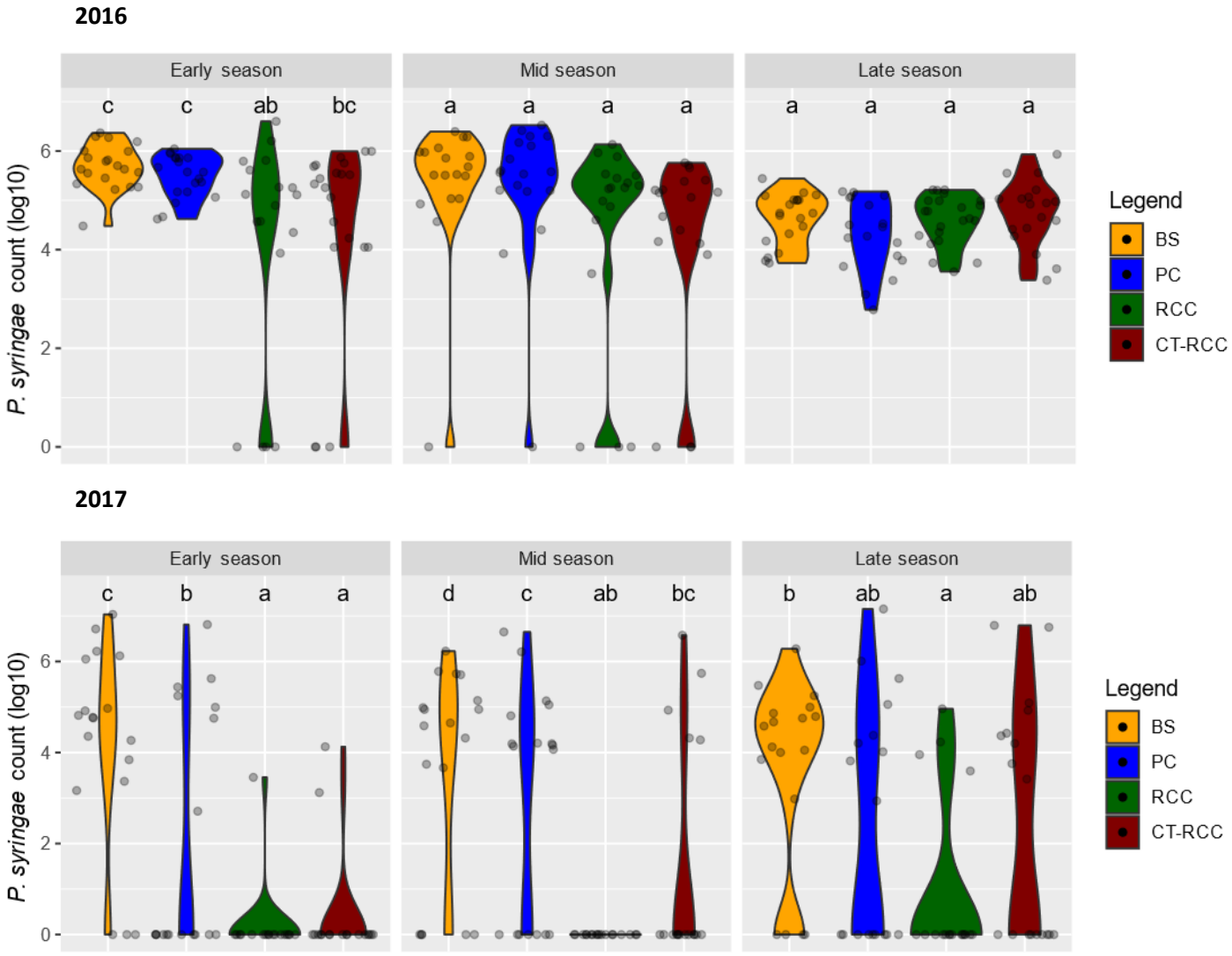

Figure 1.1 : *P. syringae* populations on squash leaves for different cover cropping practices during 2016 and 2017. Squash pathogen population sizes were estimated based on CFU count from bacterial culture of each leaves sample retrieved from 4 cropping treatments: Bare Soil (BS), Plastic Cover (PC), Rye Cover Crop (RCC) and Chemically Terminated Rye Cover Crop (CT-RCC). Different letters represent significantly different treatments ($p<0.05$) from a post-hoc test (TukeyHSD) of a linear model (*P. syringae* as a function of treatment) in 2016 or a linear mixed model (*P. syringae* as a function of treatment (fixed effect) and block (random effect) in 2017.

1.4.2 Phyllosphere microbial communities differed between sampling dates and treatments

Cover cropping treatments influenced bacterial community composition on squash leaves. Treatments also affected bacterial diversity and richness (see Supplemental Analysis 1.1 with Supplemental Figures 1.7 & 1.8). A nonmetric multidimensional scaling ordination of the overall community distance matrix suggests that squash phyllosphere samples clustered by sampling dates in both years (see Supplemental Figure 1.9); sampling date accounted for 28% (R^2 = 0.28, $p<0.001$) and 11% (R^2 = 0.11, $p<0.001$) of community compositional variation between samples for 2016 and 2017 respectively (PERMANOVA on Bray-Curtis distances among samples). Because there was an interaction between sampling date and treatments (PERMANOVA on Bray-Curtis distances among samples; sampling date * cover crop treatment interaction $P<0.001$ both years), and sampling date accounted for the majority of the effect, we thus analyzed the effect of treatments on communities separately for each date in order to summarize these complex effects.

1.4.3 Cover cropping treatments influence squash phyllosphere community diversity and composition

Bacterial community alpha diversity was significantly different among treatments for several sampling dates (linear model; Shannon index vs. cover cropping treatment, $P<0.05$; Figure 1.2). Bacterial community alpha diversity was higher for both rye treatments as compared to bare soil and plastic treatments in 2016 in the Early season sampling (Tukey HSD on linear model, $P<0.05$; Figure 1.2). No further differences were observed between treatments at the other sampling dates in 2016. In 2017, although both rye cover cropping practices resulted in significantly lower alpha diversity in Mid season sampling as compared to Bare Soil, their alpha diversity was higher as compared to plastic treatments in Early season sampling. No further differences were observed between treatments in late season sampling of 2017. Taken together alpha diversity increased early in the growing season for both rye cover cropping treatments as compared to bare soil and plastic treatments of 2016 or to the plastic treatment of 2017.

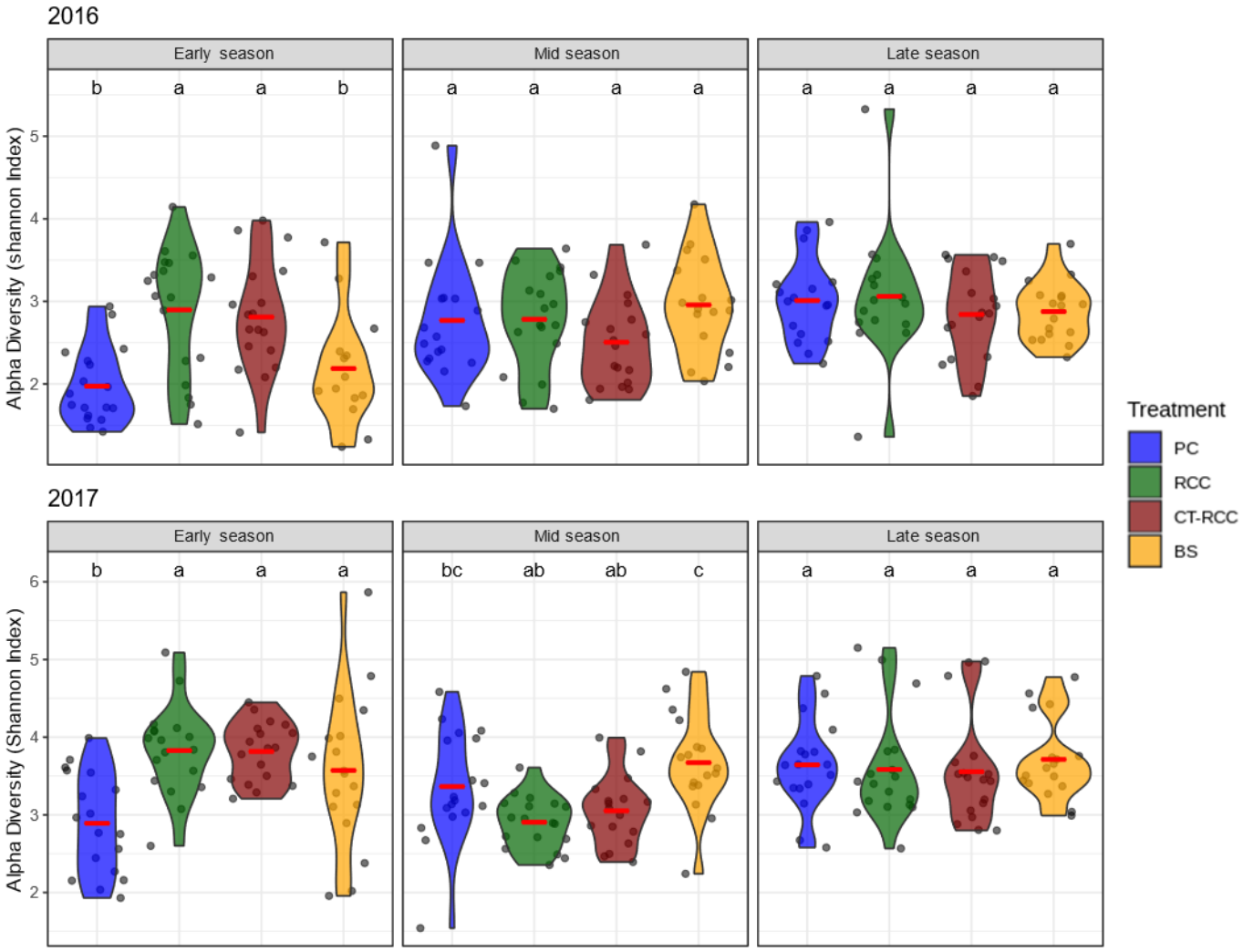

Figure 1.2 : Alpha diversity (Shannon index) of phyllosphere bacterial communities for each treatment and each sampling date during the growing season of years 2016 and 2017. Horizontal red line represents the mean. Blue: PC (Plastic Cover), green: RCC (Rye Cover Crop), red: CT-RCC (Chemically Terminated Rye Cover Crop) and yellow: BS (Bare Soil). Different letter represents significantly different treatments ($p<0.05$) from a post-hoc test (TukeyHSD) of a linear model (alpha diversity as a function of treatment).

Community composition varied among cover cropping treatments for each sampling date of 2016 and 2017 (PERMANOVA on Bray-Curtis distances for each sampling date; cover cropping effect $P<0.001$). Moreover, distances between treatment clusters visible in the ordination (Figure 1.3) suggests that treatment effects were more important for Early season sampling as compared to the other sampling dates. Differences in community composition among treatments were more pronounced in Early (PERMANOVA on Bray-Curtis distances; effect of cover cropping treatment $P_{2016\ \&\ 2017}<0.001$; $R^2_{2016} = 0.24$, $R^2_{2017} =0.31$) rather than Mid ($P_{2016\ \&\ 2017}<0.001$; $R^2_{2016} = 0.14$, $R^2_{2017}=0.30$) and Late season sampling ($P_{2016\ \&\ 2017}< 0.001$; $R^2_{2016} = 0.07$, $R^2_{2017}= 0.16$) (Figure 1.3).

Ordination of samples based on community composition also indicated that samples clustered into two compositionally distinct groups: Rye and Chemically-Terminated and Rye Cover Cropping, versus Plastic and Bare Soil (Figure 1.3). At each sampling date, sample scores on the first axis of the ordination differed significantly among treatments (linear mixed model with NMDS axis scores as a function of treatment (fixed effect) with experimental block as random effect). Rye and Chemically-Terminated Rye were different from the Plastic and Bare Soil, but not different from each other (least squares comparisons on the above linear mixed model; Supplemental Figure 1.10). Moreover, these two compositionally distinct groups (Rye and Chemically-Terminated Rye versus Plastic and Bare Soil) are more different during the Early season as compared to every other sampling date: these community compositional differences remained throughout the growing season, although they progressively decreased in magnitude (least squares comparisons intervals between these two groups are closer throughout the growing season on the first axis; Supplemental Figure 1.10). However, plastic and bare soil remained different in Mid season in 2017 and were often separated along the second axis of the NMDS ordination (Figure 1.3; estimated marginal means test of ordination axis scores; Supplemental Figure 1.10).

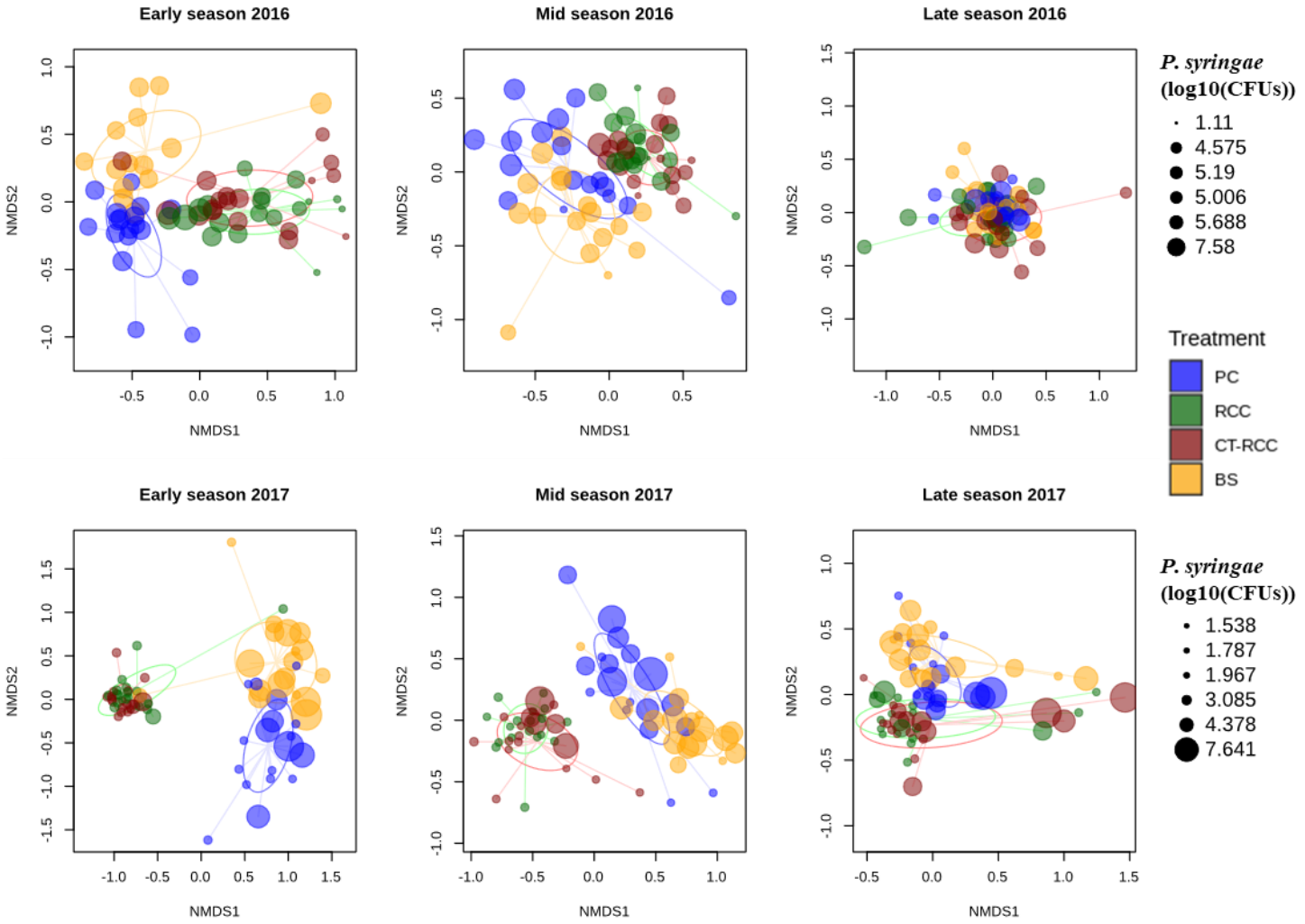

Figure 1.3 : Non-metric multidimensional scaling (NMDS) ordination of bacterial community composition in squash phyllosphere samples from different cover cropping treatments in 2016 and 2017. Each point represents a phyllosphere community; symbol size indicates the abundance of *P. syringae* colony forming units (log10(CFUs)) in that sample; colors indicate the cover cropping treatment: blue: PC (Plastic Cover), green: RCC (Rye Cover Crop), red: CT-RCC (Chemically Terminated Rye Cover Crop) and yellow: BS (Bare Soil).

1.4.4 *Sphingomonas* and *Methylobacterium* were more abundant with cover crops treatments

An analysis of differential abundance of ASVs among treatments and sampling dates identified several ASVs that were more abundant in certain treatments and at certain times. As mentioned previously, the abundance of *P. syringae* and the squash phyllosphere communities were influenced by the sampling date, thus the cover crop effect on taxa abundance was analyzed separately for each sampling date. To identify ASVs that were strongly associated with different cover cropping systems we took the top differentially abundant ASVs with the highest log_2-fold change in abundance for each treatment comparison (2017: Figure 4, 2016: Supplemental Figure 1.11). Different cover cropping treatments had several differentially abundant ASVs with log_2-fold changes in abundance between treatments ranging from -10.2 to 9.7. Overall, the contrasts of Rye versus Chemically-Terminated Rye, and Plastic versus Bare Soil consistently had few and weakly differentially abundant ASVs. Conversely, contrasts of both rye treatments versus Plastic and Bare soil exhibited more and strongly differentially abundant ASVs. In 2016, ASVs that were significantly more abundant for Rye and Chemically-Terminated Rye Cover Crop treatments included those annotated at the genus level as *Rhizobium*, *Pseudomonas* and *Saccharibacillus* during the Early season, and *Chryseobacterium* and *Sphingomonas* during the Mid season. Conversely, ASVs that were significantly more abundant in Bare Soil and Plastic Cover treatments included those annotated as *Pseudarthrobacter* during the Early season, *Exiguobacterium* and *Pseudathrobacter* during the Mid season, and *Deinococcus* during the Late season. In 2017, ASVs that were significantly more abundant for Rye and Chemically-Terminated Rye Cover Crop treatments included those annotated as the genera *Sphingomonas*, *Methylobacterium* or Hymenobactrium during the Early season, *Sphingomonas*, *Methylobacterium*, *Aureimonas* and *Microbacterium* during the Mid season, and *Chryseobacterium* and *Rhizobacterium* during the Late season. On the other hand, ASVs that were significantly more abundant in Bare Soil and Plastic Cover treatments included *Massila* and *Exiguobacterium* at Early season, *Massila*, *Exiguobacterium*, *Hymenobacter*, *Deinococcus* and *Pseudarthrobacter* at Mid season and *Deinococcus* and *Microbacterium* at Late season.

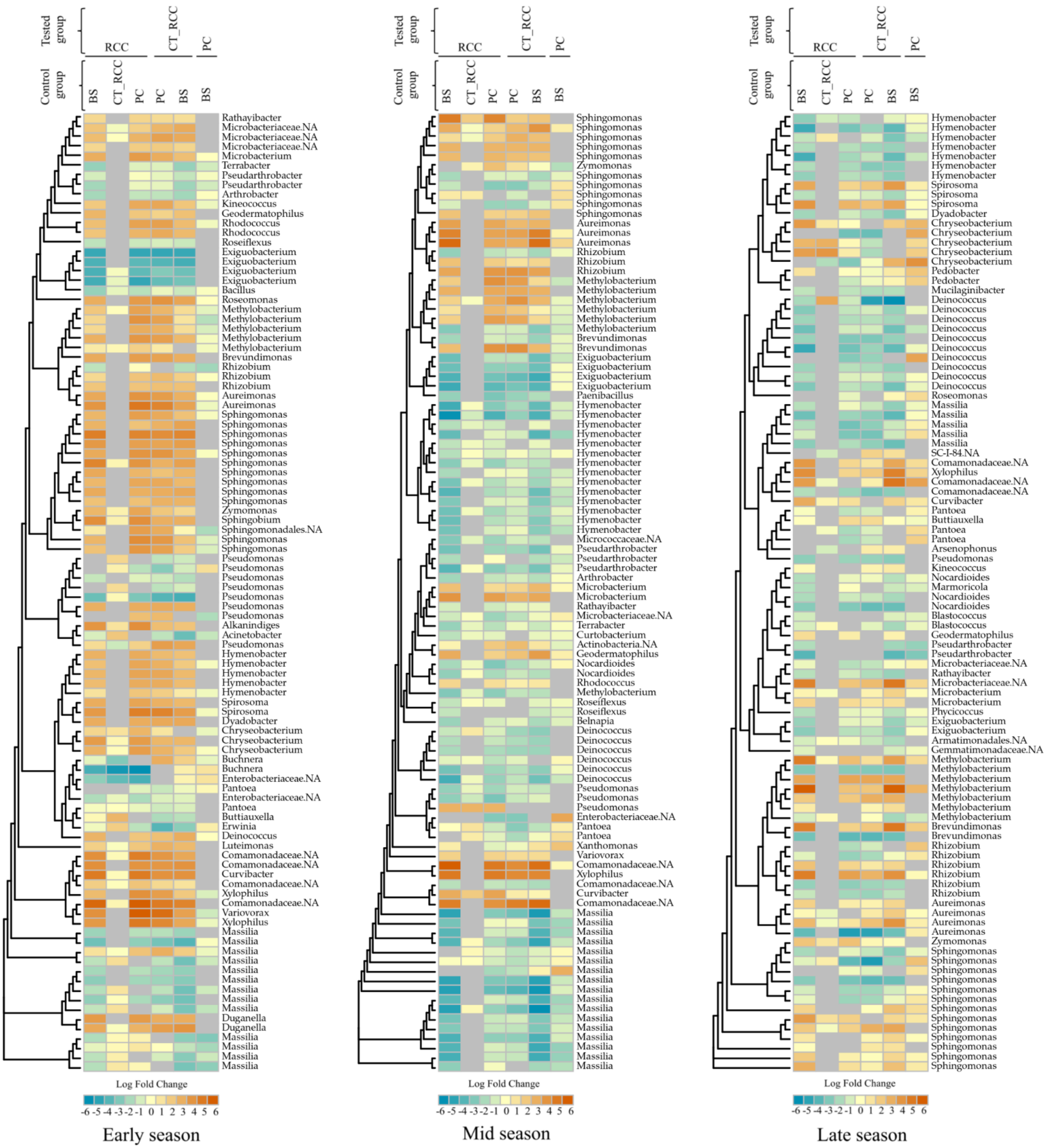

Figure 1.4: Log$_2$-fold change (LFC) heatmap of most differentially abundant ASV from DeSeq2 analysis for each sampling date of the 2017 samples. For each panel, the left track is the phylogenetic tree from

PyNAST alignment of ASVs sequence while the right track is the corresponding taxonomic genus name. Each heatmap column is a different contrast between two treatments mentioned in the header as follow: the upper name is the "tested" treatment whereas the lower name is the "control" treatment meaning that a positive LFC value represents an ASV more abundant for the tested treatment. Grey color represents no LFC for the ASV. Each number on the bottom LFC colour scale represents a level of LFC. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and yellow: BS (Bare Soil).

1.5 Discussion

Rye cover cropping reduced the abundance of *P. syringae* on squash leaves, improved the health and marketability of fruit, and shaped phyllosphere bacterial community composition and diversity. The greatest effect of cover cropping on both the phyllosphere community and *P. syringae* abundance was observed early in the growing season. *P. syringae* begins life on leaves as an epiphyte but then must colonize host tissue through stomata or wounds (Misas-Villamil *et al.*, 2013). Disease severity could be lowered if the early establishment and survival of *P. syringae* is compromised.

We found that both Rye and Chemically-Terminated Rye Cover Cropping treatments induced a strong shift in the squash phyllosphere microbiota, leading to a distinct community composition in comparison with Bare Soil and Plastic Cover treatments. The largest difference in leaf bacterial community composition associated with rye cover crops was observed early in the growing season. Our results are consistent with previous reports of homogenisation of phyllosphere community structure over time; early in the growing season, the leaf microbiota was more diverse and colonization from the soil is likely a strong driver of the phyllosphere microbiome (Copeland *et al.*, 2015). Moreover, shifts in microbial community composition are likely driven by changes in environmental conditions as well as shifts in sources of bacterial migration to the phyllosphere. Cover cropping can directly modify soil abiotic properties such as temperature and moisture and chemical properties (Villamil *et al.*, 2006). Cover cropping also likely influences bacterial dispersal sources both by promoting colonization by bacteria living on the cover crops themselves, as well as through their effects on dispersal from different potential sources such as soils (Bodenhausen *et al.*, 2013), water splash (Butterworth et McCartney, 1991) and insects (Manirajan *et al.*, 2016 ; Vega et Herrera,

2013). Taken together, we hypothesize that such local environmental shifts modify bacterial migration to the phyllosphere early in the growing season. We further speculate that an early shift in squash phyllosphere may intervene by mechanical transfer from the cover crop, when the young plant goes through the rye mulch.

In addition to the effects of cover cropping on phyllosphere microbial communities, cover cropping treatments also likely influenced environmental conditions, which may explain part of their protective effects against *P. syringae*. Cover crops influence humidity and temperature (Teasdale et Mohler, 1993). Moreover, dispersal of epiphytic bacteria is a function of humidity (Lindemann et Upper, 1985), and the structure of phyllosphere bacterial communities is significantly influenced by soil temperature (Ren *et al.*, 2015). We observed that soil moisture and temperature varied among cover cropping treatments; soil moisture was higher under rye cover crops and plastic cover relative to bare soils, and temperatures were elevated under plastic cover relative to other treatments (results not shown). Rye is also known to have allelopathic properties (Schulz *et al.*, 2013), which can influence soil microbiota (Hu *et al.*, 2018). Moreover, rye degradation can lead to decreases in soil pH (Abdollahi et Munkholm, 2014) and improve weed control (Barnes et Putnam, 1983). During this experiment, no weeds were found for the plastic cover treatment, a few grew in both rye cover treatments, while many were found in the bare soil treatment (all weeds were manually removed on a routine basis during the growing season). All of these effects of cover cropping on the abiotic and biotic environment could influence early pathogen development and interact with shifts in phyllosphere microbiota to modulate the potential protective effects of cover crops. Furthermore, since *P. syringae* may likely be seed borne (reviewed in Hirano and Upper, 2000), we inoculated seeds with this pathogen at the time of planting, but in real situations, the effect of cover crops on pathogen populations will also be a function of temporal variation in seed and soil pathogen and microbiota reservoirs.

Previous studies have reported a protective effect against pathogens by phyllosphere microbial diversity per se (Keesing *et al.*, 2010), for example where increasing diversity of *Sphingomonas* genus on leaves increased protection against *P. syringae* (Innerebner *et al.*, 2011). We did not find strong evidence for an effect of alpha diversity on its own to explain the protective effect of cover cropping against *P. syringae*; there were no overall differences in alpha diversity of phyllosphere bacteria among cover cropping treatments, although rye cover cropping did increase diversity in the early season. As neighboring plants shape phyllosphere diversity (Meyer *et al.*, 2022), we hypothesize that the presence of a cover crop might

have increases local diversity by bacterial migration from the rye material to the squash phyllosphere, early in the growing season. To properly test for a protective effect of phyllosphere diversity against *P. syringae*, future studies that directly manipulate diversity while keeping other factors constant will be required, but our results suggest that it was the composition of bacterial communities and not the diversity of the community per se that could explain the protective effects of cover cropping treatments.

Our results support the hypothesis that rye cover crops could protect against *P. syringae* by promoting the establishment of potential competitors or plant growth promoting bacteria (PGPB) on the leaf surface. This included several ASVs belonging to the genus *Sphingomonas*, which were more abundant with rye cover cropping, especially early in the growing season. Previous studies have demonstrated that *Sphingomonas* strains protect *Arabidopsis* against *P. syringae* in a controlled environment (Innerebner *et al.*, 2011). Our findings provide field-based evidence suggesting that the *Sphingomonas* clade is a potential competitor, given the increased abundance of this genus in the protective rye cover crop treatments. We also found many other taxa preferentially associated with squash under rye cover cropping treatments, including ASVs belonging to the genus *Methylobacterium* that is a phyllosphere-associated clade (Delmotte *et al.*, 2009) known to be an important PGPB in agriculture (Madhaiyan *et al.*, 2006), and non-pathogenic ASVs belonging to the genus *Pseudomonas*, which has been shown to be an antagonist of the pathogens Erwinia (Cabrefiga Olamendi *et al.*, 2007), Tobacco Necrosis Virus (Maurhofer *et al.*, 1998), and *Botrytis cinerea* (De Meyer et Höfte, 1997). Thus, rye cover crops appear to favor the establishment of potentially plant beneficial bacteria in the phyllosphere. While our results need to be followed up with more experimental tests to quantify the potential benefits of these genera, the potentially beneficial bacterial ASVs associated with rye cover crops that we have identified are already candidates for exploration of microbiome engineering approaches to directly inoculate protective bacterial strains to protect crops against pathogens (Quiza *et al.*, 2015b).

1.6 Availability of data and materials

The demultiplexed sequence data have been deposed as sequences read archive under the BioProject: PRJNA705113. The scripts used to perform analyses for the current study are available in a GitHub repository: https://github.com/RemiMaglione/Science-Communication/tree/main/Article/cover-crop-squash-phyllosphere-microbiota-2021

1.7 Acknowledgements

We thank Katherine Bisaillon, Pierre-Olivier Hebert and Jean-Baptiste Leducq for their help in the field, in the lab, with analysis and for discussions.

CHAPITRE 2

Functional characterization of bacterial communities on rye cover crop grown squash (*Cucurbita pepo*)

Cet article est en processus de soumission dans le journal Phytobiomes

Dans ce chapitre (Résumé).

Les cultures de couverture sont largement utilisées en agriculture durable, mais leurs effets sur les interactions entre les plantes et les micro-organismes, ainsi que sur le contrôle des pathogènes, ne sont pas bien compris. Dans cette étude, nous avons utilisé le séquençage métagénomique *shotgun* pour étudier l'impact des cultures de couverture de seigle sur la communauté bactérienne de la phyllosphère des plants de courge et sur leurs voies métaboliques. Nous avons constaté que les cultures de couverture de seigle ont un impact significatif sur les taxons et les fonctions de la communauté bactérienne, entraînant des améliorations potentielles dans le biocontrôle des pathogènes. Les cultures de couverture ont enrichi les voies métaboliques liées à la dégradation des composés antimicrobiens, à la biosynthèse des phospholipides et de la paroi cellulaire, suggérant que les traitements au seigle peuvent avoir influencé les interactions entre les plantes et les micro-organismes et favorisé un microbiome de la phyllosphère capable d'améliorer la santé des plantes. Nos résultats suggèrent que la réduction des symptômes de *Pseudomonas syringae* pv *lachrymans* par les traitements aux cultures de couverture est probablement due à la promotion de taxons bactériens et de voies métaboliques spécifiques dans la phyllosphère de la courge. Notre étude offre des aperçus précieux sur l'utilisation des cultures de couverture en agriculture durable et souligne l'importance de comprendre la composition taxonomique et fonctionnelle des communautés microbiennes associées au contrôle des agents pathogènes.

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2.1 Abstract

Cover crops have been widely used in sustainable agriculture, but their effects on plant-microbe interactions and pathogen control are not well understood. In this study, we used metagenomic *shotgun* sequencing to investigate the impact of rye cover crops on the phyllosphere bacterial community of squash plants and their metabolic pathways. We found that rye cover crops significantly impacted bacterial community taxa and functions, leading to potential improvements in pathogen biocontrol. Cover crops enriched metabolic pathways related to antimicrobial compound degradation, phospholipid, and cell wall biosynthesis, suggesting that rye treatments may have influenced plant-microbe interactions and selected for a phyllosphere microbiome competent to improve plant health. Our results suggest that the reduction in *Pseudomonas syringae* symptoms by cover crop treatments is likely due to the promotion of specific bacterial taxa and metabolic pathways in the squash phyllosphere. Our study provides valuable insights into the use of cover crops in sustainable agriculture and highlights the importance of understanding the taxonomic and functional composition of microbial communities associated with pathogen control.

2.2 Introduction

Cover crops are increasingly used in agricultural practices for their numerous benefits, including improved soil health (reviewed in Sharma et al. 2018), reduced erosion (Dabney *et al.*, 2001), and weed control (Haramoto et Gallandt, 2004). However, the impact of cover crops on the phyllosphere bacterial community of crops is not yet fully understood. The phyllosphere is a crucial interface between the plant and the environment, consisting of the above-ground plant parts such as leaves, stems, and fruits, and the associated microbial communities. These communities play a critical role in plant health (Liu *et al.*, 2020), growth and productivity by influencing nutrient cycling, plant hormone regulation, and pathogen control (reviewed in Vorholt 2012).

Squash (*Cucurbita pepo*) is grown worldwide and is susceptible to bacterial diseases that can significantly affect yield (Salehi *et al.*, 2019). *Pseudomonas syringae*, a bacterial pathogen that causes bacterial leaf spot, is one of the most important squash pathogens and despite attempts to biologically control this pathogen (Innerebner *et al.*, 2011 ; Lindemann et Suslow, 1987), it is primarily managed through copper application which has resulted in the development of resistance (Bender et Cooksey, 1986). However, the phyllosphere bacterial community of squash plants grown on cover crops has been shown to play a role in plant health and disease resistance (Maglione et al. 2022, Maglione et al. 2023). Therefore, in the context of the scarcity of pesticides effective against *P. syringae* on squash, the emergence of genetic resistance, and public concerns about reducing the utilisation of synthetic pesticides, an understanding of the impact of cover crops on the phyllosphere bacterial community of squash plants is of great importance.

Two sequencing approaches are currently widely used to profile bacterial communities: targeted sequencing by PCR-enriching the bacterial 16S rRNA gene prior to sequencing, and untargeted metagenomic *shotgun* sequencing. While targeted 16S sequencing is well suited for a greater number of samples due to its lower cost per sample as compared to *shotgun* metagenomic sequencing, it lacks taxonomic resolution and frequently cannot provide genus or species level identification. A fundamental limitation lies in the choice of the barcode region itself. Indeed, it has been shown that each 16S region has its own specific sequence clustering and taxonomic resolution capabilities (D'Amore *et al.*, 2016). In addition, while there are methods that attempt to infer function from 16S sequences (Aßhauer *et al.*, 2015 ; Huttenhower *et al.*, 2013), there is debate about the accuracy of these methods, and in any case these approaches generally do not work well when the microbiome being targeted is something other than the human gut microbiome due to a lack of reference data on microbes with fully sequenced genomes in most

other habitats (Lajoie et al. 2019; Douglas et al. 2020). In contrast with targeted sequencing methods, *shotgun* metagenomic sequencing is a method that involves untargeted sequencing of all the genetic material in a sample, including in our case both microbial and host DNA. As opposed to targeted sequencing methods, in metagenomic *shotgun* sequencing, DNA in the sample is not amplified nor targeted using specific primers. Thus, metagenomic *shotgun* sequencing allow the characterisation of both the taxonomic and functional composition of entire microbial communities including diverse taxa such as bacteria, archaea, fungi, and viruses (Lajoie *et al.*, 2020 ; Pavlopoulos *et al.*, 2023), de novo assembly of genes, identification of metabolic pathways (Methe *et al.*, 2020), and metagenome assembled genomes (MAG) (Su *et al.*, 2022).

In a previous study (Maglione *et al.*, 2022) we used bacterial 16S rRNA gene amplicon sequencing to evaluate the effect of cover crops on squash phyllosphere bacterial communities. We demonstrated that cover crops caused a strong shift of the bacterial community taxonomic structure and diversity, specifically early in the growing season. This effect was followed by a decrease in *P. syringae* populations leading to a reduction of the symptoms and increased fruit marketability. Moreover, rye cover crops promoted the establishment of bacterial genera known for their biocontrol capabilities. We recently used *In vivo* competition trials to show that cover crops select strains competent to reduce pathogen symptoms on squash (Maglione *et al.*, 2023). However, we were only able to characterize the impacts of cover crops on bacterial taxa at the genus level, and the impact of cover crops on microbial function in the phyllosphere remains unknown. In the current study we hypothesize that the use of rye cover crops will affect both the taxonomic and functional composition of phyllosphere microbial communities.

To test our hypothesis, we conducted a metagenomic *shotgun* sequencing analysis to compare the phyllosphere bacterial communities of squash (*Cucurbita pepo*) grown with rye cover crops (RCC) and chemically terminated rye cover crops (CT-RCC) with those grown on bare soil (BS) and under plastic cover (PC). The RCC and CT-RCC cover crop treatments were chosen as they represent the two most common rye cover crop management practices in Quebec and eastern North America, and the use of plastic cover and bare soil serves to represent common alternative squash cultivation practices. We first re-evaluated the effect of these cropping practices on squash phyllosphere microbiomes by quantifying the taxonomic diversity and composition of the phyllosphere bacterial community. We then estimated if taxonomic annotations yielded by metagenomic *shotgun* sequencing were similar to those obtained from 16S gene

amplicon sequencing. (Maglione *et al.*, 2022). Finally, we quantified the impact of cover cropping practices on the functional composition and diversity of metabolic pathways in the phyllosphere.

2.3 Material & Methods

2.3.1 Experimental design and field treatment

Samples in this study were derived from the same samples collected as part of a field experiment at the Acadie Farm at Saint-Jean sur Richelieu (45°17'48.7"N 73°20'14.8"W), QC, Canada as described in Maglione et al. 2022. Briefly, the field plots of this experiment were designed to compare different cropping systems on squash (*Cucurbita pepo*; variety: "végétale améliorée"; source: Norseco, Canada) and associated phyllosphere bacteria consisted of 6 replicates of 4 treatments fully randomized in blocks: Rye Cover Crop (RCC) where rye was rolled on the ground, Chemically Terminated Rye Cover Crop (CT-RCC) where winter rye (Secale cereale; variety: Gauthier; source: Secan Association, Canada) was chemically terminated with glyphosate (Roundup, WeatherMax^{MD}, Bayer, Canada) at a rate of 2.16 kg a.e. ha^{-1} before rolling, Plastic Cover (PC) where plastic was applied on the ground, and Bare Soil (BS).

2.3.2 Microbial DNA collection

As previously described (Maglione *et al.*, 2021), microbial communities of the phyllosphere were collected from squash on July 13, 2017. Briefly, each sample consisted of a mix of young and old leaves harvested from the squash canopy of an individual plant into sterile roll bags. As sampling replicates, 3 samples were retrieved per plot for a total of 72 samples (3 samples x 4 treatments x 6 replicates). Microbial cells were then retrieved by washing each leaf sample with 110ml of a sterile saline solution (0.85% NaCl). 100ml of the wash solution was split between two 50ml tubes and centrifuged at 11,500g for 20 min. After centrifugation, the aqueous phase was removed and the pellet was stored at -80°C prior to further analysis.

2.3.3 DNA extraction and Illumina sequencing

DNA was extracted from each pellet using DNAeasy PowerSoil kits (Qiagen, Valencia, MD, USA), according to the manufacturer's instructions, and used for metagenomic *shotgun* sequencing. DNA concentration was quantified on a Qubit 3.0 (Invitrogen, Waltham, Massachusetts, USA). DNA quality was assessed on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Based on DNA quality and quantity, an

equal amount of DNA from each sample was pooled together for replicates 1, 2 and 6 of each treatment. Thus, 12 DNA samples (3 replicates x 4 treatments) were *shotgun* sequenced (2x100 paired-end sequencing on an Illumina NovaSeq at McGill University and Génome Québec Innovation Centre, Montreal, QC, Canada).

2.3.4 Sequence processing & annotation

Sequence count and quality were assessed with FastQC version 0.11.9 (Andrews et al. 2010). Sequences were processed by first removing remaining sequencing adapters with bbduk tool from bbmap version 38.86 (https://sourceforge.net/projects/bbmap/), using the built-in adapters database, with the following parameters: ktrim=r k=23 mink=11 hdist=1 tpe tbo (Bushnell, 2014). To remove host sequence contamination, Illumina reads were aligned to the C. *pepo* genome v4.1 with Bowtie2 (Langmead et Salzberg, 2012) using paired-end mode with the sensitive preset. We kept 1 890 578 756 reads that did not map to the C. *pepo* genome with --un parameter (see Supplemental Table 2.1) and used them as input for the Metagenome-Atlas pipeline to finalize sequence decontamination (Kieser *et al.*, 2020). For that purpose, we ran only the quality control step that performed host decontamination by a comparison of reads against the squash genome with Bowtie2, and some further data purity enhancement: removal of PCR duplicates and known contaminants, and error-correction of overlapping paired-end reads. Filtered sequences from the Metagenome-Atlas pipeline were then used for both functional and taxonomic annotation. Functional annotations were done with the HUMMAnN pipeline version 3.0.1 (Beghini *et al.*, 2021, p. 3) on the full UniRef90 database (Suzek *et al.*, 2007) by using default parameter except: --search-mode uniref90. Taxonomic annotation was done with the Kraken2 (version 2.1.2) and Bracken (version 2.5) tools (Lu *et al.*, 2017 ; Lu et Salzberg, 2020 ; Wood *et al.*, 2019). Metabolic pathway annotations were retrieved based on UniRef code annotations using the MetaCyc database with HUMMAnN default parameters (Karp *et al.*, 2002). In the present study, we defined the functional composition of communities as the list of metabolic pathways present in the bacterial community and their respective abundances, and defined functional diversity as the distribution of metabolic pathways in the community that quantifies the number of different pathways (pathway richness) and their relative abundances (pathway evenness). Taxonomic annotations were done with the standard Kraken2 database that included bacterial, archaeal, and viral domains from the complete genomes in RefSeq of NCBI database (Kraken 2 database: PlusPFP 19/9/2022, based on NCBI release 254). The taxonomic annotation revealed that more than 99% of the annotated sequences belonged to the domain Bacteria (see Supplemental Table 2.2). Further taxonomic analyses were thus conducted focusing solely on Bacteria.

2.3.5 Data analysis

All data analysis was conducted in R 4.2.0 (R Core Team, 2023). Additional visualisations were created using ggplot2 3.4.1 (Wickham, 2011).

2.3.5.1 Effect of cover cropping treatments on bacterial taxonomic and functional diversity

Kraken2/Bracken results were imported into R with the package File2mico 0.4.0 (Liu *et al.*, 2022). Taxonomic diversity analyses at the species rank were performed using the R packages Phyloseq 1.42.0 (McMurdie and Holmes, 2013), Picante 1.8.2 (Kembel et al., 2010), and Vegan 2.6-4 (Oksanen et al., 2007). In order to assess the impact of treatments on the taxonomic and functional pathway diversity and composition of bacterial communities on squash leaves, samples were randomly rarefied to 3,279,280 (taxonomic annotations) or 346,136 (functional annotations) sequences per sample (Supplemental Figure 2.1). These thresholds were chosen in order to preserve the greatest number of samples with the necessary number of taxa or pathways to adequately represent the majority of the diversity in each sample (Supplemental Figure 2.1). The Shannon index was used to evaluate the consistency of the relative abundance distributions of taxa or functions (alpha diversity, Haegeman et al., 2013). The impact of treatments on taxonomic alpha diversity at the species rank was assessed using a post-hoc test (Tukey's HSD) of a linear model (alpha diversity as a function of treatment). Major gradients in taxonomic or functional composition were evaluated with Principal Component Analysis (PCA) ordination from variances of Hellinger-transformed (Legendre et Gallagher, 2001) rarefied counts. We partitioned the variance in phyllosphere bacterial taxonomic or functional composition explained by treatment using permutational ANOVA (PERMANOVA) analysis based on Hellinger transformed Euclidean distances.

2.3.5.2 Effect of sequencing methods on taxonomic diversity

Since the samples used for this study were the same as our previous study where we quantified bacterial community composition using amplicon sequencing of the 16S rRNA gene (Maglione *et al.*, 2021), we directly compared taxonomic annotations between 16S amplicon sequencing and metagenomic *shotgun* sequencing approaches on the same samples to evaluate the effect of sequencing methods on taxonomic composition estimation. Since only replicates 1, 2 and 6 were *shotgun* sequenced for the current metagenomic project (see methods; DNA extraction and Illumina sequencing), 16S sequences from those replicates were used for the comparative analysis. As large discrepancies exist between the total number of sequences per dataset, taxa abundances were normalized as relative abundances per dataset. All

subsequent comparisons were performed at different taxonomic ranks ranging from phylum to species. We performed correlation tests (Pearson correlation) to quantify the correlation between phylum relative abundances for each sequencing approach (amplicon vs metagenomic *shotgun* sequencing) globally and for each treatment. The ggvenn R package version 0.1.10 was used to represent shared annotations between sequencing methods using Venn diagrams (Yan, 2023).

2.3.5.3 Differential abundance analysis of metabolic pathways

Differential abundance analysis of metabolic pathways among treatments (cover crops versus non-cover crops) was performed with ANCOM-BC 2.0.2 (Lin et Peddada, 2020) with default parameters except for the following: lib_cut = 1000, struc_zero = TRUE, conserve = TRUE and global = TRUE. P-values were adjusted with the default Holm method (Holm, 1979). Pathways with abundance differences with an adjusted p-value below 0.05 were considered as significantly different. The abundance of differentially abundant pathways were then normalized as a percentage of total abundance, prior to visualization with the R package complexHeatmap (Gu *et al.*, 2016).

2.4 Results

2.4.1 Cover cropping treatments influence squash phyllosphere taxonomic diversity and composition

Cover cropping treatments influenced bacterial taxonomic composition on squash leaves. The PCA ordination of taxonomic annotations indicated that the squash phyllosphere samples clustered taxonomically by treatment (Figure 2.1.A). The first axis of the PCA accounted for 41% of the total taxonomic variation among samples: the rye treatments (RCC and CT-RCC) and non-rye treatments (PC and BS) were separated along this first axis. Treatment also accounted for 57% (R^2 = 0.57, p=0.004) of community taxonomic compositional variation at species rank among samples (PERMANOVA on Euclidean distances among samples). According to the PERMANOVA, treatment effects on community composition were significant at all taxonomic ranks (Supplemental Table 2.3). Moreover, bacterial community taxonomic alpha diversity was significantly different among treatments (linear model; Shannon index vs. cover cropping treatment, P<0.05; Figure 2.1.B). Finally, bacterial community taxonomic alpha diversity was higher for both rye treatments as compared to plastic treatments (Tukey HSD on linear model, P<0.05; Figure 2.1.B), but there were no significant differences in alpha diversity between bare soil and the other treatments. The same results were observed for taxonomic species richness analyses (Supplemental Figure 2.2).

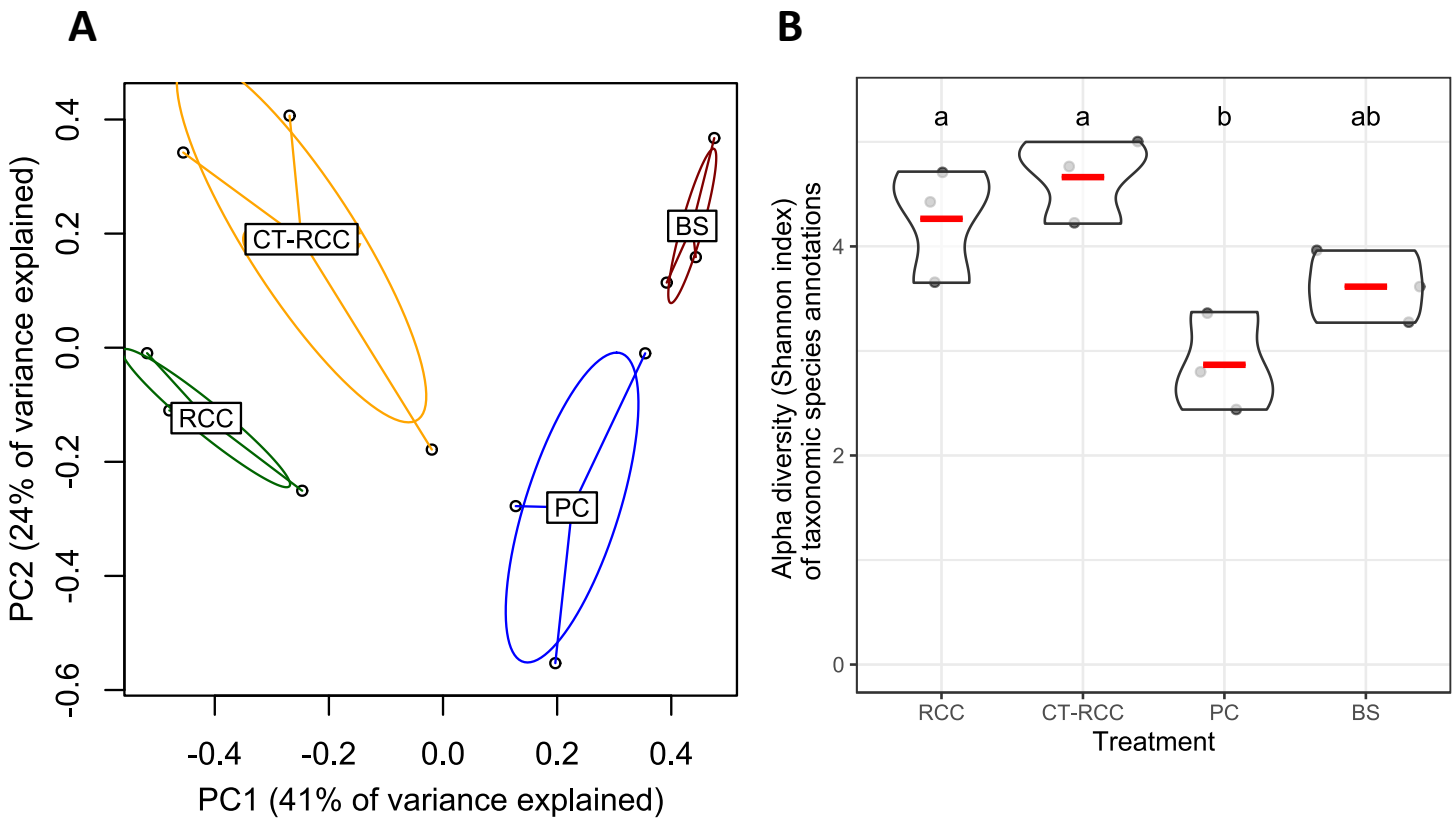

Figure 2.1: Impact of cover crop treatments on bacterial taxonomic community diversity in the phyllosphere of squash. A: Principal Component Analysis (PCA) ordination plot of taxonomic annotations at species rank obtained from metagenomic *shotgun* sequencing of bacterial communities present on the phyllosphere of squash. Ellipses represent treatment clusters at 95% confidence interval. Treatments: blue = plastic cover (PC), green = rye cover crop (RCC), yellow = chemically terminated rye cover crop (CT-RCC), and red = bare soil (BS). B: Alpha diversity (Shannon index) of taxonomic annotations at the species rank of bacterial communities present in the phyllosphere of squash. The horizontal red line within each violin indicates the mean. The letters above each violin represent post-hoc tests performed with Tukey's HSD analysis on the linear regression of taxonomic alpha diversity as a function of treatment. Different letters indicate a significant difference between treatment groups (p value < 0.5).

2.4.2 Sequencing methods do not influence overall taxonomic composition of squash phyllosphere

A comparison of taxonomic annotations of phyllosphere bacterial communities based on 16S rRNA gene amplicon sequencing and metagenomic *shotgun* sequencing of the same samples performed on the taxonomic abundance at phylum rank showed that bacterial community relative abundance were highly correlated between the two sequencing methods, with major phyla belonging to *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria* showing similar high relative abundances between the two datasets (Figure 2-2.A, linear model; Phylum relative abundance vs. dataset and treatments: $R=0.88$, $P<0.0001$). A correlation test of the abundance of phyla between sequencing methods was significant for all major phyla (Figure 2.2.B; Pearson correlation test: $p<0.001$). This correlation was also consistent among treatments (Supplemental Figure 2.3). However, the correlation between taxon abundances based on 16S and metagenomic *shotgun* sequencing broke down at the species rank (Supplemental Figure 2.4). At the species rank, the different sequencing methods only shared 93 species-level annotations representing 1.6% of identified species (Supplemental Figure 2.5), and *shotgun* sequencing had 18 times more taxonomic annotations at the species level compared with targeted amplicon sequencing. Moreover, taxonomic alpha diversity (Shannon index) was not significantly different between sequencing methods (post-hoc test performed on linear regression of alpha diversity as a function of sequencing methods, see Supplemental Figure 2.6). Taken together, the two sequencing methods yielded broadly similar results for taxonomic annotations up to the genus rank.

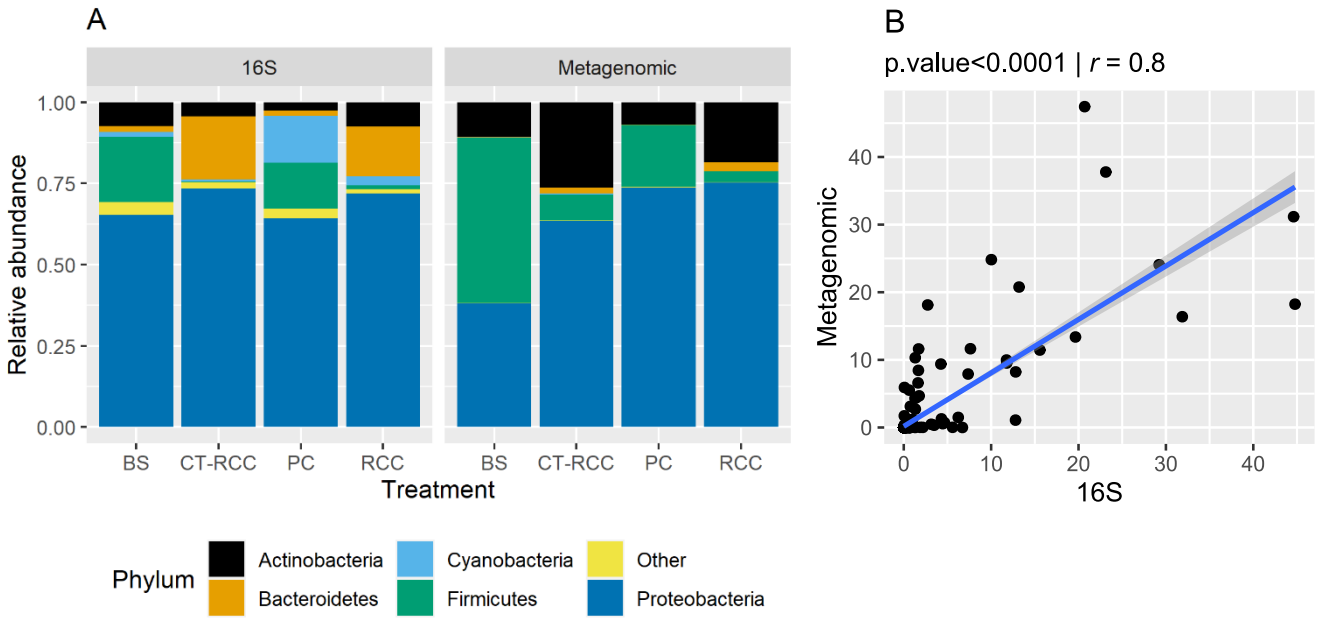

Figure 2.2: Comparison of bacterial community relative abundances at the taxonomic phylum rank in different treatments between 16S rRNA gene amplicon sequencing and metagenomic *shotgun* sequencing. A: Stacked bar plots of relative bacterial community abundance at taxonomic rank phylum for each treatment and sequencing methods. The bar plot on the left is based on 16S sequencing data from a previous study (Maglione et al., 2022) while the one on the right is based on metagenomic sequencing data from the current study. B: Correlation plot of overall phylum abundances between 16S and metagenomic sequencing methods. Blue line: linear regression. Grey zone: 95% confidence interval. Pearson correlation test results (p-value and estimates r) are shown in the title.

2.4.3 Cover cropping treatments influence squash phyllosphere metabolic pathways diversity

Cover cropping treatments had a strong effect on phyllosphere microbial metabolic pathways diversity (Figure 2.3; PERMANOVA on Euclidean distance of Hellinger transformed counts; effect of cover cropping treatment R^2=0.33; p=0.007). The first PCA axis accounted for 41% of the total variation in metabolic pathways among samples and was related to cover crop treatment (Figure 2.3). Sample scores on the first axis of the ordination differed significantly among treatments (linear model with PCA axis scores as a function of treatment, p=0.0016). The rye treatments (RCC and CT-RCC) were different from non-rye treatments (PC and BS) along this first axis (post hoc test (TukeyHSD) on the above linear model, Supplemental Table 2.4). We thus analyzed the effect of the treatments on metabolic pathways by grouping the rye and non-rye treatments according to these two PCA clusters in order to summarize these complex effects. A differential abundance analysis conducted with ANCOM revealed that several metabolic pathways were differently abundant between cover crop and non-cover crop treatments: 43 metabolic pathways out of a total of 638 pathways (6.7%) were differentially abundant among treatments (Figure 2.4; p<0.05). Moreover, 35 (5.5%) metabolic pathways were more abundant for cover crop treatments and 8 (1.3%) for non-cover crop treatments. Finally, 22 (3.4%) of metabolic pathways enriched for cover crop treatments belonged to metabolic degradation, 11 (1.7%) to metabolic biosynthesis and 2 (0.3%) to generation of metabolite precursors.

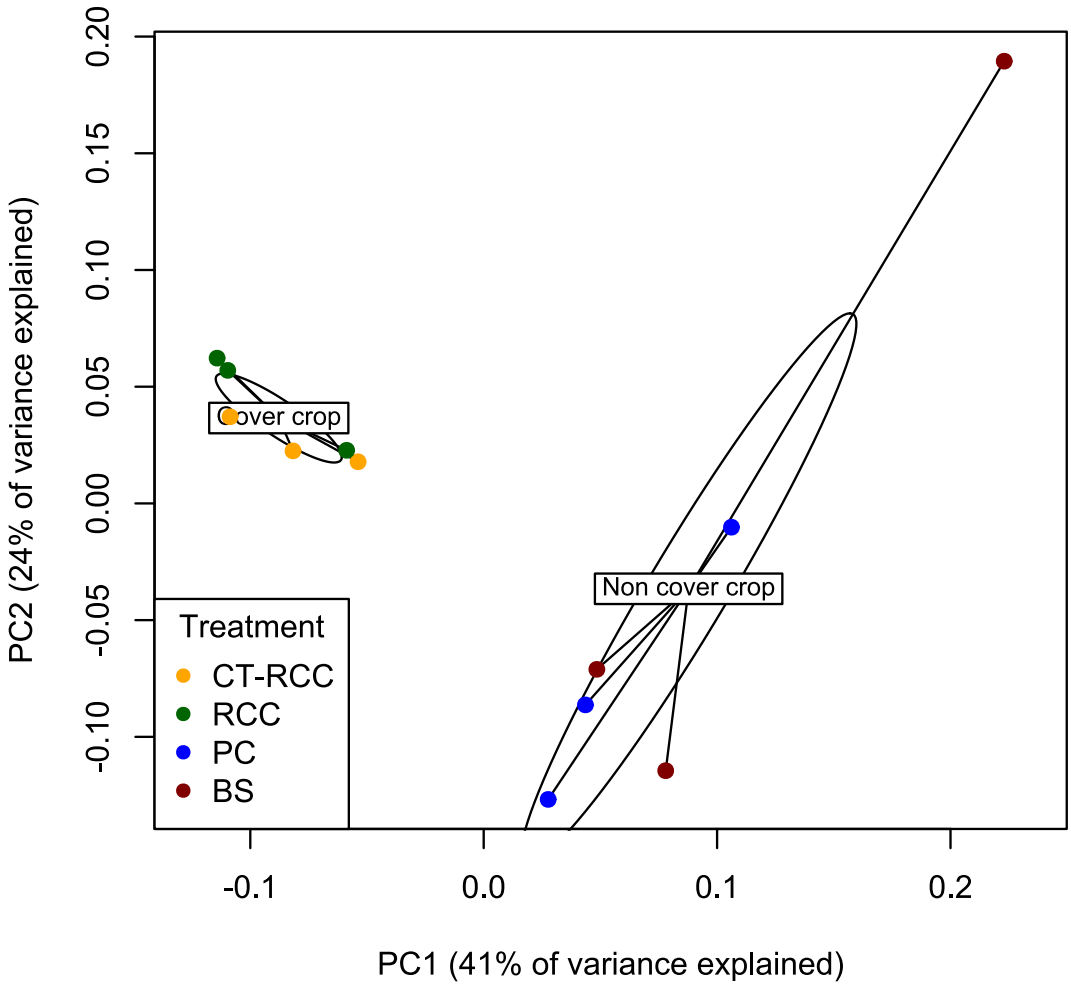

Figure 2.3: Impact of cover crop treatments on bacterial metabolic pathways in the phyllosphere of squash. Metabolic pathway annotations (MetaCyc pathways annotated using HUMMAnN software) were done for each of the four treatments (blue = plastic cover [PC], green = rye cover crop [RCC], yellow = chemically terminated rye cover crop [CT-RCC], and red = bare soil [BS]). The ellipses represent 95% confidence intervals around samples from cover crop (RCC and CT-RCC) and non-cover cover (PC and BS) treatments.

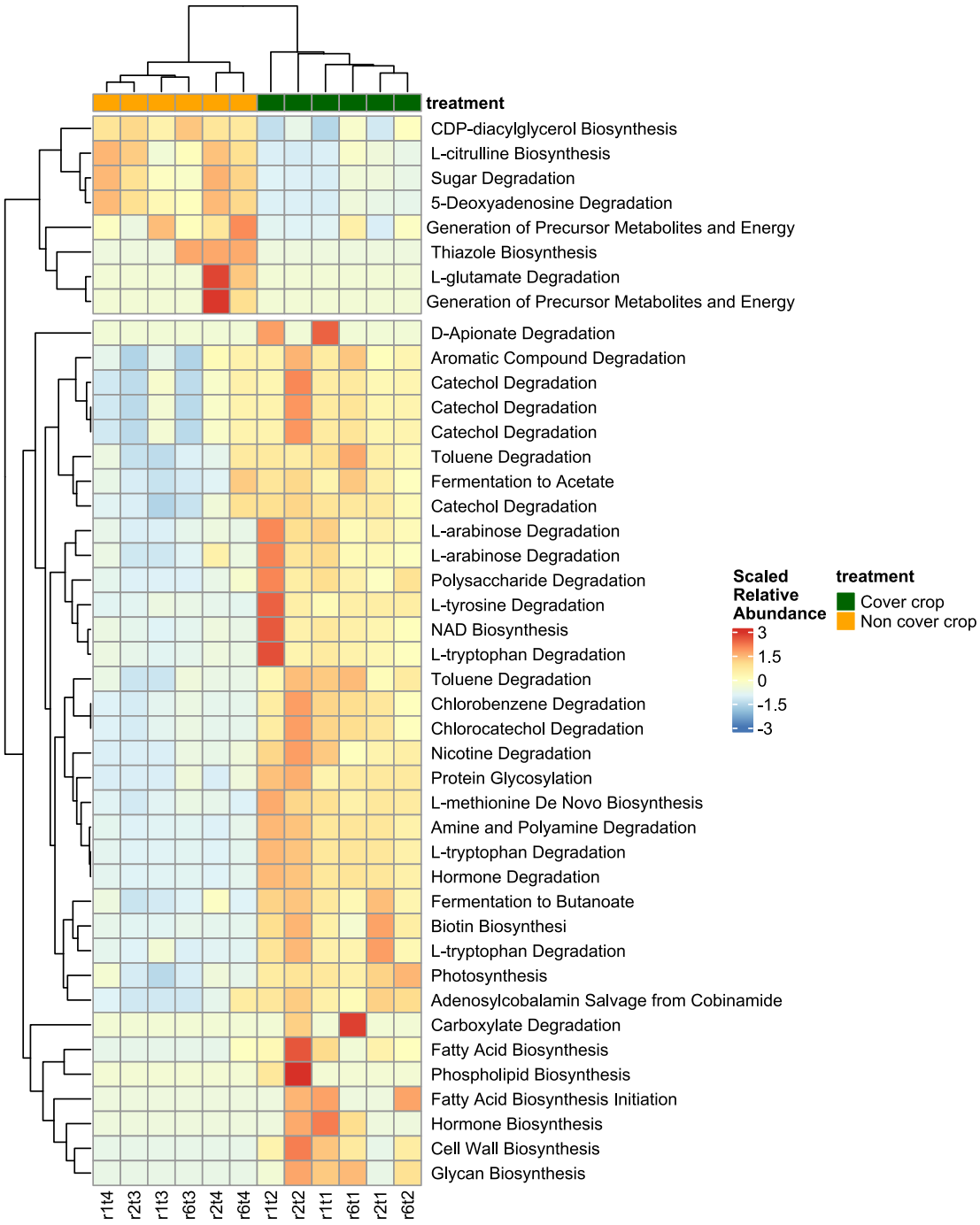

Figure 2.4: Differentially abundant phyllosphere microbe metabolic pathways between cover crop and non-cover crop treatments. Pathways that are differentially abundant are clustered based on similar patterns across samples. Abundance is represented by color intensity, red indicating higher abundance and blue indicating lower abundance. The first group of treatments ("Cover crop") includes the rye cover crop (RCC) and the chemically terminated rye cover crop (CT-RCC), while the second group ("Non cover crop") includes the plastic cover (PC) and the bare soil (BS) treatments. The differential abundance analysis

was performed using Analysis of Composition of Microbiomes (ANCOM) and pathway abundances were normalized to relative abundance (%). Color intensity represents centered and scaled transformations of relative abundances to improve readability of differences among samples.

2.5 Discussion

2.5.1 Cover crop effects on microbial taxonomic composition and diversity

In comparison to the bare soil and plastic cover treatments, both cover crop and chemically-terminated cover crop treatments generated a significant shift in the squash phyllosphere microbiota, resulting in a different taxonomic composition of phyllosphere bacterial communities among cropping treatments. Our findings are consistent with our prior observations of shifts in phyllosphere community taxonomic structure during the growing season and in response to cover crops (Maglione *et al.*, 2021). As we previously demonstrated, this shift was followed by a decrease in pathogen populations and an improvement of fruit health and marketability. Thus, our current results confirm the potential of using cover crops to promote beneficial squash phyllosphere communities. Moreover, taxonomic alpha diversity and species richness were lower for plastic cover treatments as compared to both rye cover crop treatments, suggesting that black plastic cover leads to negative impacts on the establishment or survival of bacterial populations in the phyllosphere.

2.5.2 Cover crops influence phyllosphere microbial metabolic pathways

Analysis of metabolic pathways revealed that cropping treatments impacted the functional composition of the phyllosphere bacterial community. As functional composition patterns were similar between the two cover crop treatments and between the two non-cover crop treatments, we compared pathway abundance between rye (RCC and CT-RCC) versus non rye (BS and PC) cropping treatments with a differential abundance analysis. Our results showed that the use of rye cover crop treatments, both RCC and CT-RCC, significantly enriched certain bacterial metabolic pathways in the phyllosphere of squash plants. Specifically, we observed an increase in the abundance of pathways involved in metabolic degradation and biosynthesis. Taken together with the shift of community and functional composition followed by a reduction of *P. syringae* populations and symptoms, we hypothesize that these metabolic pathway enrichments may explain the previously reported reduction of *P. syringae* symptoms when squash are grown on cover crops.

Both rye cover crop treatments (RCC and CT-RCC) had a higher relative abundance of potentially beneficial bacterial metabolic pathways, such as those associated with nitrogen fixation and plant growth promotion, compared to bare soil and plastic cover treatments. Specifically, rye cover crop treatments enriched metabolic pathways for NAD biosynthesis (from tryptophan) and L-tryptophan degradation. Catabolism of

L-tryptophan is an important source of NAD+; both NAD and tryptophan are well known for their role in electron transport to nitrogenase, part of the nitrogen fixation pathway (Bermudes et Benzing, 1991 ; Bothe *et al.*, 2010). By promoting biosynthesis of fatty acids, biotin, and methionine, metabolic pathways related to the production of essential nutrients, rye cover crops may have contributed to the growth and survival of bacteria and plant health.

Analysis of enriched metabolic pathways in cover crop treatments suggests that these treatments may have promoted the growth and metabolic activity of bacteria involved in degrading and utilizing complex organic compounds such as aromatic compounds, chlorobenzene, toluene, and catechol. These compounds have been reported to be implicated in anti-microbial activity. Specifically, toluene is commonly used as organic contaminants to test phytotoxicity (Weyens *et al.*, 2011). *Pseudomonas putida* has been intensively studied for its toluene degradation activity (Worsey et Williams, 1975 ; Zylstra et Gibson, 1989) and bacterial engineering to remediate toluene contamination is an active area of research (Weyens *et al.*, 2013). Some chlorobenzene compounds have been reported to have an antimicrobial activity against bacteria and fungi (Arslan, Loğoğlu, and Öktemer 2006). Finally, catechol – a phenolic compound synthetized in plants (Kuiters et Sarink, 1986) – has been described by Kocaçalışkan et. al (2006) to have antibacterial activity. Based on these observations, we hypothesize that cover crops may improve bacterial establishment in the squash phyllosphere by promoting metabolic pathways that help degrade antimicrobial compounds.

Many of these phenolic compounds are hypothesized allelochemicals that are used in pathogen biocontrol (Kocaçalışkan et. al 2006). We propose that the use of cover crops may help bacterial establishment in the squash phyllosphere even when faced with the use of naturally produced biocontrol compounds, since biocontrol compounds applied to the plant have a short half-life due to their biodegradability they are thought to be more toxicologically and ecologically safe than synthetic substances (Vyvyan, 2002). Rye has been known for decades to have sustainable weed control effects, thanks to its important allelopathic capability (Barnes et Putnam, 1987 ; Schulz *et al.*, 2013). We hypothesize that the protective effect of rye cover crops could possibly be explained by the fact that they produce allelopathic compounds that protect against weeds and pathogens, while simultaneously selecting for competent phyllosphere bacteria to degrade those compounds. Taken together, this may suggest a future research direction to evaluate the potential use of both cover crops and allopathic compound-based products to improve pathogen biocontrol.

Chlorobenzene, toluene, and catechol degradation pathways are related to the breakdown of these aromatic compounds that are commonly found in plant residues, which may suggest an important role of higher availability of plant-derived organic matter in rye treatments. For instance, Wu et al. (2023) recently showed that toluene emission increased when soils and microorganisms interacted with wheat residue left on the soil. Moreover, Hamer and Marschner (2002) revealed that catechol decreased dissolved organic carbon originating from lignin, a major component of plant cell walls, causing a priming effect defined as short-term effects on soil carbon content from mineralisation of organic matter by microorganisms (reviewed in Kuzyakov, Friedel, and Stahr 2000). Here, we demonstrated that cover crops enriched bacterial metabolic pathways linked to catechol degradation. We thus hypothesise that the use of rye cover crops could help to promote a microbiome competent to decrease catechol and improve soil carbon content resilience. Indeed, increasing catechol degradation before the growing season would leave other compounds more potent for carbon solubilisation and its availability during the rest of the growing season. This would also help further organic inputs, with a cropping system primed with a microbiome ready for catechol degradation. We recommend a future study focused on the effects of cover crops on organic soils versus conventional soils to test this hypothesis. Furthermore, as our study was phyllosphere-centered, enrichment of metabolic pathways involved in aromatic compound degradation could also indicate potential dispersal of catechol breakdown competent bacteria from the rye material and the soil to plant leaves, highlighting the complex interactions that exist among these bacterial reservoirs. Thus, further investigation of bacterial dispersal will be required to fully grasp the mechanisms of the beneficial effects of rye cover crops. Taken together, the enrichment of metabolic pathways related to aromatic compound degradation highlights an increase of plant residue and the importance of ensuring that the cover crop is killed before starting the growing season.

2.5.3 Comparison of 16S and metagenomic *shotgun* sequencing taxonomic annotations

Metagenomic sequencing methods yielded similar taxonomic annotations at the phylum rank compared with 16S rRNA gene sequencing. Although tools exist to impute functions from 16S rRNA gene sequences (Aßhauer *et al.*, 2015 ; Huttenhower *et al.*, 2013), we previously demonstrated (Lajoie et al. 2019) that it is challenging to impute plant phyllosphere community microbial functions based on 16S rRNA gene sequencing. The taxonomic composition and diversity results based on metagenomic *shotgun* sequencing were similar as compared to the analysis of the traditional 16S rRNA gene sequencing data from the same samples, but the metagenomic sequencing approach used in this study provided insights into the functional genes and metabolic pathways present in these bacterial communities. However, it is important

to note that 99% of our taxonomic annotations were attributed to the bacterial kingdom, and overall, only 14% of sequences could be annotated functionally, highlighting some limitations of untargeted sequencing to capture the diversity of non-bacterial organisms present in the whole phyllosphere community. These limitations are likely due to the poor representation of non-bacterial taxa in reference databases used for taxonomic and functional annotations. Finally, sequences assembly were challenging: recovered MAG poorly captured a significant number of sequences, and didn't yield any exploitable functional information between samples (data not shown). But overall, metagenomic *shotgun* sequencing provided a more comprehensive evaluation of the effect of cropping practices on the phyllosphere microbiome by providing both taxonomic and functional information about microbial communities.

2.6 Availability of data and materials

The demultiplexed sequence data have been deposed as sequences read archive under a future BioProject. The scripts used to perform analyses for the current study are available in a GitHub repository: https://github.com/RemiMaglione/Science-Communication/tree/9bf48013a45d67748657936dfe2107ce69be3006/Article/2023_1

2.7 Acknowledgements

We thank Katherine Bisaillon for her help in the field.

CHAPITRE 3

Winter rye cover crops shelter competent squash phyllosphere bacteria to reduce *Pseudomonas syringae* pv. *lachrymans* growth and angular leaf spot symptoms

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Dans ce chapitre (Résumé).

Les cultures de couverture, une pratique de conservation des sols, peuvent contribuer à réduire la pression de la maladie causée par *Pseudomonas syringae*, considéré comme l'un des agents pathogènes bactériens les plus importants pour les plantes. Nous avons récemment démontré que la structure de la communauté bactérienne de la phyllosphère (surface des feuilles) changeait lorsque la courge (*Cucurbita pepo*) était cultivée avec un traitement de culture de couverture de seigle d'hiver (*Secale cereale*), suivie d'une diminution des symptômes de la tache angulaire des feuilles (ALS) sur la courge causée par *P. syringae* pv. *lachrymans* (*Psl*). L'application d'agents de biocontrôle est une pratique agricole connue pour atténuer les pertes de récolte dues aux maladies microbiennes. Dans cette étude, nous avons testé l'hypothèse que certaines bactéries de la phyllosphère préférentiellement sélectionnées lorsque la courge est cultivée sur des cultures de couverture pourraient être isolées et utilisées comme agent de biocontrôle pour réduire les symptômes de l'ALS. Nous avons cultivé des courges au cours d'une expérience de terrain de deux ans en utilisant quatre pratiques agricoles différentes : sol nu, cultures de couverture, cultures de couverture chimiquement terminées et couverture plastique. Nous avons échantillonné les feuilles de courge à trois dates différentes chaque année et constitué une collection de souches bactériennes cultivables isolées des feuilles de courge et du matériel de culture de couverture de seigle d'hiver. Chaque souche isolée a été identifiée par séquençage du gène ARNr 16S et utilisée dans des essais de croissance du pathogène *In vitro* (boîte de Petri) et de contrôle des symptômes *In vivo* (serre). Quatre souches bactériennes appartenant aux genres *Pseudarthrobacter*, *Pseudomonas*, *Delftia* et *Rhizobium* ont montré qu'elles inhibaient la croissance de *Psl* et le développement des symptômes de l'ALS. Finalement, l'efficacité du contrôle des symptômes de toutes les souches était plus forte sur les feuilles plus âgées. Cette étude met en lumière l'importance de l'isolement bactérien à partir de sources de cultures de couverture pour favoriser le contrôle des maladies.

3.1 Abstract

Cover crops, a soil conservation practice, can contribute to reducing disease pressure caused by *Pseudomonas syringae*, considered one of the most important bacterial plant pathogens. We recently demonstrated that phyllosphere (leaf surface) bacterial community structure changed when squash (*Cucurbita pepo*) was grown with a rye (Secale cereale) cover crop treatment, followed by a decrease of angular leaf spot (ALS) disease symptoms on squash caused by *P. syringae* pv. *lachrymans*. Application of biocontrol agents is a known agricultural practice to mitigate crop losses due to microbial disease. In this study, we tested the hypothesis that some phyllosphere bacteria promoted when squash are grown on cover crops could be isolated and used as a biocontrol agent to decrease ALS symptoms. We grew squash during a two-year field experiment using four agricultural practices: bare soil, cover crops, chemically terminated cover crops, and plastic cover. We sampled squash leaves at 3 different dates each year and constructed a collection of cultivable bacterial strains isolated from squash leaves and rye cover crop material. Each isolated strain was identified by 16S rRNA gene sequencing and used in *In vitro* (Petri dish) pathogen growth and *In vivo* (greenhouse) symptom control assays. Four bacterial isolates belonging to the genera *Pseudarthrobacter*, *Pseudomonas*, *Delftia* and *Rhizobium* were shown to inhibit *P. syringae* pv. *lachrymans* growth and ALS symptom development. Strikingly, the symptom control efficacy of all strains was stronger on older leaves. This study sheds light on the importance of bacterial isolation from cover crops sources to promote disease control.

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3.2 Introduction

Global food production must increase by 2050 in order to respond to the world's population growth (Baldos et Hertel, 2014), and food security is still threatened by crop pathogens. It is estimated that 16% of farm gate value losses are due to fungal and bacterial pathogens (Oerke and Dehne 2004). The emergence of new diseases under climate change pressure will likely increase this percentage (Savary *et al.*, 2012). Of all the pathogens associated with bacterial diseases, *Pseudomonas syringae* is one of the most important and has been studied since the beginning of the previous century (Van Hall, 1902).

Comprising at least 56 different pathovars (Bull *et al.*, 2010), *P. syringae* can infect a wide spectrum of plant hosts. Contamination by this pathogen is regulated by two life cycles: 1) an epiphytic phase, where the pathogen establishes itself within the phyllosphere (Hirano et Upper, 2000a), defined as all aboveground plants organs, including leaves, stems, flowers and fruits; and 2) an apoplastic phase, where the pathogen colonizes the internal plant tissues via stomata openings (Romantschuk et Bamford, 1986) or wounds (Misas-Villamil *et al.*, 2013), followed by development in the apoplastic space (Helmann *et al.*, 2019). During its epiphytic phase, where the successful increase in its population size leads to the emergence of the disease (Beattie et Lindow, 1995), *P. syringae* shares its living space with other microorganisms. Although the effects of *P. syringae*-microorganisms-plant interactions on the population dynamics of this pathogen remain poorly characterized, numerous binary interaction studies have shown the biocontrol potential of some microorganisms against *P. syringae* (Xin *et al.*, 2018). Copper-based compounds have been historically used as a method to control bacterial diseases, including *P. syringae* infections, in various crops. However, there are concerns associated with the use of copper including its toxicity and the emergence of resistant pathogen populations (Bender et Cooksey, 1986). Conversely, biocontrol agents are a promising avenue for integrated pest management. Many studies have shown the possibility to control populations of *P. syringae* by modulating the plant's induced systemic resistance elicited through recognition of microbe-associated molecular patterns (reviewed in Pieterse et al. (2014) and Van Wees et al. (2008)). Along with induced systemic resistance, microbial competition is a valuable way to regulate pathogen populations and ultimately manage plant diseases. Indeed, Innerebner et al. (2011) demonstrated that applying several strains of *Sphingomonas* on the leaf surfaces of *Arabidopsis thaliana* could inhibit *P. syringae* populations and disease symptoms. Since direct competition by application of a biocontrol agent is possible, future studies should turn to the identification of new reservoirs harboring these beneficial microorganisms. Moreover, DiLegge et al. (2019) demonstrated that

a biocontrol agent targeting one pathogen could be used to control other pathogens, as a generalist behavior.

The establishment of the plant microbiome can be influenced by vertical transmission among generations, where microbial colonization of the seed expands to the rest of the plant (Ringelberg et al., 2012; Shade et al., 2017). It can also occur through horizontal transmission, spreading from environmental sources such as the soil and neighboring plants via wind, precipitation or animal vectors (Frank *et al.*, 2017). These environmental reservoirs constitute a valuable source of biocontrol agents (Müller et al. 2016). The soil is known to be a reservoir of plant-protective bacteria effective against *P. syringae* (Hossain et al., 2008), and the phyllosphere could also be an attractive reservoir for the isolation of competent microorganisms in the fight against this pathogen (Innerebner *et al.*, 2011). In a previous study, we demonstrated that the use of winter rye cover cropping in squash (*Cucurbita pepo*) production, which is defined by the growth of rye to cover the soil for environmental benefits rather than for its harvest, could reduce symptoms of angular leaf spot disease (ALS) caused by *Pseudomonas syringae* pv. *lachrymans* (Maglione *et al.*, 2021). This decrease in symptoms was accompanied by an increase in populations of potential biocontrol agents, some of them belonging to the genus *Sphingomonas*. Thus, the systematic isolation of bacteria from the phyllosphere of squash or from rye cover crops could provide a source of potentially plant-protective microorganisms.

In this study we build upon our observation of a plant-protective effect of rye cover cropping practices for squash cultivation, to identify potential biocontrol agents that protect squash against ALS caused by the pathogen *P. syringae* pv. *lachrymans*. We hypothesized that we would be able to isolate plant-protective bacteria from the phyllosphere of squash and rye used as a cover cropping treatment. We isolated and identified the best bacterial candidates with antagonist effects on *P. syringae* pv. *lachrymans* growth and ALS disease symptom suppression efficacy on squash. We first isolated bacteria from the phyllosphere of squash cultivated on cover crops and from the rye cover crops using a culture-dependent approach and characterized their taxonomic and phylogenetic identity by 16S rRNA gene sequencing. We then performed an *In vitro* competition assay between the isolated bacteria and *P. syringae* pv. *lachrymans* from squash. To evaluate whether these isolates could inhibit the growth of other pathogens, we extended this *In vitro* assay to include three other pathogens: *Xanthomonas hortorum* from *Lactuca sativa*, *Xanthomonas campestris* from *Brassica oleracea* and *P. syringae* pv. *phaseolicola* from *Phaseolus vulgaris*.

Finally, four of the best-performing bacterial antagonist candidates were used in an *In vivo* disease symptom control assay in a greenhouse to assess their potential to biocontrol ALS disease on squash.

3.3 Material & Methods

3.3.1 Experimental design and microbial collection

All samples in this study were derived from the same samples collected as part of a field experiment at the Acadie Farm at Saint-Jean sur Richelieu (45°17'48.7"N 73°20'14.8"W), QC, Canada as described in Maglione et al. (2021). Briefly, the field plots of this experiment designed to compare different cropping systems on squash (*Cucurbita pepo*; variety: "végétale améliorée"; source: Norseco, Canada) and associated phyllosphere bacteria consisted of 6 replicates of 4 treatments fully randomized in blocks: Rye Cover Crop (RCC) where rye (Secale cereale; variety: Gauthier; source: Secan Association, Canada) was rolled on the ground, Chemically Terminated Rye Cover Crop (CT-RCC) where rye was chemically terminated with glyphosate (Roundup, WeatherMax^{MD}, Bayer, Canada; at a rate of 2.16 kg a.e. ha^{-1}) before rolling, Plastic Cover (PC) where plastic was applied on the ground, and Bare Soil (BS) (see Supplemental Figure 3.1).

Bacteria were collected at four different time points (referred as pre, early, mid and late season) during the 2016 and 2017 growth season (2016: pre=June 13, early=July 12, mid=August 1 and late=September 1; 2017: pre=June 12, early=July 13, mid=August 1 and late=September 8), from microbial wash of squash leaves and rye cover crop plant material, collected as previously described by Maglione et al. (2021) (see Supplemental Methods 3.1 for a microbial collection overview). Humidity and temperature were measured during the entire season, and we reported their values at time of sampling in Supplemental Table 3.1. Small weather differences existed at the time of isolation, but as numerous environmental conditions co-vary throughout the growing season, it was not possible to isolate the effect of a single environmental condition on the abundance and isolation of certain microbial taxa.

3.3.2 Microbial culture and isolation

Isolation of bacteria was done in parallel to our experiment described in Maglione et al., (2021). Each wash sample was diluted three times using 10-fold dilutions, from 1:1 to 1:1x10^3, and a volume of 1 mL of the diluted sample placed on culture medium (10% Tryptic Soy Agar (TSA; Sigma-Aldrich, Saint-Louis, MO, USA)) as follows: each Petri dish contained 4 spread drop lines for each of the four dilutions. After 4 days of growth at 28 C°, colony-forming units (CFUs) were isolated based on shape and color. Specifically, each selected CFU was sub-cultured on a new culture medium (10% TSA). Purified CFUs were then cryopreserved at -80°C in a solution of tryptic soy broth (30g/L) and glycerol (100mL/L).

3.3.3 Microbial DNA extraction and sequencing

DNA extraction was performed on thawed material using 50 µl of each cryopreserved bacterial culture using the DNAeasy PowerLyzer PowerSoil kit (Qiagen, Ville, MD, USA) according to the manufacturer's protocol. Amplicon libraries were prepared using either Sanger sequencing or Illumina MiSeq sequencing. The V5–V6 region of the bacterial 16S rRNA gene was targeted using the chloroplast-excluding primers 799F and 1115R (Chelius et Triplett, 2001 ; Redford *et al.*, 2010). 25 microliter PCR reactions consisted of 5 µL 5x HF buffer (Thermo Scientific, Waltham, MA, USA), 0.75 µL DMSO, 0.5 µL dNTPs (10 mM each), 0.25 µL Phusion Hot Start II polymerase (Thermo Scientific), 1 µL each primer (5 µM), 1 µL of genomic DNA, and 15.5 µL molecular-grade water (IDT, Coralville, IA, USA). Negative controls contained 1µl of sterile water (IDT, Coralville, IA, USA) instead of 1 µL of genomic DNA. Amplicon libraries were normalized using the SequalPrep kit (A1051001, Life Technologies, Burlington, CA) according to the manufacturer's protocol and then sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA), using V2 chemistry (MS-102-2003; 500 cycles, 2x251 base pairs) or at the Genome Québec service platform (McGill, QC) using the Sanger dideoxynucleotide sequencing protocol (Sanger *et al.*, 1977). To increase the accuracy of taxonomic annotation, DNA of four bacterial isolates – MC16-285, MP17 005, MP17 115 and MP17 308 - were amplified by targeting the full-length 16s rRNA gene with primers 27F and 1492R and Sanger sequenced at the Genome Québec service platform.

3.3.4 Sequence processing and analysis

Sequences were processed by first removing remaining sequencing adapters with the bbduk tool from bbmap version 38.86 (https://sourceforge.net/projects/bbmap/), using the built-in adapters database, with the following parameters: ktrim=r k=23 mink=11 hdist=1 tpe tbo (Bushnell, 2014). Cleaned sequences were demultiplexed with deMulMe (a pipeline that uses Sabre (2013) at its core alignment algorithm) allowing one mismatch on the barcode sequence (https://github.com/RemiMaglione/genomicScript/tree/master/deMulMe). Sequence barcodes were removed with cutadapt version 2.10 in paired-end mode (Martin, 2011). All subsequent data processing and computations were done in R version 4.1.2 (RC Team, 2013) with DADA2 version 1.18 (Callahan *et al.*, 2016) and graphs were produced using the ggplot2 version 3.3.5 package (Wickham, 2016, p. 2). Sequences were trimmed and quality filtered with default DADA2 parameters except: trimLeft = c(38,38), truncLen = c(230, 210), maxEE=c(2,3); trimleft was set to remove both PCR primers and barcodes used for library preparation, truncLen and maxEE were set to yield filtered sequences with an average PHRED

quality score of 30 or greater. Amplicon sequence variants (ASVs) were constructed from filtered sequences with the following set of built-in DADA2 functions and their default parameters except: functions dada in pseudo-pooling mode, mergePairs with minOverlap = 30, collapseNoMismatch with minOverlap = 240, and removeBimeraDenovo with method="pooled". The ASVs with sequence length >=50 nucleotides were then taxonomically annotated by assignTaxonomy using the SILVA128 database (Quast *et al.*, 2013 ; Yilmaz *et al.*, 2014). Traces of the full length 16S rRNA gene Sanger sequences of the four isolates were assembled into fasta format using the GEAR genomics server with the Pearl tool (https://www.gear-genomics.com, Rausch et al. 2020; 2019; Untergasser et al. 2021). Assembled Sanger sequences were taxonomically annotated with online NCBI BLAST tools by comparison with the RefSeq genome database (fasta sequences are accessible in Supplemental Data).

3.3.5 Data analysis

3.3.5.1 Phylogenetic analysis

Phylogenetic trees were constructed to evaluate the phylogenetic and taxonomic diversity of bacterial isolates among different sampled materials and dates. ASV sequences were aligned with pyNAST 1.2.2 (Caporaso *et al.*, 2010a), an algorithm included in the align_seq.py QIIME 1.9 tool (Caporaso *et al.*, 2010b), against a built-in pre-aligned database of sequences (Greengenes core set (DeSantis *et al.*, 2006)) and with the default parameters. FastTree 2.1.11 (Price *et al.*, 2010) was used to construct a phylogenetic tree with the pyNAST aligned sequences, in default mode (JTT+CAT substitution model). Phylogenetic trees were visualized with the ggtree R package (Yu *et al.*, 2017). Bacterial phylogenetic relationships were assessed with standardized effect sizes of mean pairwise phylogenetic distances (Webb *et al.*, 2008) provided by the R package picante 1.8.2 (Kembel *et al.*, 2010).

3.3.5.2 Risk group classifications for bacterial candidate selection

Risk group classifications were attributed to isolated bacteria based on their taxonomic annotations. Specifically, we used ePATHogen - Risk Group Database (https://health.canada.ca/en/epathogen) to assess the biosafety of each isolated bacterial strain, whenever taxonomic annotations were available at the genus or species level. Only isolated bacteria belonging to risk group 1 were selected for downstream *In vitro* and *In vivo* assays (see Figure 3.1 for the full-assay workflow).

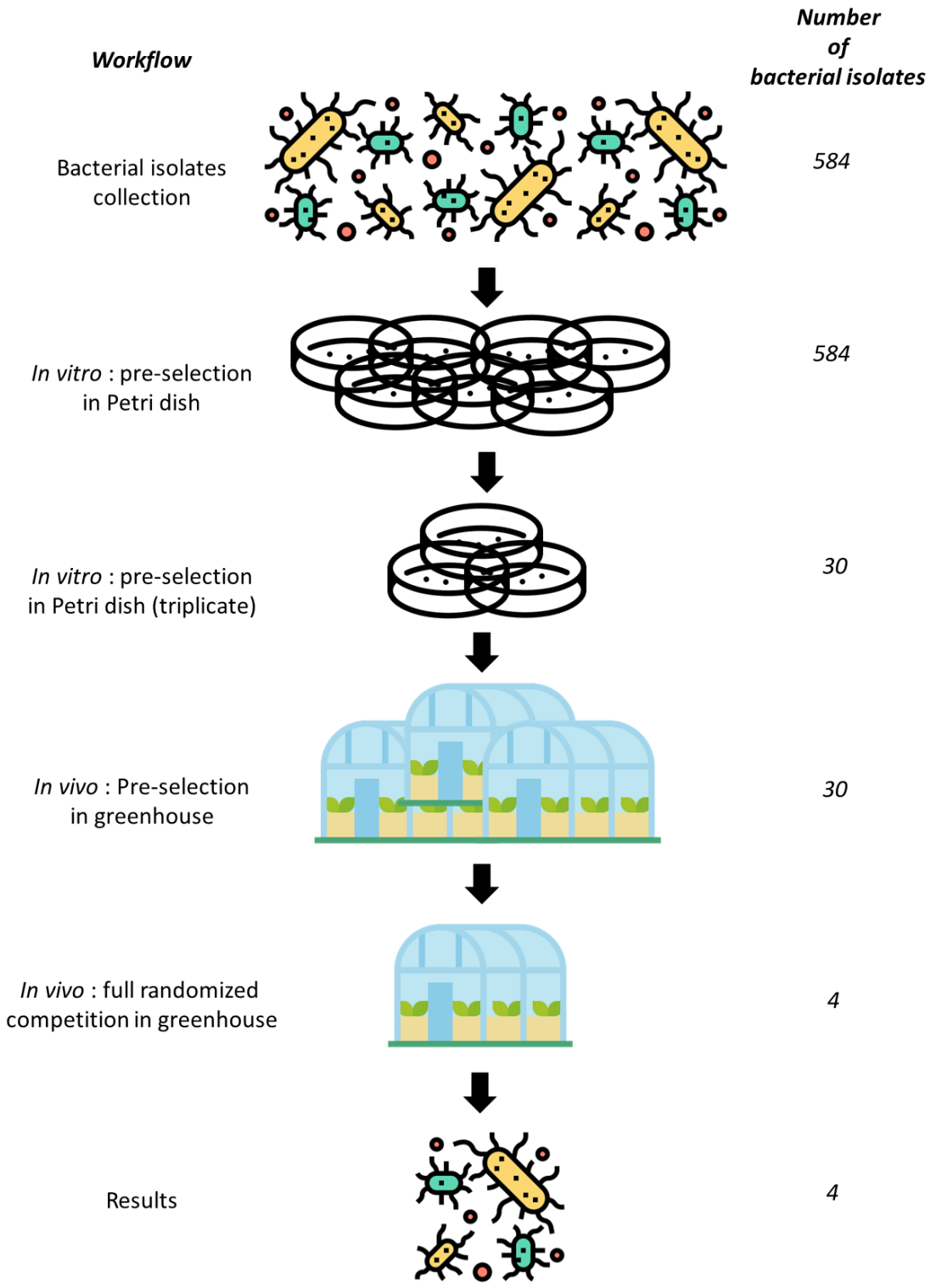

Figure 3.1: Workflow from bacterial isolation to disease suppression validation. This diagram represents the overall workflow used in this study. On the left: workflow step names, on the right: number of bacterial isolates obtained at each step.

3.3.6 *In vitro*: pre-selection and competition assay on Petri dish

Risk group 1 bacterial isolates selected for the *In vitro* pre-selection assay were plated on Petri dishes containing 10% Tryptic Soy Agar (TSA) media at an Optical Density (OD) target of 0.2 (in this study, all ODs were measured with a 600 nm wavelength). After four days of growth at 28°C, 4 drops of squash, lettuce, broccoli, and bean leaf pathogens (*P. syringae* pv. *lachrymans*, X. *hortorum*, X. *campestris* and *P. syringae* pv. *phaseolicola*, respectively) were deposited at the same time on the top of the culture media, at an OD target of 0.09. Pathogen growth inhibition was assessed by measuring the distance between the edges of the pathogen growth (bottom edge) and the bacterial candidate growth line (top edge) using a ruler under a 60X binocular microscope (see Supplemental Figure 3.2). To better represent the pathogen's inhibition, we transformed each measure as follows:

$$\text{Percentage of inihibition} = \frac{\text{Distance between edges of the pathogen growth and the antagonist}}{\text{Distance between the initial pathogen drop and the antagonist}} * 100$$

During this *In vitro* pre-selection assay, thirty bacterial isolates exhibited inhibition against *P. syringae* pv. *lachrymans* growth and were further selected for the *In vitro* competition assay.

For the *In vitro* competition assay, each pairwise combination of selected bacterial isolates versus the four target pathogens was replicated in 3 different Petri dishes, with the same protocol described in the *In vitro* pre-selection assay. To evaluate pathogen inhibition over time, we performed an ANOVA on the linear regression of inhibition percentage as a function of the day of the measurement. Based on the ANOVA results, three growth inhibition patterns were defined: inhibition decaying over time, inhibition increasing over time, and stable inhibition over time. We defined significant inhibition increases or decreases over time corresponding to a significantly positive or a negative slope ($p<0.05$), while a stable inhibition over time was supported by a flat slope with non-significant P-value. The 30 bacteria, that exhibited the highest pathogen growth inhibition during this *In vitro* assay, were further selected for *In vivo* pre-selection and symptoms control assays under greenhouse conditions.

3.3.7 *In vivo* pre-selection assay in greenhouse

To maintain the genetic integrity of isolated strains, fresh cultures of each bacterial antagonist candidate were prepared from stock stored in a -80°C freezer by plating on a Petri dish containing 10% TSA media.

After 4 days of cultivation at 28°C, bacterial suspensions were prepared by diluting bacteria in sterile water at an OD target of 0.2. This solution was diluted twice at $1x10^{-1}$ and $1x10^{-2}$. Thus, 3 different concentrations were used for the antagonist suspension inoculation. Dilution success was assessed by spectroscopy on a NanoDrop 2000C (Thermo Scientific, Waltham, MA, USA) with sterile cuvettes. 12 mL of each bacterial suspension was sprayed with an airbrush on the canopy of 6 young squash plants at a time. Specifically, squash plants were seeded in Promix (Premier Tech Home and Garden, Rivière-du-Loup QC, Canada) and germinated in Conviron growth chambers (Conviron, Winnipeg, MB, Canada). Germinated squash plants from the same batch of seeds used in the 2016 and 2017 experiment were then transplanted in Promix and grown in a greenhouse for 6 days before antagonist inoculation. Squash plants with 3 developed leaves after the cotyledons and the 4^{th} leaf in development were selected. A 15 mL Falcon tube was used as an airbrush reservoir for antagonist candidate inoculation. The spraying was performed under a fume hood, with a work surface sterilized with the application of 95% alcohol and air dried between each inoculation. In addition, 18 squash plants were sprayed with sterile water used to prepare the antagonist suspension as negative antagonist control, and a further 18 squash plants were not inoculated as a negative experimental control. Inoculated plants were randomly placed in a complete random block design in a greenhouse and cultured for 4 days before a pathogen application. *P. syringae* pv. *lachrymans* was the pathogen used for this *In vivo* pre-selection assay. It was cultivated on Petri dish containing media with TSA 10% and rifampicin (Sigma-Aldrich, Saint-Louis, MO, USA) at 28°C for 2 days. On inoculation day, a pathogen suspension was prepared with sterilized water at an OD equal to 0.09 (measured by Spectroscopy on a NanoDrop 2000C), for a concentration of $1X10^{8}$ CFU/mL diluted to obtain a final concentration of $1X10^{5}$ CFU/mL. The squash canopy was inoculated with a pathogen suspension volume of 2 L directly in the greenhouse with a custom spray ramp harboring multiple nozzles to cover the whole width of the workspace. Half of the total plants were inoculated with water as negative pathogen control. To summarize, 558 plants were used for this experiment: 3 plants * 30 antagonist candidates * 3 antagonist dilutions * 2 pathogen conditions inoculation (pathogen or sterile water) + 9 plants inoculated only with pathogen + 9 plants inoculated only with water (see Supplemental Figure 3.3). Symptoms were evaluated 7 days after *P. syringae* pv. *Lachrymans* inoculation by counting leaf lesions (yellow spots). Dry weight of aboveground plant biomass was measured after 2 days in a dryer at 72°C.

3.3.8 *In vivo* symptoms control assay in greenhouse

During this experiment, the same inoculation workflow as described in the initial *In vivo* pre-selection assay was performed with some modifications: four antagonist bacterial strains MC16-285, MP17-005, MP17-

115 and MP17-308 belonging to the genera *Pseudarthrobacter*, *Pseudomonas*, *Delftia* and *Rhizobium*, respectively, were chosen from the *In vitro* and *In vivo* pre-selection assay (results not shown) and used at a single concentration (OD of 0.12 for a concentration of $1X10^8$ CFU/mL diluted to obtain a final concentration of $1X10^5$ CFU/mL). These four bacterial isolates were chosen based on their antagonism capability observed in the *In vitro* test and symptoms control the *In vivo* pre-selection assay, their pathogen inhibition profile on the TSA media from the *In vitro* test and their belonging to different genera. The 4 antagonist bacteria were also mixed at equal concentration to create a fifth antagonist treatment referred to as the "mix" treatment. Squash plants were arranged in 12 complete randomized blocks, half with pathogen treatments, half with water as negative pathogen control. Blocking effects were defined by row and by column (Supplemental Figure 3.4), referred to hereafter as "block row" or "block column" respectively. Those two parts of the experiment were set up 1.5 m away from each other to avoid cross contamination. Six squash plants were grown per tray (see supplemental Figure 3.4). Another 30 plants were added as negative controls: 15 received water (the solution used to resuspend the antagonist) and the pathogen while another 15 received only water, referred to respectively as negative antagonist control and negative experiment control. Taken together, a total of 390 squash plants were used for this experiment: 6 squash plants * 5 antagonist treatments * 2 pathogen conditions (pathogen or sterile water) * 6 repetitions (blocks) + 15 negative antagonist control + 15 negative experiment control (see Supplemental Figure 3.4). Symptoms were evaluated 7 days after *Pseudomonas syringae* pv. *lachrymans* inoculation by counting pathogen lesions (yellow spots) on each of the 4 leaves next to the cotyledons on each plant. Plant dry weight was then assessed as previously described in the initial *In vivo* pre-selection assay. To evaluate antagonist treatment and leaf age effects on symptom count we used a generalized linear model implemented in the R package lme4 1.1-27.1 (Bates *et al.*, 2007, p. 4) as follows:

$$\text{Symptom count} \sim \text{antagonist} * \text{leaf} + (1|\text{block row}) + (1|\text{block column})$$

A post-hoc test (Tukey's Honestly Significant Difference (HSD)), provided by R package emmeans 1.7.2 (Lenth *et al.*, 2018), was performed on this model to assess antagonist and leaf effects on these raw symptom counts. Since symptom counts varied among leaf ages for the water antagonist control, we normalized the antagonist symptoms count for the other treatments by comparison with the symptom count on water antagonist controls for each leaf by calculating the $\log_2$-ratio of symptom counts for each antagonist on a given leaf versus the mean symptom count for the antagonist water treatment on the same leaf. We added a constant value to the numerator and denominator of this log-ratio to allow

calculation of the normalized symptom count when there were zero symptoms for either the antagonist or the antagonist water treatment:

$$\text{Normalized symptom count} = \log2\left(\frac{\text{symptom count}_{\text{Antagonist X | leaf Y}} + 1}{\text{mean }(\text{symptom count}_{\text{Antagonist Water | leaf Y}} + 1)}\right)$$

To assess the effect of the antagonism candidate on each leaf, we then used a linear mixed model to evaluate the impact of the antagonist and leaf on this normalized symptom count:

$$\text{Normalized symptom count} \sim 0 + \text{antagonist} \times \text{leaf} + (1|\text{block row}) + (1|\text{block column})$$

Then, we performed a post-hoc test (Sidak test), provided by R package emmeans 1.7.2 on this model for a selected set of pairwise comparisons: Antagonist_X leaf_Y with $\text{Antagonist}_{water}$ leaf_Y.

3.4 Results

3.4.1 Bacterial isolation and sequencing identification

We isolated 584 colonies from squash leaves and rye cover crop material to create a collection of bacterial strains from our cover crops experiment. From this bacterial isolate collection, we sequenced part of the 16S rRNA gene for a total of 334 and 250 bacterial isolates for the years 2016 and 2017 respectively. Sequenced bacterial isolates were dominated by the class *Gammaproteobacteria* for both experimental years, followed by *Actinobacteria* in 2016 (Figure 3.2, 2016 panel) and *Alphaproteobacteria* in 2017 (Figure 3.2, 2017 panel) (χ^2 test; $p<0.0001$). Moreover, bacterial isolates were unevenly distributed among taxonomic classes among sampling dates (Figure 3.3, see also Supplemental Figure 3.5 for taxonomic distribution at Order rank). Strikingly, when compared by sampling date, more *Gammaproteobacteria* were isolated from rye cover crop material, and early in the 2016 and 2017 growing season (χ^2 test; $p=0.0128$). However, the number of isolated *Gammaproteobacteria* was not different between sampling dates for the squash leaves (χ^2 test; $p=0.5938$). As opposed to *Gammaproteobacteria*, fewer bacteria belonging to *Actinobacteria* were isolated early in both growing seasons from rye cover crop material (χ^2 test; $p<0.0001$), and fewer *Alphaproteobacteria* early in both growing seasons were isolated from squash (χ^2 test; $p=0.0129$) and rye cover crop material (χ^2 test; $p=0.0121$). Finally, strains isolated early in the growing season were more closely phylogenetically related than expected for each isolation year according to a test of the standardized effect size of mean pairwise phylogenetic distances in communities (standardized effect size of mean pairwise phylogenetic distances in communities SES_{MPD}: $Z= -3.06$; $p=0.003$).

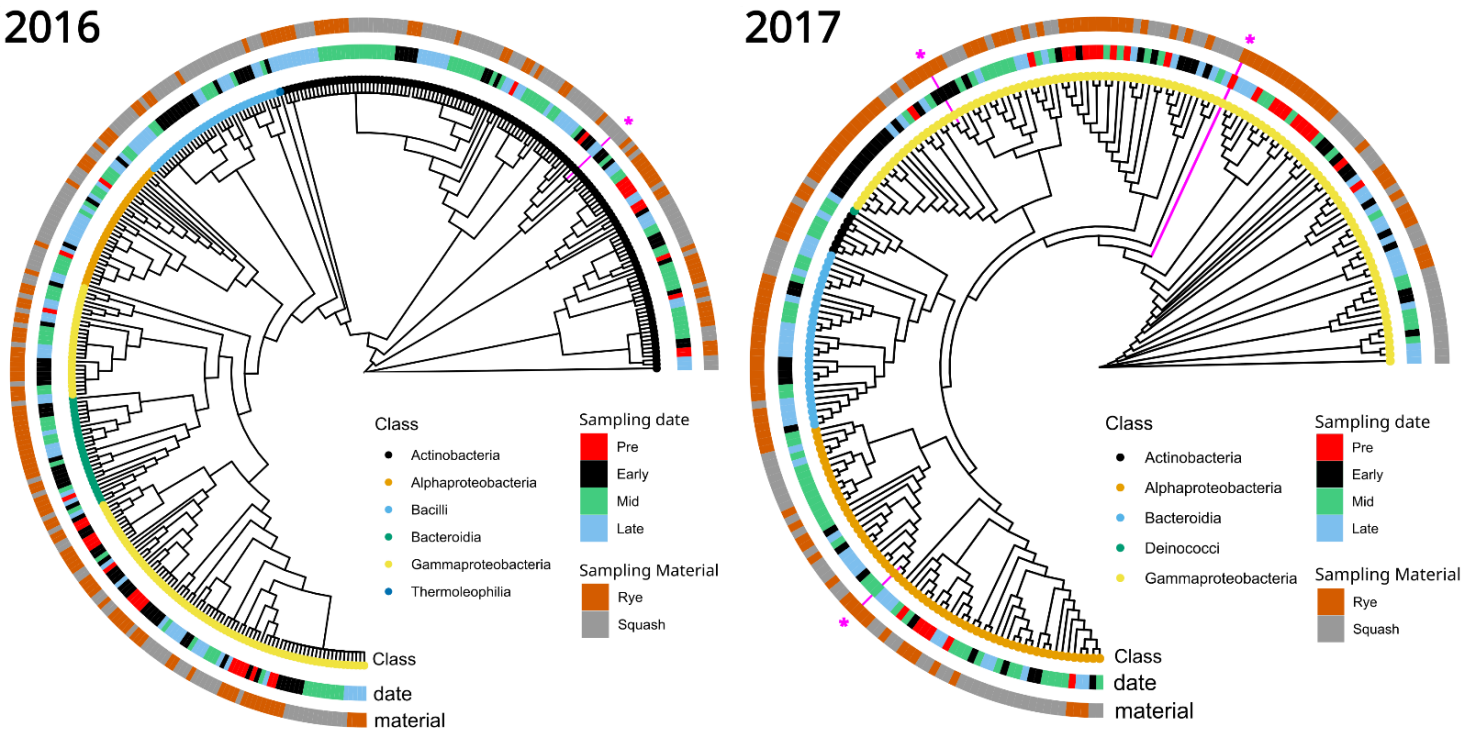

Figure 3.2: Phylogenetic tree of bacterial isolates from winter rye cover crops and squash leaves collected during growing season 2016 and 2017. Phylogenetic trees were constructed with FastTree2 from a PyNast alignment 16S gene sequence for each isolate to the default pre-aligned database of sequences (Greengenes core set). Innermost circle from the center: taxonomic annotation at class rank retrieved from SILVA database with the RDP classifier. Middle circle; Sampling date; red: pre-season, black: early season, green: mid-season, blue: late season. Pre-season represents the sampling of rye cover crop material before squash seeding, whereas other sampling dates are stages of squash growing season.

Outermost circle: Sampling material; brown: rye, grey: squash. Purple lines and stars identify the isolates competent to reduce *P. syringae* pv. *lachrymans* growth and ALS symptoms.

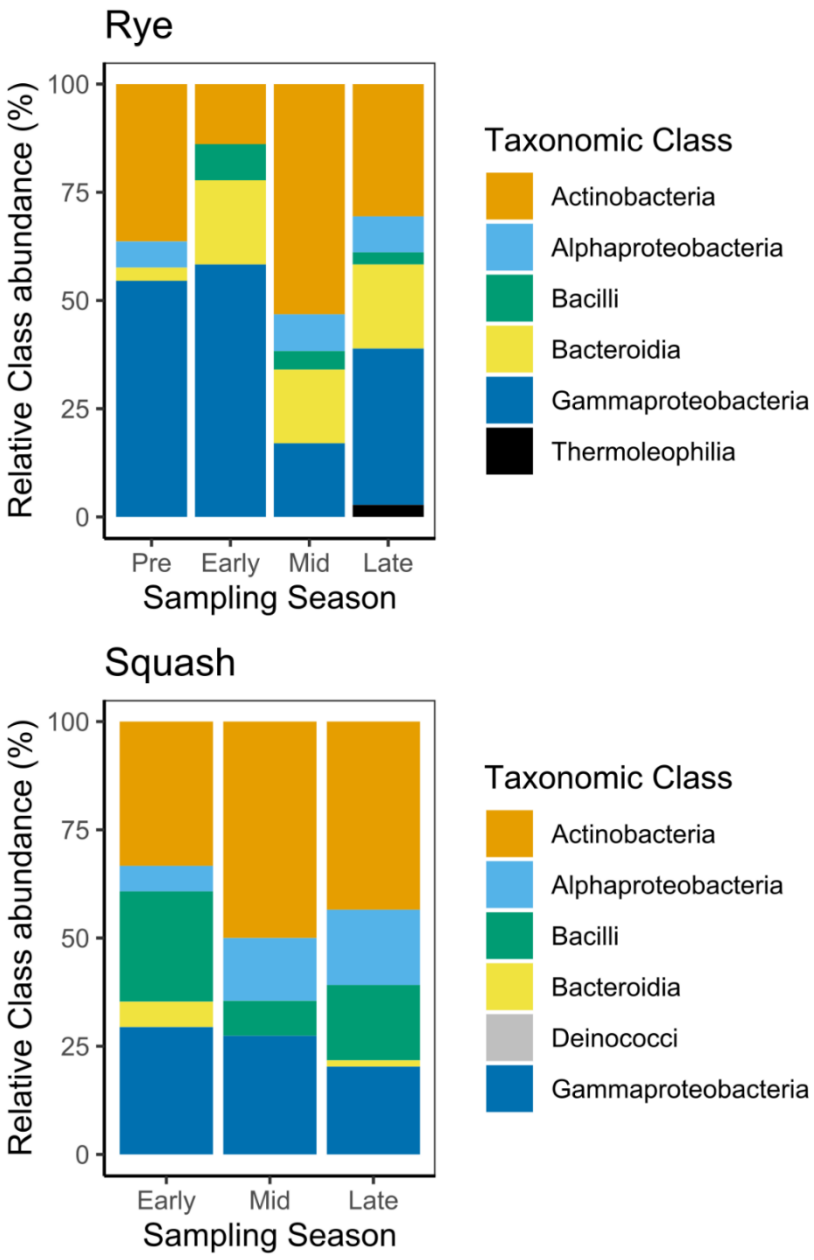

Figure 3.3: Relative number of the 584 isolates belonging to different taxonomic classes. Annotation of bacteria at the taxonomic class rank, isolated from rye cover crop (top panel) or squash leaves (bottom panel) for each sampling date, Early, Mid or Late. Relative class abundance was computed based on the sum of sequence abundances per class across both growing seasons 2016 and 2017.

3.4.2 Screening for effective inhibitors of pathogen growth

All strains from the bacterial isolate collection were tested for inhibition of *P. syringae* pv. *lachrymans* growth to eliminate isolates without any antagonism effect, and 30 isolates were kept for an *In vitro* competition assay on Petri dishes conducted in triplicate for each isolate (see workflow in Figure 3.1). The results from this triplicate assay confirmed the ability of all 30 bacterial isolates to inhibit *P. syringae* pv. *lachrymans* (Supplemental Table 3.3). During this *In vitro* competition assay, three *P. syringae* pv. *lachrymans* antagonism profiles were observed: inhibition decaying over time, inhibition increasing over time and inhibition stable over time (Figure 3.4 provides data for 4 selected isolates described below). These inhibitory profiles reflect a given pathogen's ability to grow on TSA. We defined this profile based on the statistical significance of the linear regression of *P. syringae* pv. *lachrymans* growth over time. A significant relationship between *P. syringae* pv. *lachrymans* counts and time with a positive regression slope denoted an increasing antagonism profile, a significant effect over time with a negative regression slope indicated a decreasing antagonism effect, and a non-significant effect over time indicated a stable inhibition over time (significance threshold; $p<0.05$).

To evaluate the "generalist potential" of each strain (i.e., the ability to inhibit the growth of different pathogens), this assay was performed using three other pathogens: *Xanthomonas hortorum* from *Lactuca sativa*, *Xanthomonas campestris* from *Brassica oleracea* and *P. syringae* pv. *phaseolicola* from *Phaseolus vulgaris* (Supplemental Table 3.3 and Supplemental Figure 3.6).

The 30 antagonists identified were more frequently isolated from a cover crop treatment (Supplemental Figure 3.7). In 2016, strains that showed antagonist effects against *P. syringae* pv. *lachrymans* were significantly more frequently isolated from both cover crop treatments as compared to bare soil and plastic cover treatments, according to a Chi-squared test ($p=0.012$). Moreover, in 2017, all competent strains were isolated from a cover crop treatment. Finally, taxonomic annotations of isolation events were not significantly different between cover crop treatments for both experimental years, according to a Chi-squared test (2016; $p=0.78$ and 2017; $p=1$).

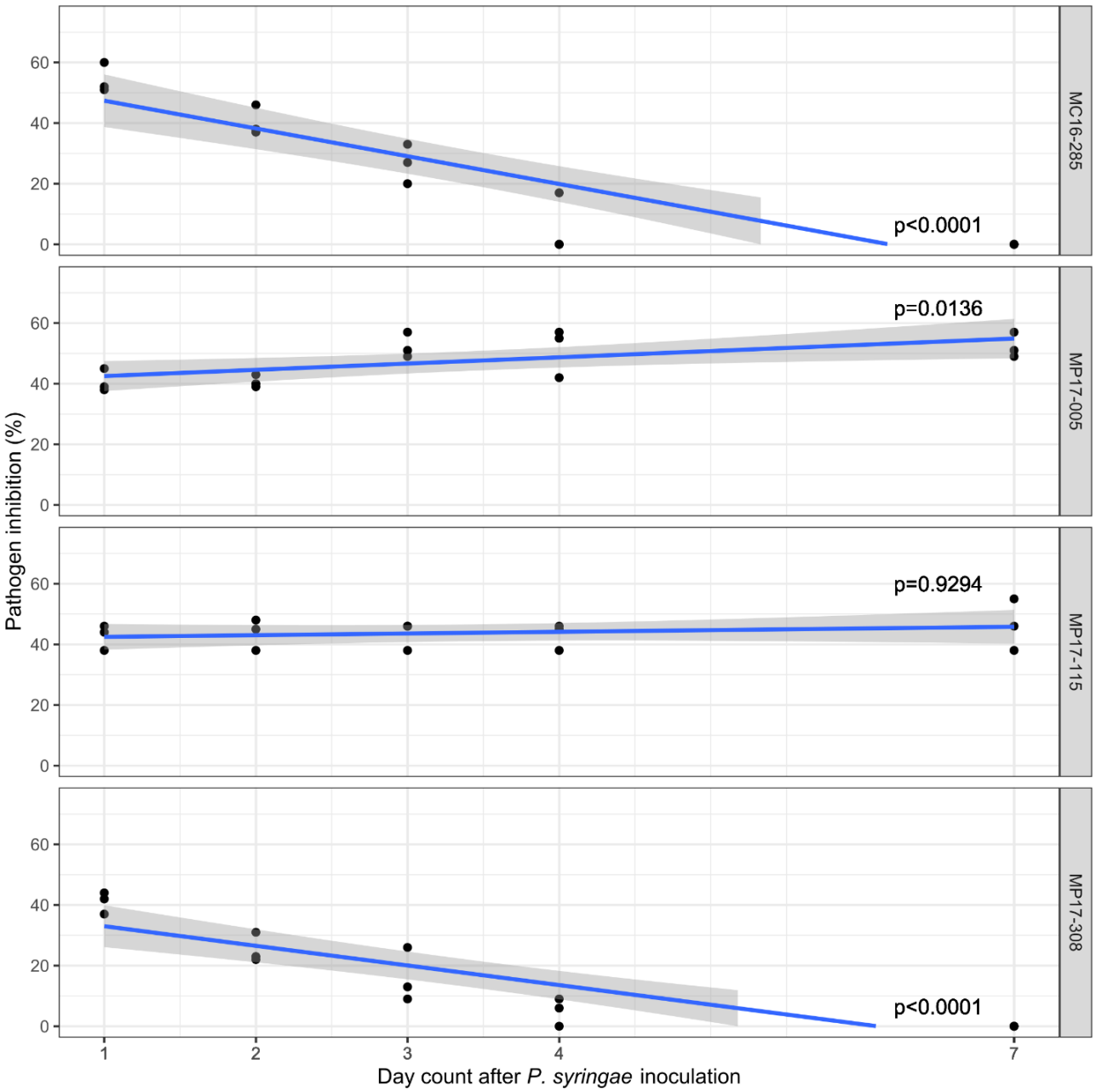

Figure 3.4: Inhibition of *P. syringae* pv. *lachrymans* growth by antagonist candidates over time. Inhibition was assessed by measuring the distance between the antagonist streak and first visual sign of pathogen growth at five time points after *P. syringae* pv. *lachrymans*. inoculation as presented in the legend: 1, 2, 3, 4 and 7 days (d). Pathogen growth inhibition is presented as a percentage as described in the Methods. Linear regression is represented by the blue line and standard error by grey shading. Linear models of inhibition over time are indicated by the P-value: significant inhibition increase or decrease over time corresponds to a positive or negative slope supported by a significant P-value < 0.05

This pool of 30 *P. syringae* pv. *lachrymans* antagonists identified during the *In vitro* competition assay was then used in an *In vivo* pre-selection assay conducted in a greenhouse (data not shown, see diagram in Supplemental Figure 3.3 for overview of the *In vivo* pre-selection test in greenhouse). From these 30 candidates, four bacterial isolates were selected for a follow-up *In vivo* symptoms control assay in greenhouse. These four bacterial isolates were selected based on their different *In vitro* pathogen growth inhibition profiles, their symptoms suppression observed during the *In vivo* pre-selection assay, and their belonging to distinct phylogenetic groups at the genus rank. Specifically, one of these selected antagonist candidates (MC16-285) belongs to the genus *Pseudarthrobacter* and was isolated from squash leaves during the 2016 growing season, while the others (MP17-005, MP17-115 and MP17-308) belong to the genera *Pseudomonas*, *Delftia* and *Rhizobium*, respectively, and were isolated from rye cover crop material during the 2017 growing season (Table 3.1; see also Figure 3.2 for phylogenetic placement of these strains). Sanger sequencing of full length 16s rRNA genes allowed MP16-285 and MP17-115 to be annotated taxonomically at the species level (as *Pseudarthrobacter chlorophenolicus* and *Delftia acidovorans* respectively) whereas MP17-005 and MP17-308 taxonomic annotations remained undetermined at the species rank due to matching more than one species. As highlighted in Figure 3.4, MC16-285 and MP17-308 showed a decreasing inhibition profile against the pathogen over time (linear model, $p<0.001$), while MP17-115 and MP17-005 revealed a stable ($p=0.9294$) and increasing ($p=0.0136$) *P. syringae* pv. *lachrymans* inhibition profiles, respectively. Taken together, bacteria isolated from rye cover crop material and squash leaves during the growing season 2016 and 2017 inhibited growth of *P. syringae* pv. *lachrymans* during the *In vitro* assay and reduce disease symptoms throughout the *In vivo* pre-selection trial.

Table 3.1: Taxonomic identification of *P. syringae* pv. *lachrymans* antagonist candidate bacterial isolates selected for follow-up study in pathogen growth inhibition and disease control assays. Taxonomic annotations were based on comparison with the SILVA database with using the RDP classifier, as implemented in DADA2 software. Species-level taxonomic annotations include multiple species annotations when the isolate sequence was a 100% match to multiple species within a genus.

Isolate	**Class**	**Order**	**Family**	**Genus**	**Species**
MC16-285	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Pseudarthrobacter*	*chlorophenolicus*
MP17-005	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*orientalis/viridiflava*
MP17-115	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Delftia*	*acidovorans*
MP17-308	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Rhizobium*	*fabacerum/ tumefaciens*

3.4.3 Biological control effect confirmed with an *In vivo* greenhouse disease assay

As described above, four bacterial isolates were selected for an *In vivo* greenhouse disease assay with 5 repetitions of fully randomized blocks (Figure 3.5). A TukeyHSD post-hoc test performed on a generalized linear mixed model (Poisson family) confirmed that lesion counts were significantly lower for plants treated with each of these bacterial candidates or their mix as compared to the water control (Tukey's HSD, all $P<0.01$). No symptoms were observed on plants treated with the negative pathogen control treatment. While the effects of the MP17-115 and NP17-308 antagonists on ALS symptoms were lower on average than the effects of MC16-285 and MP17-005, these differences were not statistically significant (TukeyHSD, $P>0.05$). ALS lesion counts were not statistically different between the individual bacterial isolates and their mix, indicating that mixing bacteria with different *P. syringae* pv. *lachrymans* antagonism characteristics at an equal concentration had a suppressive effect that was the average of the constituent strains rather than an additive effect. Finally, no antagonist effect was observed on plant dry weight according to a linear mixed model ($p= 0.071$; Supplemental Figure 3.8). Taken together, the results indicate the four bacterial isolates and their mix inhibited ALS symptoms during this *In vivo* greenhouse disease assay.

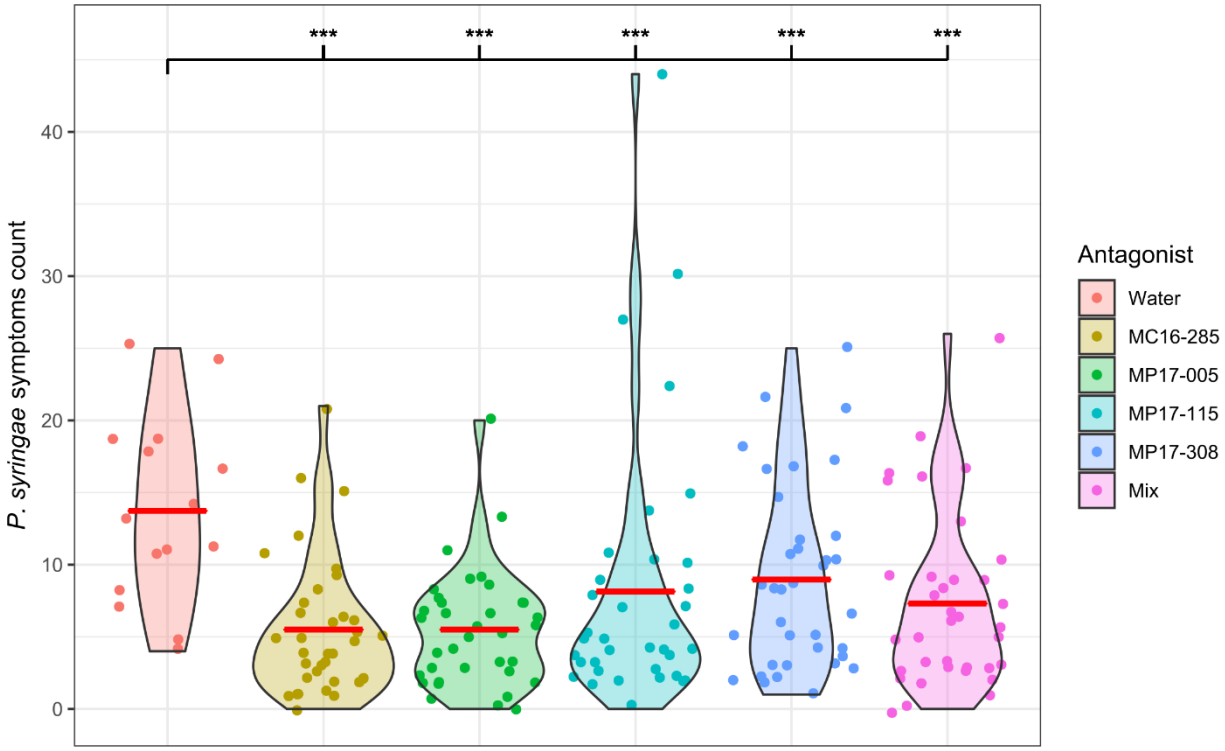

Figure 3.5: ALS lesion counts on squash leaves caused by *P. syringae* pv. *lachrymans* as a function of antagonist treatment. Symptoms were identified by counting yellow spots on each of the four selected leaves for the following treatments: water control (negative antagonist control); bacterial isolates MC16-285, MP17-005, MP17-115, MP17-308 and the mix of bacterial isolates. Horizontal red lines represent the mean of each distribution. Significant symbols represent significant differences (***: p <0.0001) in pairwise comparisons between *In vitro* antagonist and water control from a post-hoc test (Tukey's HSD) of a generalized linear mixed model (Poisson distribution) with ALS symptoms as a function of antagonist treatment as fixed effect, and blocks and plant batches as random effects (see Methods).

3.4.4 Biological control of ALS disease symptoms was a function of leaf age

Leaf age, defined as leaf number starting with the first leaf at the apex, influenced both the amount of disease symptoms observed and the efficacy of disease suppression by each strain (Figure 3.6 and Supplemental Table 3.2). The younger the leaf, the fewer the ALS symptoms (spots) observed. A post-hoc test (Tukey's HSD) performed on a generalized mixed model (Poisson family) confirmed that lesion counts were significantly lower for younger leaves as compared to older leaves (Tukey's HSD, $P<0.05$) for all tested conditions, including water as negative antagonist control (Figure 3.6.A).

Since the water antagonist treatment exhibited differences in *P. syringae* pv. *lachrymans* counts among leaves, we normalized the lesion counts for each leaf by taking the $\log_2$-ratio of the lesion counts for plants treated with an antagonist isolate versus lesion counts for water treatment by leaf age (see Methods). Antagonists isolates significantly reduced lesion counts compared with the water treatment on older leaves (the two leaves closest to the cotyledons), while no significant difference were observed in lesion counts between bacterial treatments and the water treatment on the younger leaves (the two leaves farthest from the cotyledons at inoculation). These findings were based on a linear mixed model analysis of normalized symptom counts per leaf (Figure 3.6.B). Taken together, squash plants had fewer ALS disease symptoms when inoculated with bacterial antagonists as compared to water control plants, younger leaves had fewer normalized symptoms overall than older leaves, and antagonist isolates were more effective at reducing ALS disease symptoms on older leaves.

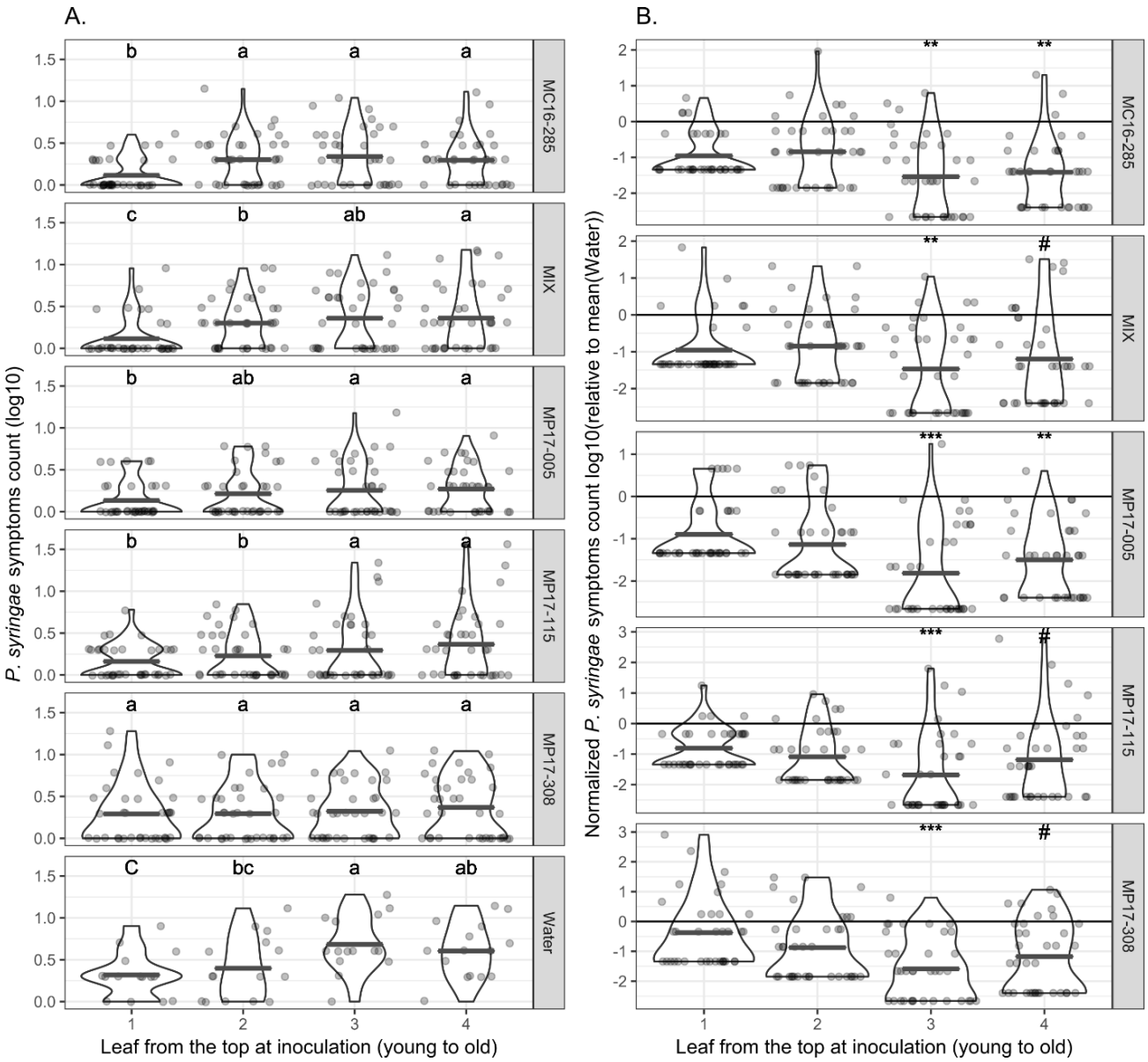

Figure 3.6 : ALS lesion counts as a function of leaf age for each antagonist treatment in the *In vivo* symptoms control assay. Counts are shown for each assessed leaf, from younger leaves (leaf 1) to older leaves (leaf 4). **A**. Violin plot of lesion counts (log_{10}). Horizontal black lines represent the mean of lesion counts (log_{10}). Different letters represent significantly different treatments ($p<0.05$) based on a post-hoc test (Tukey's HSD) of a generalized linear mixed model (Poisson distribution) lesion counts as a function of treatment (fixed effect) and blocks (random effects) **B**. Violin plot of log-ratio normalized lesion counts comparing symptom counts for each leaf with mean symptom count of the water treatment for each leaf age. Symbols represent leaves with significantly lower normalized symptom counts compared to the expected value of zero (antagonist and water effects on symptom counts are equal) according to the Sidak test based on the linear mixed model, as follows: #=p<0.1, *=p<0.05, **=p<0.01 and ***=p<0.001).

3.5 Discussion

We isolated a total of 584 bacterial strains from the squash phyllosphere and rye cover crop material in order to create a bacterial isolate collection. Strains from the phylum *Gammaproteobacteria* dominated this bacterial isolate collection for bacteria isolated at the start of the season (at Pre and Early sampling date, both years). This pattern was consistent for both the squash phyllosphere and the rye cover crop material. The prevalence of this clade is also consistent with previous studies that isolated plant-associated microorganisms, such as the collection of 2,131 CFUs isolated from *Arabidopsis thaliana* by Bai et al., (2015) in which the *Gammaproteobacteria* were highly abundant among phyllosphere isolates.

We demonstrated that 30 of these isolated bacterial strains were effective antagonists of *P. syringae* pv *lachrymans*. These bacterial isolates also inhibited the growth of other bacterial pathogens including *X. hortorum*, *X. campestris* and *P. syringae* pv *phaseolicola*, indicating a potential generalist effect of these antagonists.

During an *In vivo* greenhouse disease symptom assay, we confirmed the efficacy of four effective antagonists of *P. syringae* pv. *lachrymans* and their mixture in reducing ALS lesions on squash. These isolates belonged to genera or species whose biocontrol activity has already been described. These include *Rhizobium* which is a genus containing numerous plant symbionts including some that are known to improve resistance to *P. syringae* (Díaz-Valle *et al.*, 2019). *Delftia acidovorans* is an antagonist of several rice pathogens (Han *et al.*, 2005) and has plant growth promoting activity in rice (Han *et al.*, 2005), canola and soybean (Perry *et al.*, 2017). One of the strains was identified as *Pseudarthrobacter chlorophenolicus*, an environmental microbe that has not been previously identified as having anti-pathogen properties, but rather as having plant growth-promoting abilities (Issifu *et al.*, 2022). Strain MP7-005 was identified as a *Pseudomonas* species with highest sequence similarity to *P. orientalis* and *P. viridiflava*. Interestingly, *P. orientalis* strains have shown promise as a potent antagonist against bacterial pathogens of apple (Zengerer *et al.*, 2018), whereas *P. viridiflava* strains have been reported to be plant pathogens (Hu *et al.*, 1998). Interestingly, this *Pseudomonas* isolate displayed an increase of *P. syringae* pv. *lachrymans* inhibition over time during the *In vitro* test. Given the involvement of secretion systems of *Pseudomonas* in controlling plant disease (Chen *et al.*, 2016), we speculate that an inhibitor secreted by the antagonist may radiate from the colony over time and cause the observed growth inhibition, although this hypothesis remains to be tested. Overall, these results highlight that further work to genomically and ecologically

characterize these strains will be necessary since taxonomic identification is ultimately limited in what it can tell us about the properties of bacterial strains.

We showed in a previous publication that shifts in the squash phyllosphere microbiome and the abundance of the *Gammaproteobateria* and *Pseudomonas* clades were more important at the start of the production season, followed by a reduction in populations of *P. syringae* pv. *lachrymans* observed on squash grown with cover crops on the field (Maglione *et al.*, 2021). In the present study, a majority of the 30 strains that showed antagonist activity against the pathogen during the *In vitro* test were isolated from a rye cover crop treatment (86.7% in 2016 and 100% in 2017). Moreover, three of the four confirmed *P. syringae* pv. *lachrymans* antagonists were isolated from rye cover crop material, and one was retrieved from the phyllosphere of squash grown on a cover crop. Taken together, those findings emphasize the important role of cover crops as a source of biocontrol agents. Our discovery supports the importance of the protective role of growth with cover crops at the start of the growing season as previously noted (Maglione *et al.*, 2021).

We also reported in this previous study a dominance of bacterial isolates belonging to the *Gammaproteobateria* and *Pseudomonas* clades, which we have shown to have biocontrol potential during our *in vivo* disease control assay. Two of the isolates found to reduce disease symptoms belonged to the genera *Rhizobium* and *Pseudomonas*; these genera were also reported to be more abundant in cover crop treatments in our previous study (Maglione *et al.*, 2021), providing further evidence that cover crops are an important biocontrol source. Finally, as our previous study showed that fruit health and marketability increased in concert with reductions in *P. syringae* pv. *lachrymans* populations when squash plants were grown with cover crops, we speculate that reduction of ALS disease symptoms observed in the current greenhouse assay may provide plant health improvement when inoculated with the four potential antagonists originally isolated in a cover cropping context. As we only measured dry weight as plant response proxy during this *In vivo* assay and did not measure *P. syringae* pv. *lachrymans* population sizes directly, we suggest that further metrics directly related to plant health and pathogen populations should be monitored in future plant inoculation experiments. Moreover, as pathogen growth inhibition effects were observed during the *In vitro* assay, we presume the same effect is at play in the *In vivo* experiment. However, our measurements only indicate a decrease in disease symptoms, and we cannot exclude the possibility that reductions in disease symptoms were caused by indirect mechanisms such as induced systemic resistance triggered by the antagonist candidate inoculation rather than direct effects on

pathogen populations. To improve future studies involving the inoculation of the four candidates, direct in planta competition assays will be required. This would involve quantitatively assessing the abundance or growth of both the antagonist and the pathogen populations, employing methods like CFU counting, qPCR, or fluorescent labeling.

Mixing antagonists did not increase biocontrol effects over *P. syringae* symptoms compared with the effect of individual antagonists. Xu et al. (2011) reported that only 2% of the scientific articles included in their metanalysis demonstrated a synergy of biocontrol effect between the different isolates composing the inoculate mixtures. In our study, the bacterial strains competent to reduce the symptoms of *P. syringae* pv. *lachrymans* on squash had an antagonist effect equivalent to the effect of their mixture. Our results suggest that in the context of microbiome engineering for biocontrol purposes, more research is required to understand how different potential biocontrol strains might interact to determine the effects of bacterial mixtures versus inoculation with individual strains, but at least for the strains we examined there was no clear benefit of using a mixture of competent strains compared with using the best-performing individual strains.

We demonstrated that leaf age has an impact on antagonist efficiency at reducing ALS disease symptom counts. Overall, antagonist effects were correlated with leaf age where older leaves were significantly better protected from disease symptoms; younger leaves had fewer symptoms overall, even in the water antagonist control treatment. In contrast, Kus et al. (2002) reported that older leaves had fewer symptoms overall; however, it is essential to note a nuanced distinction in our findings. In our study, we did not observe a general reduction in symptoms on older leaves; rather, when compared with the control treatment, antagonist inoculation led to more substantial decreases in symptoms on older leaves compared to younger leaves. Age-related resistance is known to provide resistance to pathogens in other systems, even in fruits (Mansfeld *et al.*, 2020). Our studies only focused on squash leaves but we found the opposite phenomenon for epiphytic bacteria, where younger leaves had fewer symptoms. However, our measure of symptoms was normalized by comparing pathogen-treated and control leaves at each leaf age, which implicitly accounts for variation in leaf area with age. We did not measure leaf area nor consider it explicitly in our analyses, and thus leaf expansion might have accounted for some of the age-related effects we observed. This distinction between a per-leaf and per-area effect of the antagonists would be very interesting to investigate in a future project. The composition of the leaf microbiome is known to shift with leaf age (Wagner *et al.*, 2016). We hypothesize that microbiome shifts over time may promote the

beneficial effect of biocontrol agents on leaves for young plants. Finally, in a related study we similarly observed that the biocontrol effect of cover crops on *P. syringae* pv. *lachrymans* were greater at the start of the growing season (Maglione *et al.*, 2021). In this study, we have demonstrated a striking effect of leaf age on inoculum efficacy for relatively young squash plants (i.e., at the four-leaf developmental stage). Early inoculation seems to be required to increase protective effects, suggesting the potential for priority effects (Debray et al. 2022) to play a role in community assembly on squash leaves as early introduction of bacterial strains from rye cover crop material and the squash phyllosphere community increases the protective effect of those strains. Extending our previous study (Maglione *et al.*, 2021) and the present experiment with population analyses on young and old leaves will be necessary to understand the role of leaf maturation on pathogens and microbial biocontrol agents.

3.6 Conclusion

Using a culture-based approach, we created a bacterial isolate collection from the squash phyllosphere and rye cover crop material, and we identified several candidate bacterial antagonists of *P. syringae* pv. *lachrymans* based on *In vitro* competition and *In vivo* disease suppression assays. From an initial pool of 30 potential antagonists that inhibited *P. syringae* pv. *lachrymans* growth, we demonstrate that four bacterial strains reduced ALS disease symptoms during an *In vivo* assay in a greenhouse and these bacterial isolates should be considered for their biocontrol capability. Individual strains were just as effective as a mixture of strains indicating that the protective effects of these strains were non-additive. However, limited by the resolution of 16S rRNA gene amplicon sequencing to taxonomically identify isolated strains, these antagonists could not all be identified to the species level. We suggest that genomic characterisation should be conducted for these isolates along with biochemical analysis to identify potential compounds that could be used in biocontrol applications. Our study provides evidence cover crop material may serve as an important reservoir of potential biological control agents and that bacterial isolate collections from cover crops can be rapidly developed and exploited to identify biocontrol agents to protect crop species against pathogens as a means of improving crop yields and ultimately food security.

3.7 Availability of data and material.

The demultiplexed sequence data have been deposited as sequence read archives under BioProject PRJNA858269. The scripts and supplemental raw data tables used to perform analyses for the current study are available in a GitHub repository (https://github.com/RemiMaglione/Science-Communication/tree/main/Article/2022_1).

3.8 Acknowledgements

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CONCLUSION

4.1 Résumé

L'approche des cultures de couvertures est une pratique agriculturale de plus en plus utilisée par les producteurs et les recherches à leurs sujets sont en plein essor. Les connaissances autour des bénéfices qu'elles apportent évoluent, notamment pour leur contrôle des mauvaises herbes et leur amélioration de la santé des sols. Cependant, aucune recherche n'avait encore exploré leur potentiel de contrôle de maladies bactériennes, en général, et de la maladie de la tâche angulaire des courges causée par *Pseudomonas syringae* pv *lachrymans*. Le travail présenté ici apporte donc une exploration originale des cultures de couverture en contexte de contamination de ce pathogène, en combinant l'étude des dynamiques des communautés de la phyllosphère de courge, des approches culture-dépendantes, et des tests de compétitions subséquents, et cultures indépendantes, avec des techniques de séquençage de nouvelles générations.

L'objectif principal de ce doctorat était d'améliorer les connaissances de l'impact des pratiques de cultures de couverture sur le microbiome de la phyllosphère de la courge et leur potentiel de biocontrôle sur le pathogène *P. syringae*. Pour supporter ce but, l'objectif du premier chapitre visait à décrire (1) la diversité taxonomique des communautés bactériennes associées à la phyllosphère de courge en condition de culture de couverture, (2) la diminution potentielle d'abondance de *P. syringae* à la surface des feuilles de courge et (3) des paramètres agronomiques tels que la santé des plantes et la qualité marchande des fruits à la récolte. Le premier chapitre nous a donc permis de dresser un profil inédit des communautés bactériennes de la phyllosphère de courge en contexte de culture de couverture et de leur bénéfice pour des applications futures. Pour explorer davantage leur impact sur le phytobiome, l'objectif du deuxième chapitre était d'étudier l'effet de cette technique sur les fonctions des communautés de la phyllosphère de courge avec un séquençage métagénomique de type *shotgun*. Ceci nous a majoritairement permis d'affiner et de préciser nos connaissances fondamentales sur l'effet potentiellement combiné de biocontrôle du pathogène et d'élicitation des défenses de la plante lors de l'implémentation de culture de couverture. Finalement, l'objectif du troisième chapitre a été d'isoler par culture des bactéries de la phyllosphère de courge en contexte de culture de couverture et d'évaluer leur potentiel de biocontrôle de *P. syringae* avec des tests de compétition *In vitro* et *In vivo*. Ce dernier chapitre a donc apporté une contribution notable au domaine agronomique pour de futures applications dans des stratégies de gestion

en lutte intégrée. Afin d'ancrer nos travaux dans une réalité de production de courge, nous avons construit nos expérimentations en champs avec les différentes pratiques agriculturales suivantes : culture de couverture de seigle d'hiver, culture de couverture de seigle d'hiver préalablement chimiquement exterminé, paillis de plastique noir et sol nu.

4.2 Chapitre 1 : Les cultures de couvertures améliorent la qualité marchande des courges et façonne le microbiome de la phyllosphère de courge.

Bien que l'on observe une augmentation de recherches autour des bénéfices apportés par les cultures de couvertures, notamment avec le contrôle des mauvaises herbes (Teasdale, 1996) et l'amélioration de la santé des sols (Blanco, 2023), aucune étude n'avait exploré leur effet sur les communautés bactériennes de la phyllosphère. Dans ce premier chapitre nous avons conduit une étude combinant, (1) le profilage des communautés bactériennes du phytobiome de courge par séquençage d'amplicons, (2) le suivi de l'abondance du pathogène *P. syringae* et (3) l'évaluation de la santé des plantes et la qualité marchande des courges à la récolte, pour 4 pratiques agriculturales incluant les cultures de couvertures, et ce pour 3 dates clefs durant la saison de production. Ce cadre expérimental nous a donc permis de proposer une exploration originale des cultures de couverture sur paillis de seigle avec l'évaluation simultanée de l'identité et abondance de taxon bactérien, de la dynamique quantitative et temporelle du pathogène et des caractères agronomiques.

Le premier chapitre nous a ainsi offert de nouvelles connaissances sur les avantages de la culture sur couvert végétal de seigle. En effet, cette technique permet de diminuer l'abondance *P. syringae* sur les feuilles de courge et d'améliorer la santé globale des fruits et leur qualité marchande. De plus, les couverts végétaux induisent un changement des communautés bactériennes de la phyllosphère, durant toute la saison de production. Les effets observés du couvre-sol de seigle à la fois sur la communauté de la phyllosphère et sur la population de *P. syringae* sont plus marqués en début de saison de production, indiquant une fenêtre d'intervention propice sur ce pathogène. D'autre part, les paillis de seigle permettraient de sélectionner préférentiellement des bactéries appartenant à des genres dont certains membres ont été décrits dans de précédentes études comme compétents pour lutter contre des pathogènes : on retrouve notamment les genres *Sphingomonas* (Innerebner *et al.*, 2011) et *Methylobacterium* (Delmotte *et al.*, 2009 ; Madhaiyan *et al.*, 2006), mais aussi des espèces non pathogènes appartenant au genre *Pseudomonas* (Cabrefiga Olamendi *et al.*, 2007 ; De Meyer et Höfte, 1997 ; Maurhofer *et al.*, 1998). Finalement nous avons émis l'hypothèse que les changements de composition de

la communauté bactérienne de la phyllosphère associés au couvert végétal de seigle pourraient être expliqués par une modification de migration bactérienne entre les différents réservoirs en présence et les conditions environnementales locales.

4.3 Chapitre 2 : Des voies métaboliques bactériennes au service de la santé des plantes préférentiellement sélectionnées avec les cultures de couverture

Dans le premier chapitre, l'effet des cultures de couverture sur la composition de la communauté de la phyllosphère de courge a pu être évalué, cependant, elle ne l'a été que partiellement dû aux limitations de la technique d'analyse par séquençage d'amplicons. Ainsi, dans le deuxième chapitre de cette thèse, nous avons voulu décrire, grâce à un séquençage métagénomique de type *shotgun*, l'interaction complexe existant entre les traitements de culture de couverture, les communautés microbiennes de la phyllosphère de courge et leurs voies métaboliques dans le contexte de l'amélioration de la santé des plantes et du contrôle des pathogènes. Les changements significatifs observés dans la composition taxonomique et fonctionnelle des communautés bactériennes de la phyllosphère de la courge démontrent l'influence considérable des stratégies de couverture végétale.

Plus particulièrement, l'enrichissement différentiel de certaines voies métaboliques en réponse aux traitements de culture sur paillis végétale souligne davantage la dynamique des interactions bénéfiques entre les plantes et micro-organismes. En effet, l'augmentation significative de l'abondance de certaines voies métaboliques bactériennes spécifiques, telles que celles liées à la dégradation de composés organiques complexes comme les composés aromatiques, le chlorobenzène, le toluène ou le catéchol, et à la biosynthèse, notamment des acides gras, biotine et méthionine, pourrait jouer un rôle clé dans la réduction observée des symptômes de *P. syringae*. Ces fonctions ont déjà été rapportées dans la littérature comme impliquées dans des stratégies de résistance à certains pathogènes. Nous pouvons aussi ajouter à cette stratégie de défense présentée dans l'introduction, celle de la production de métabolite. En effet, il parait vraisemblable que l'enrichissement de certains métabolites du phyllobiome et de sa plante hôte ait stimulé les fonctions de dégradation métabolique. De plus, les interactions proposées liant les couvertures végétales et les communautés bactériennes à la dégradation de certains composés antimicrobiens offrent de nouvelles perspectives pour les stratégies de biocontrôle. Les voies métaboliques identifiées associées à la dégradation des composés allélopathiques renforcent les avantages mulitples des couvertures végétales. Nous pensons qu'il serait possible d'utiliser à la fois des couvertures végétales et des composés allélopathiques du seigle pour améliorer le biocontrôle des

pathogènes. Ceci fournirait une voie potentielle pour améliorer les rendements des cultures et réduire le besoin d'intrants chimiques ouvrant ainsi la voie à une approche plus durable sur le plan agroécologique. Enfin, la comparaison entre le séquençage métagénomique *shotgun* et le séquençage du gène ARNr 16S prouvent l'importance de choisir des méthodes appropriées pour l'analyse fonctionnelle des communautés de la phyllosphère. Le profil complet fourni par les jeux de données métagénomiques *shotgun*, englobant à la fois des informations taxonomiques et fonctionnelles, a permis d'améliorer la compréhension des effets des cultures de couvertures. En effet, nous avons pu montrer qu'il y avait plus d'assignation taxonomique au rang phylogénétique de l'espèce avec cette approche de séquençage. Les assignations fonctionnelles ont quant à elles pu mieux affiner les changements opérés par les cultures de couvertures sur la communauté bactérienne de la phyllosphère de courge.

En résumé, ce chapitre a contribué à l'accumulation des connaissances entourant certains avantages des couvertures végétales pour favoriser les interactions entre les plantes et les microbes, améliorant ainsi la santé des cultures et réduisant la pression des pathogènes. La toile complexe des interactions entre les couvertures végétales, les communautés microbiennes et les voies fonctionnelles ouvre des pistes de recherche futures pour affiner et développer des stratégies durables de protection et de productivité des cultures.

4.4 Chapitre 3 - Des bactéries isolées en contexte de culture de couverture pour combattre *P. syringae*

Dans les précédents chapitres, nous avons montré que les cultures de couvertures avaient un impact positif sur la santé des plantes et permettraient de sélectionner préférentiellement certains taxons, et fonctions associées. Cependant, nous n'avons pas validé si ceux-ci avaient un impact direct sur le contrôle de la maladie. L'objectif de ce troisième et dernier chapitre a été de (1) isoler par culture les bactéries de phyllosphère de courge en contexte de culture de couverture et (2) valider par un test de compétition *In vitro* et un test de contrôle de maladie *In vivo* en serre, le potentiel antagoniste des isolats. Nous avons donc réussi à isoler 584 souches bactériennes depuis la phyllosphère de courge et le seigle du couvert végétal et démontrer l'efficacité d'un sous-ensemble de 30 isolats pour réduire la croissance de *P. syringae* durant un test *In vitro*. Ce potentiel de biocontrôle a été validé pour 4 de ces souches dans un essai en serre et démontre leur potentiel de protection contre les symptômes de la maladie chez les plantes de courge. D'autre part, les isolats appartenant au phylum des *Gammaproteobacteria*, largement présents au début de la saison, ont prédominé dans cette collection d'isolats bactériens, illustrant leur potentiel de biocontrôle généraliste. Des antagonismes ont également été observés contre les autres pathogènes.

Xanthomonas hortorum, *Xanthomonas campestris* et *Pseudomonas syringae* pv *phaseolicola* mis à l'étude dans notre test *In vitro*, appuyant la polyvalence du spectre d'action de ces isolats. Ces découvertes illustrent une fois encore l'importance des dynamiques en place en début de saison de production, qui apparaissent clairement comme décisives pour lutter contre l'établissement du pathogène et l'implantation d'une communauté bactérienne performante pour améliorer la santé des plantes. D'autre part, l'efficacité de 4 antagonistes, confirmée lors de tests de compétition *In vivo* sur des plantes de courge en serre, met en avant le potentiel de biocontrôle de souches bactériennes appartenant à des genres et espèces déjà connues pour leurs activités de promotion de la croissance des plantes et de biocontrôle. Une majorité des souches antagonistes isolées proviennent des paillis de seigle, confirmant leur rôle crucial en tant que source d'agents de biocontrôle. Les tests de compétition en serre ont également révélé qu'augmenter la diversité de l'inoculum d'antagonistes n'augmentait pas l'efficacité de biocontrôle des symptômes de *P. syringae* lorsque comparés aux effets des isolats utilisés individuellement. Ces résultats suggèrent la nécessité de recherches approfondies pour comprendre comment différentes souches de biocontrôle pourraient interagir pour optimiser les effets potentiels des mélanges bactériens. Ceci apporte de nouvelles contraintes à considérer lors d'ingénierie du microbiome pour la production et l'application de bio-inoculant. Finalement, l'âge des feuilles a été identifié comme un facteur influençant les symptômes de *P. syringae* ainsi que l'efficacité des antagonistes. Nous avons pu montrer que l'effet des antagonistes est corrélé avec l'âge des feuilles de courge : la protection apportée par nos 4 antagonistes était plus importante sur les feuilles plus âgées. Cependant, ces corrélations semblent inverses avec d'autres systèmes d'inoculation et chez des hôtes différents. Ainsi, des recherches approfondies seront nécessaires afin de bien comprendre l'interaction en l'âge des feuilles, les pathogènes et leurs antagonistes. Cette relation a des implications importantes pour la mise en œuvre de stratégies de biocontrôle qui devront intégrer l'âge des feuilles dans le schéma d'interaction complexe de ces systèmes pour optimiser au mieux la gestion de la santé des plantes.

En somme, cette thèse contribue de manière inédite à nos connaissances du potentiel des cultures de couverture pour le biocontrôle des maladies bactériennes de la phyllosphère, mettant en évidence l'importance de son interaction avec le phyllobiome et suggérant des pistes prometteuses pour optimiser les stratégies d'amélioration de la santé des plantes.

4.5 Limites

Durant cette thèse nous avons utilisé une approche de profilage des communautés par séquençage du gène 16S. Cependant cette technique manque de précision lors de l'annotation taxonomique (Ren *et al.*, 2022). Tout d'abord, nous avons seulement ciblé un sous-fragment du ce gène ribosomique, la région V5-V6 : amplifier d'autres régions de ce gène ribosomique produiraient des profils différents (Sperling *et al.*, 2017). Ce constat se retrouve aussi quand d'autres gènes biomarqueurs cibles sont mis à l'étude (Calvó *et al.*, 2005 ; Vos *et al.*, 2012). Finalement, cette approche de séquençage d'amplicons ne donne que peu d'informations fonctionnelles (Aßhauer *et al.*, 2015 ; Douglas *et al.*, 2020 ; Huttenhower *et al.*, 2013). C'est dans cet objectif que nous avons soumis les échantillons du chapitre 1 à un séquençage métagénomique *shotgun* afin de couvrir l'ensemble du contenu génique de la communauté bactérienne. Bien que cette technique nous ait permis d'augmenter le pouvoir de détection taxonomique et d'explorer les voies métaboliques, elle est tout aussi limitée que l'approche par amplicons dans la correction des biais d'estimation d'abondance (Edgar, 2017 ; Starke et Morais, 2019), introduits par exemple lors des différentes étapes de laboratoire et de choix des algorithmes d'analyse (McLaren *et al.*, 2019), et restreintes par la complétion des bases de données (Loeffler *et al.*, 2020 ; R. Marcelino *et al.*, 2020). Ces biais induisent notamment une estimation relative de l'abondance et limite donc l'accès à une évaluation absolue. Finalement, ces deux approches sont toutes les deux limitées à appréhender la bio activité des microorganismes étudiés. En effet, nous n'avons dressé qu'un profil présence-absence. Ceci introduit deux limitations majeures : nous ne sommes pas en position de déterminer l'activité génique ou le statut de viabilité de l'organisme - vie, dormance ou mort. Nous retiendrons donc que l'analyse des dynamiques façonnant les communautés de microorganismes dépend du choix de la méthodologie : ceci implique notamment un défi majeur dans l'obtention de consensus scientifiques.

Notre étude est restreinte à un seul modèle d'étude et un seul type de paillis : la courge cultivée sur paillis de seigle. D'autres plantes réagiraient différemment à d'autres paillis (Cazzaniga *et al.*, 2023). Ce type de restriction peut aussi se retrouver au niveau microscopique puisque nous n'avons étudié qu'un seul domaine des microorganismes composant le microbiome de la phyllosphère, les bactéries. Ainsi, principalement à cause des limitations des bases de données, mais aussi de la profondeur de couvertures en séquences nécessaire à leur détection (Ren et Jin, 2016), les champignons et les virus n'ont pas été considérés durant ce projet doctoral. Ce parallèle peut aussi se faire sur les relations environnementales. En effet, bien que nous ayons répliqué notre modèle expérimental sur deux années consécutives, il a été réalisé sur un seul climat et des sols propres au sud du Québec (région agricole appartenant à la

Montérégie), limitant possiblement l'extrapolation de certains résultats, comme le potentiel contrôle de maladie par l'implantation de culture de couverture.

L'évaluation des symptômes durant notre test *In vivo* a été restreinte à des observations visuelles par un décompte de tache. De plus, nous avons assumé une corrélation entre l'antagonisme *In vitro* et un contrôle des symptômes de la maladie *In vivo*. Ces deux points nous indiquent que les tests en serres souffrent d'un manque d'une mesure directe de l'abondance et de la croissance du pathogène, mais aussi des antagonistes. D'autre part, le choix des isolats candidats pour ce test *In vivo* a été basé sur leur performance dans un test *In vitro* avec un seul type de milieu et de condition de culture, et leurs mécanismes d'action n'ont pas été explorés. Cette limitation du milieu de culture se retrouve aussi au moment l'isolation initiale des bactéries de la phyllosphère, réalisée avec un milieu unique de culture, là où Bai et al. (2015) ont constitué une banque bien plus conséquente depuis la phyllosphère d'*Arabidopsis thaliana* avec pas moins de sept milieux différents. Finalement, limité par le temps et le moyen disponible, seulement 4 des 30 isolats ayant montré une activité antagoniste lors du test de compétition sur Pétri ont été soumis à l'étude pour notre test en serres; dus aux limitations de la méthode de séquençage mentionnée précédemment, ces isolats n'ont même pas été tous taxonomiquement identifiés jusqu'à l'espèce.

Pour aller encore plus loin, notre étude en serre s'est concentrée sur une réduction potentielle de symptôme, lié au biais de corrélation avec l'antagonisme observé dans le test *In vitro*, mais nous n'avons pas exploré les mécanismes sous-jacents, comme une défense indirecte apportée par l'élicitation des mécanismes de défense de la plante de type ISR. D'autre part, bien que nous ayons essayé d'évaluer un certain niveau de complexité d'inoculum avec un mix des quatre candidats, nous n'avons examiné ni un niveau de complexité supérieur avec une communauté complète (Vorholt *et al.*, 2017), ni les différentes interactions bactéries-bactéries en jeu. De plus, l'effet lié à l'âge des feuilles est biaisé par deux stades d'inoculation de feuilles qui pourraient être considérés par la littérature comme jeune : nous n'avons pas fait d'inoculation sur des feuilles réellement âgées. Finalement, les conditions contrôlées de serres diffèrent des conditions réelles de champ, ne laissant que des hypothèses quant au potentiel antagonisme réel de nos candidats.

Il existe des dynamiques complexes façonnant les communautés composant le microbiome de la phyllosphère (Vorholt, 2012b), comme l'interaction avec l'environnement et les différentes sources de

microorganismes (Lennon et Jones, 2011 ; Zarraonaindia *et al.*, 2015). Pour le premier nous avons utilisé de nombreux capteurs et stations météo dispersées dans nos parcelles expérimentales. Cependant, bien qu'ayant généré une grande quantité de données, nous avons restreint leur utilisation au point d'échantillonnage, avec une moyenne journalière, ignorant l'effet des cultures de couvertures sur les variations journalières ou de l'humidité sur la composition de la communauté microbienne. Pour le second, il aurait été pertinent de prélever plusieurs sources d'échantillons représentant les différents réservoirs potentiellement à l'action, comme le sol, l'air et l'eau des précipitations. Cependant, la présente étude ne s'est concentrée que sur l'étude du microbiome à la surface des feuilles de courges.

4.6 Future recherche

Au vu du bénéfice majeur des cultures de couverture dans la production agricole, il est essentiel de continuer les recherches sur son potentiel de biocontrôle afin de garantir une sécurité alimentaire à faible impact sur l'environnement. Des expériences futures sont donc nécessaires pour compléter nos travaux et au vu des résultats et limites actuelles, elles devraient se concentrer sur les objectifs décisifs suivants.

4.6.1 Caractérisation complète des bactéries antagonistes sélectionnées par les couverts végétaux

Nous proposons tout d'abord de répliquer notre procédure de test *In vivo* actuel avec les 26 candidats de notre banque d'isolats ayant déjà montré une activité antagoniste durant le test *In vitro*. Cependant, aux vues des limites de nos observations visuelles de symptôme de maladie, nous conseillons aussi d'utiliser une technique liant directement la croissance du pathogène et les antagonistes par comptage de CFUs, qPCR ou marquage direct (fluo- ou bio-luminescence). Pour aller plus loin, nous suggérons d'étoffer la banque d'isolat en utilisant des milieux de culture supplémentaires à celui employé dans le présent projet (TSA), comme ceux de l'étude de Bai et al. (2015) ayant déjà fait l'objet d'une bonne efficacité d'isolation de bactérie de la phyllosphère.

4.6.2 Explorer l'impact des cultures de couverture sur les fonctions de la plante hôte et du microbiome de la phyllosphère

Il est indéniable que les cultures de couverture permettent de sélectionner préférentiellement des bactéries compétentes pour contrôler la maladie de la tâche angulaire. Cependant, afin de mieux cerner les dynamiques en place, il faudrait explorer l'impact de cette technique sur la réponse de la plante en général, et de ses défenses en particulier. Nous proposons de réaliser une étude de transcriptomique pour

suivre les changements fonctionnels qui s'opère dans la plante hôte lors de l'implantation des paillis végétaux. D'autre part et dans la même lignée, faisant suite à nos découvertes de voies métaboliques positivement éliciter lors de l'implantation des cultures de couverture (fixation d'azote, utilisation et dégradation de composé aromatique, etc.), de futures études métatranscriptomiques, et métabolomiques, devrait être conduites afin de valider si les gènes microbiens candidats sont effectivement surexprimés et, détecter les composés liés à ces voies métaboliques, respectivement.

4.6.3 Quantifier les changements de migration bactérienne en contexte de cultures de couverture

Afin de mieux cerner les changements de dynamique écologique induits par l'implantation des cultures de couverture, nous proposons d'échantillonner différentes réservoirs sources de bactérie : la phyllosphère de courge et le paillis végétal de seigle tel que présenté dans les différents chapitres, mais aussi le sol, l'eau des précipitations et l'air environnant la phyllosphère des courges. Pour évaluer statistiquement les relations de causalité entre les différents réservoirs, nous suggérons d'employer la méthode d'analyse des pistes en écologie développée par Dr. Shipley (2016). L'analyse des pistes permet de décomposer les effets directs et indirects des variables exogènes (comme les pratiques de culture de couverture) sur les variables endogènes (comme la diversité microbienne des différents réservoirs). Ces analyses sont rendues possibles par une approche de modélisation par équation structurelle supportée des tests statistiques de maximum de vraisemblance (*maximum likelihood*). Afin de bien explorer ces dynamiques écologiques en contexte de culture de couverture, il serait pertinent d'évaluer la migration spatio-temporelle des communautés de la phyllosphère entre ces différents réservoirs. Deux outils pourraient être mis à l'épreuve. Tout d'abord, BioMico qui a montré une bonne efficacité à retracer l'origine des communautés bactériennes issues de différents organes humains et à prédire les transitions saisonnières des communautés bactériennes côtières (Shafiei *et al.*, 2015). Ce logiciel apprend de l'assemblage des communauté sur une partie du jeu de donné et permet de prédire avec une approche bayésienne la source des autres échantillons. Nous citerons ensuite Ichnaea, un logiciel développé dans le but d'identifier les sources de contaminations fécales de l'eau (Sánchez-Mendoza, 2012). Ce dernier utilise quant à lui un algorithme de mutation adaptative de la chaîne de Markov à évolution différentielle afin de prédire efficacement les sources de pathogènes.

4.6.4 Améliorer les méthodes métagénomique avec implantation de procédures protocolaires rigoureuses

Durant nos expérimentations nous avons été restreints par certaines limitations et biais induits par les techniques de profilage par séquençage employées (amplicons ou métagénomique). Cependant, nous recommandons que de futures études incluent des spike-in (ajout d'une communauté synthétique de concentration connue (Tourlousse *et al.*, 2017) de l'extraction d'ADN à l'analyse computationnelle en passant par la préparation des librairies, tel que suggéré par McLaren et al. (2019). L'implantation de ces bonnes pratiques devrait être accompagnée d'une standardisation dans les approches métagénomiques, qui fait pour l'instant défaut à cette jeune science, afin d'optimiser l'émergence de consensus scientifique dans ce domaine.

4.6.5 Approche holistique et systématique

Pour aller plus loin sur l'envergure d'application des cultures de couverture, nous suggérons de tester différentes plantes d'hôte, avec différents types de sols et conditions climatiques. Dans la même logique, notre étude ayant été restreinte au domaine bactérien, il faudrait aussi étendre les travaux futurs sur les autres domaines, champignons et virus. Pour obtenir une approche holistique complète, nous recommandons aussi d'inclure systématiquement des données agronomiques exhaustives et relevés physico-chimiques : par exemple température et humidité au niveau de la phyllosphère, mais aussi pH, contenu NPK au niveau du sol. Finalement, nous conseillons un rapprochement accru des scientifiques du monde des agronomes et producteurs afin d'identifier tout changement environnemental impliquant un apport bénéfique, notamment par l'implantation de nouvelles pratiques, et de réaliser une isolation du microbiome systématique : généraliser ces isolations permettrait de constituer une banque internationale d'agent de biocontrôle et de bio-inoculant. Avec un tel projet, il serait possible de prémunir nos systèmes de production aux défis futurs : lutte contre l'émergence de maladies, diminution des contraintes énergétiques et climatiques.

ANNEXE A

Matériel supplémentaire du Chapitre 1

Supplemental Table 1.1: Mean weight (g) of squash leaves samples for each sampling date during years 2016 and 2017. The mean weights (g) are reported with their standard deviations, from the weight distribution of the pooled samples.

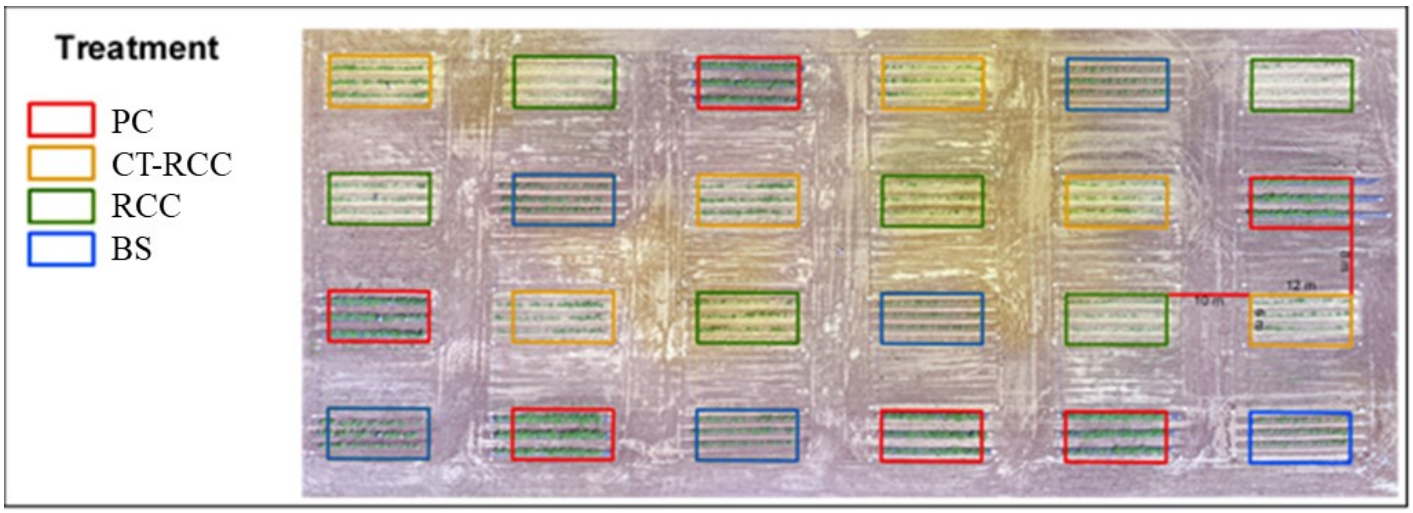
	Weight (g)	
Season	**2016**	**2017**
Early	16.91±5.34	20.38±4.50
Mid	11.49±10.86	19.21±3.74
Late	21.62±4.14	22.44±5.54
All	16.81±8.32	20.67±4.82

Supplemental Table 1.2: Mean counts (± standard deviation) of *P. syringae* colony forming units (CFUs) recovered from squash leaves grown in different cover cropping practices. Means are reported as log10-tranformed number of colonies (± standard deviation) for the four cropping practices at the three seasons of sampling each year.

Mean CFUs (log10) ± standard deviation				
Year/ Season	**Rye Cover Crop**	**Chemically-Terminated Rye Cover Crop**	**Plastic Cover**	**Bare Soil**
2016				
Early	4.06 ± 2.33	4.46 ± 1.97	5.49 ± 0.43	5.69 ± 0.45
Mid	4.38 ± 2.1	4.17 ± 1.99	5.29 ± 1.49	5.33 ± 1.42
Late	4.6 ± 0.5	4.75 ± 0.67	4.29 ± 0.74	4.64 ± 0.52
2017				
Early	0.19 ± 0.81	0.4 ± 1.18	1.98 ± 2.65	4.19 ± 2.2
Mid	0 ± 0	1.44 ± 2.43	2.93 ± 2.5	3.58 ± 2.38
Late	0.93 ± 1.81	2.43 ± 2.63	2.4 ± 2.62	3.59 ± 2.1

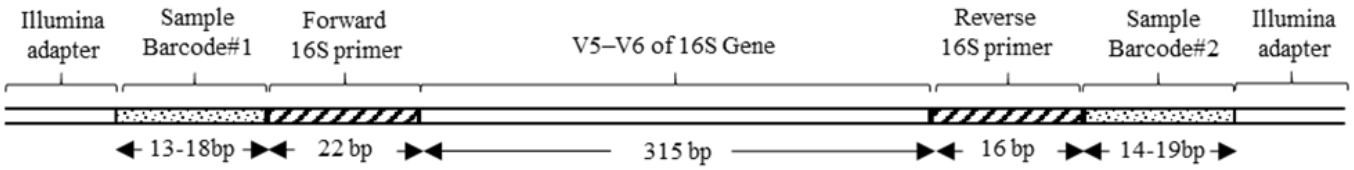
Supplemental Table 1.3: Ratios of transformed *P. syringae* CFUs counts (recovered from squash leaves) between different cover cropping practices at each sampling date during 2016 and 2017. Ratios of significantly different treatments according to Tukey HSD; *: p<0.05; **p<0.01;***p<0.001. italic value: no *P. syringae* CFU were recovered at mid season in 2017 for RCC treatments. CFU counts were transformed using the formula 10^mean(log10(CFU count)) in order to be able to calculate ratios for CFU counts of zero.

Ratios of transformed *P. syringae* CFUs counts						
2016	**CT-RCC/RCC**	**PC/RCC**	**BS/RCC**	**PC/CT-RCC**	**BS/CT-RCC**	**BS/PC**
Early	2.51	**26.9***	**42.08***	10.74	16.8	1.56
Mid	0.61	8.05	8.99	13.19	14.74	1.12
Late	1.4	0.48	1.08	0.35	0.78	2.25
2017	**CT-RCC/RCC**	**PC/RCC**	**BS/RCC**	**PC/CT-RCC**	**BS/CT-RCC**	**BS/PC**
Early	1.62	**60.71***	**9871.12***	**37.43***	**6086.54*****	**162.59***
Mid	27.27	858.06***	3796.51***	31.47	**139.22****	4.42
Late	31.56	29.48	**459.35****	0.93	14.56	15.58

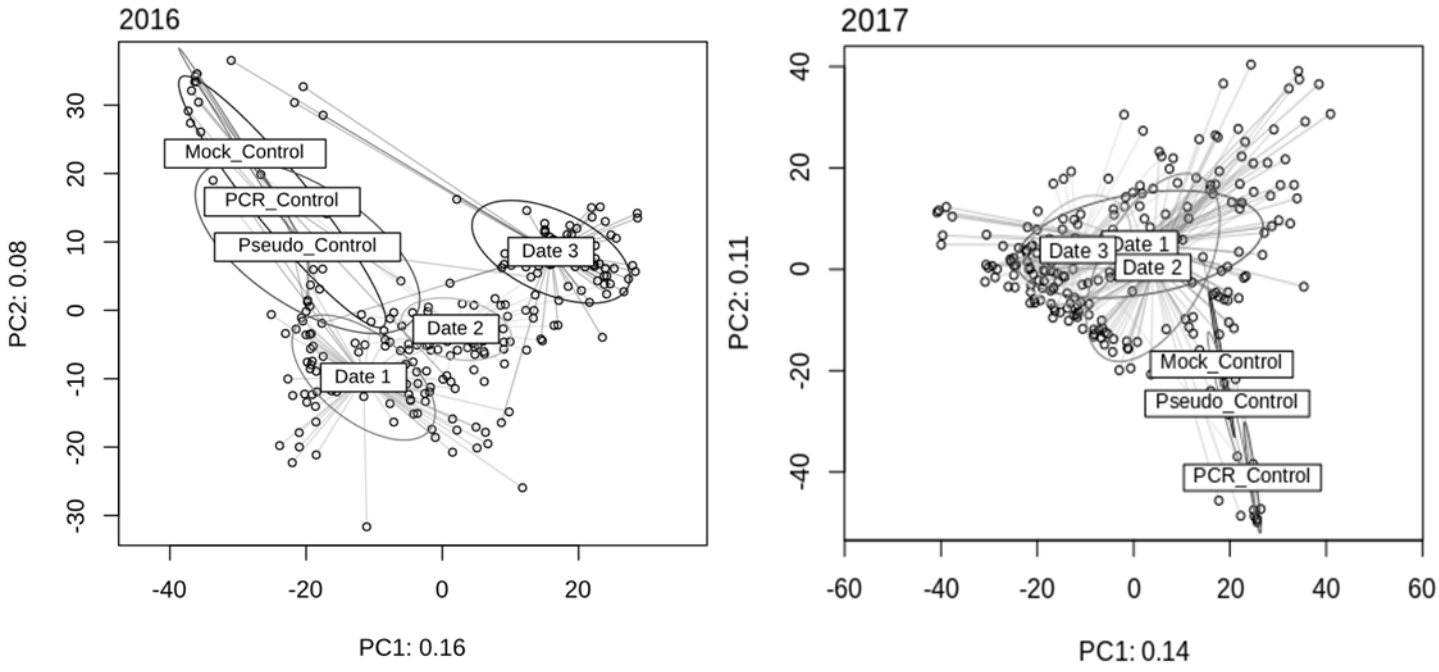


Supplemental Figure 1.1: Aerial photo of the experimental field with cover cropping treatments indicated for each experimental plot. Each experimental plot is 12m long by 6m wide. Plots within the same column are separated by 8-10m between plots of the same row. Each column defines a block of four randomized treatments. Each treatment was applied on 3 raised-bed lines of squash within each experimental plot. Treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and yellow: BS (Bare Soil).

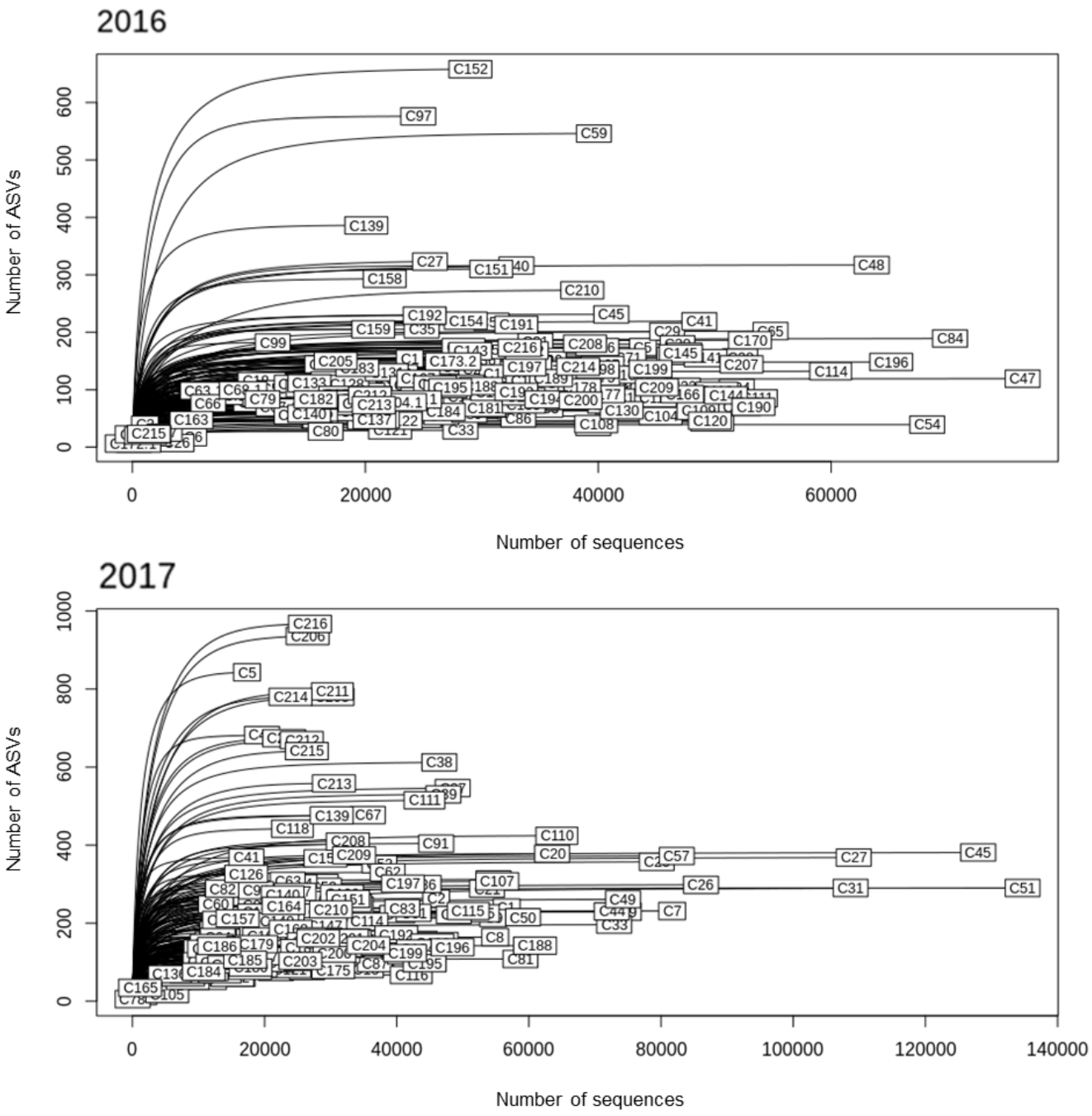
Supplemental Figure 1.2: Photos of cover crop treatments. Left-upper panel present the process of rolling the rye on the ground with the roller crimper. The right-upper panel highlights the squash seeding process directly through the rye cover crop. The left- and right-bottom panels show squash growing in cover crop treatments at mid season in 2018 (July 18 and July 31 respectively).



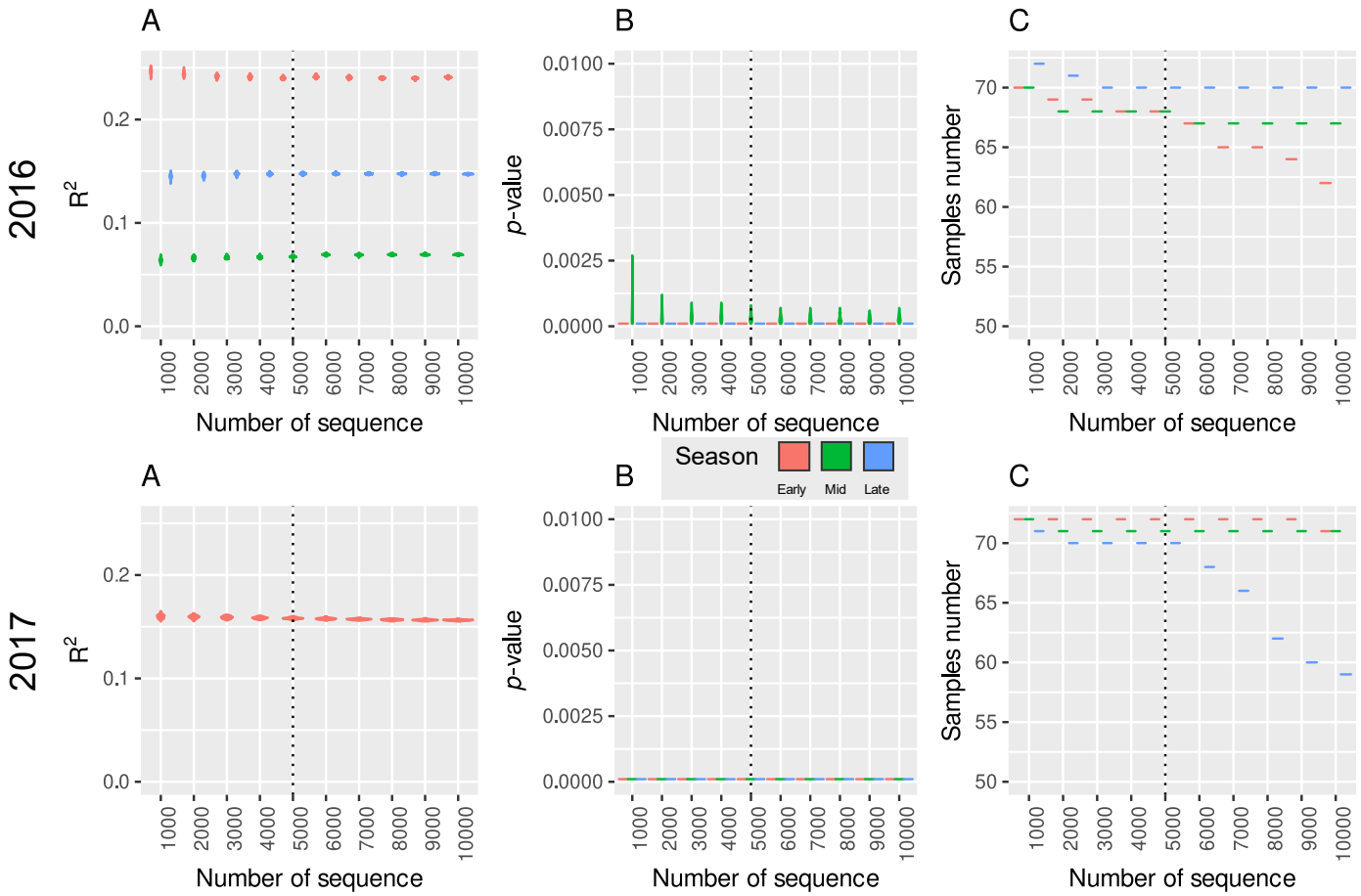
Supplemental Figure 1.3: Structural overview of 16S amplicons. Each amplicon owns a dual indexing (combination of Barcode#1 and #2) for each sample. Barcode lengths are variable between samples.



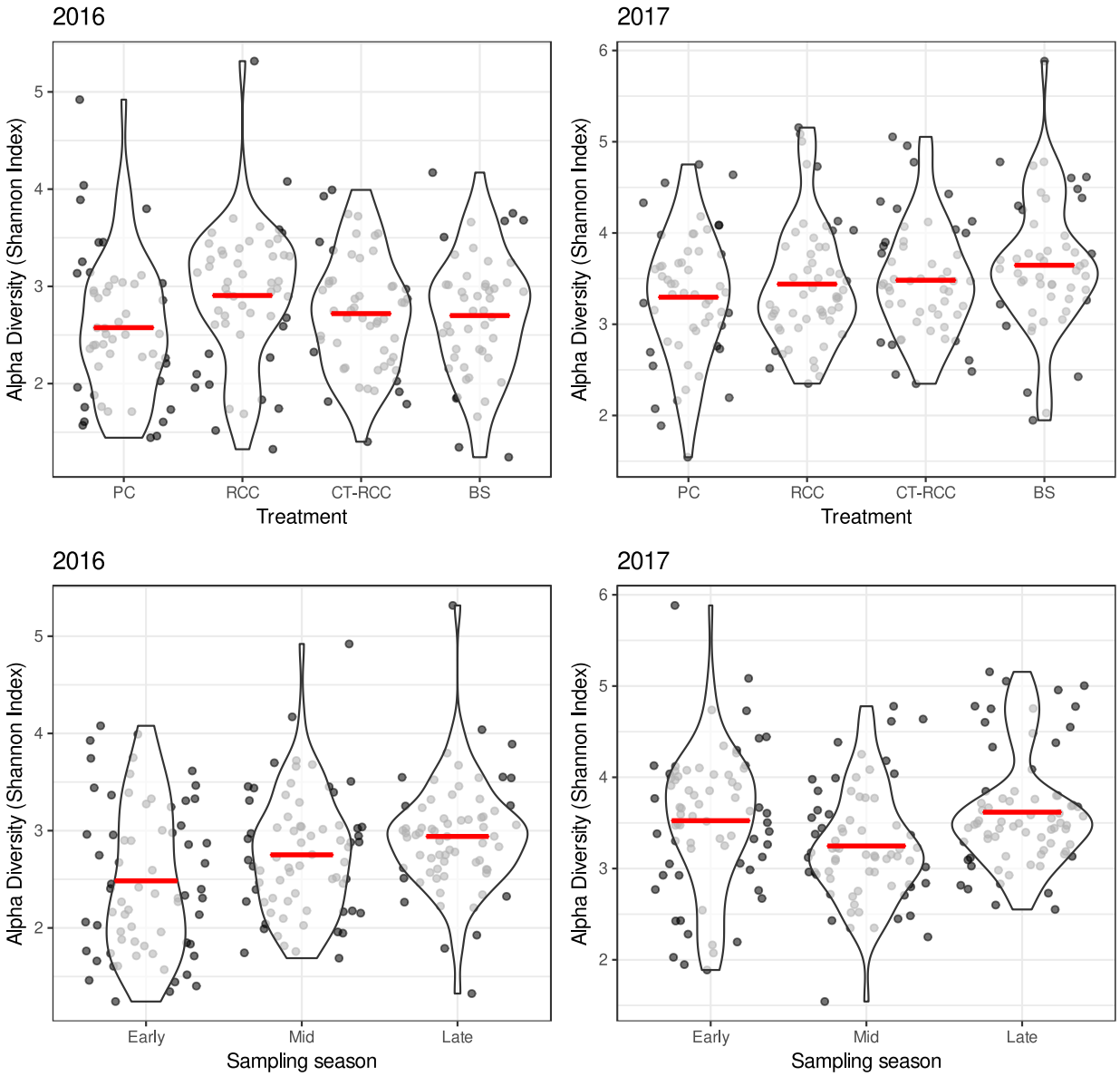
Supplemental Figure 1.4: Principal component analysis ordination among phyllosphere samples and control samples for the growing season 2016 and 2017. Ellipses represent standard deviation around samples from each category. Control samples, including positive controls Mock_Control (known mix of 5 bacterial isolates) and Pseudo_Control (*P. syringae* isolate) and negative control PCR_Control (water), are distinct compositionally from the phyllosphere samples. Corresponding sampling dates are as follows: Early=Date 1, Mid=Date 2, Late=Date 3.



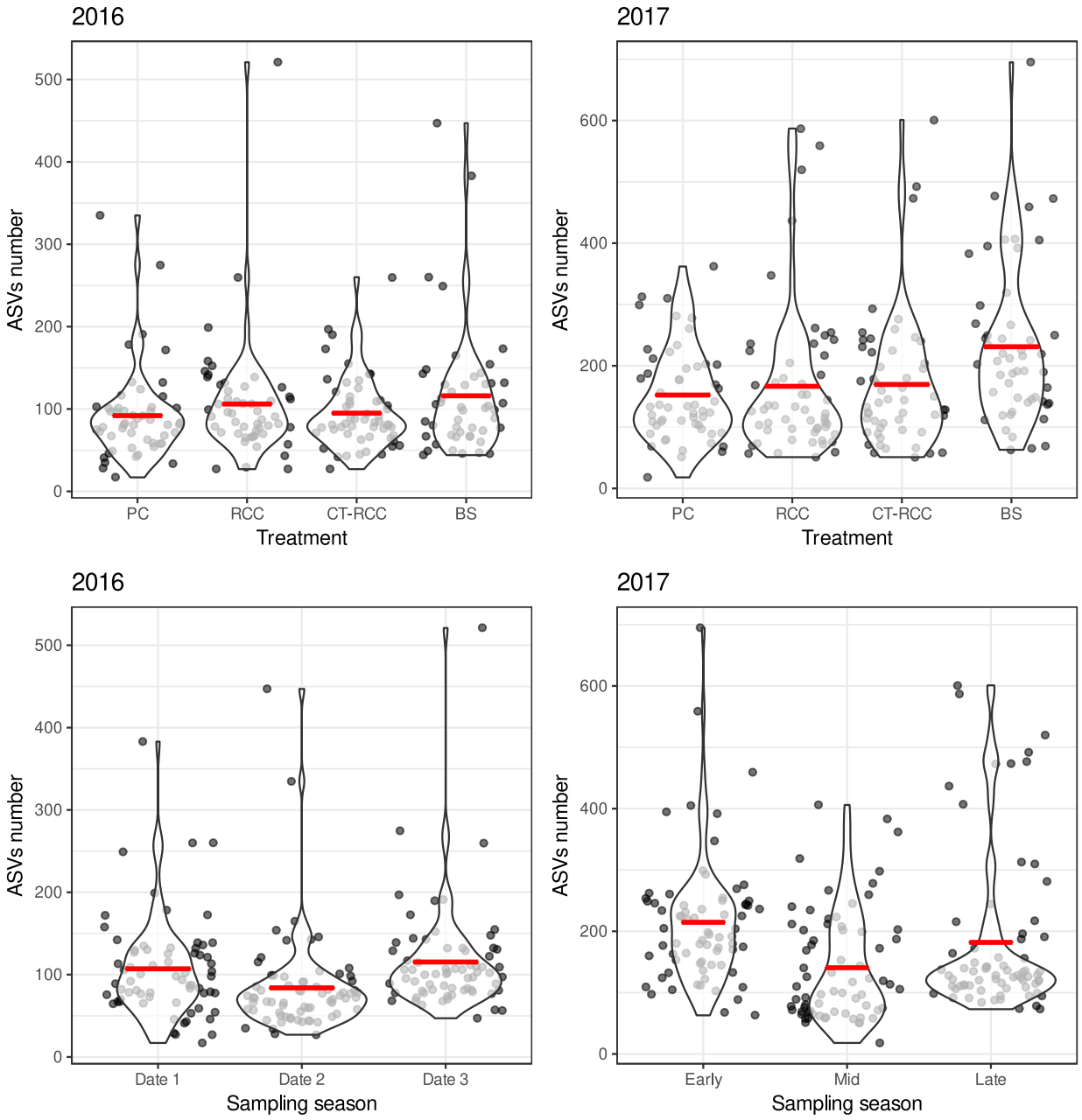
Supplemental Figure 1.5: Rarefaction curves (ASVs versus number of sequences/sample) for each squash sample of growing season 2016 and 2017. Each label represents a different sample. The flat section of each curve indicates that enough sequences have been reached to capture the majority of ASVs for a given sample.



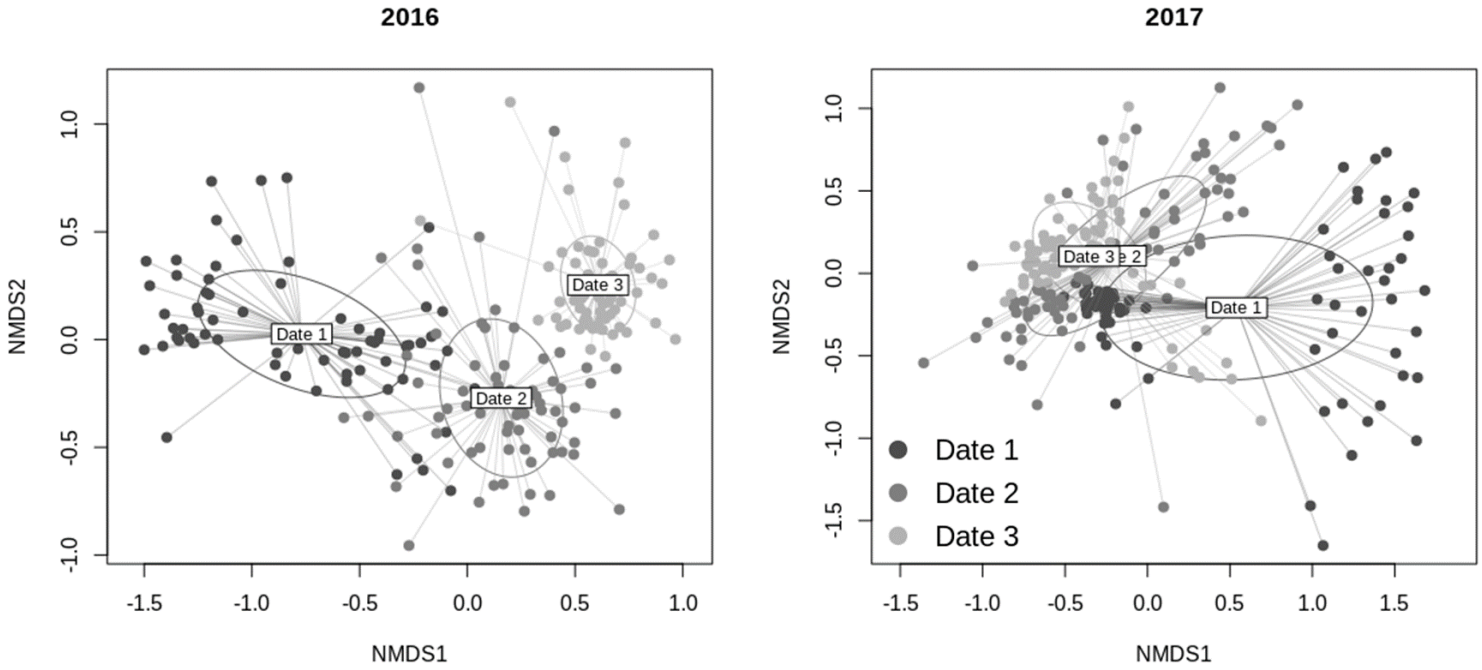
Supplemental Figure 1.6: Plot of rarefaction levels effects on PERMANOVA results and sample number. 100 iteration of rarefaction levels, from 1000 to 10000 random sampling per sample, were tested to evaluate their effects on: R^2 (A), the coefficient of determination representing the proportion of the variable variation explained by the model, and the p-value (B), of the PERMANOVA (per sampling date effect of treatment on community structure), and remaining sample number after rarefaction (C). The vertical dotted line represents the chosen rarefaction level for our study. Each data point represents a given value (R^2, p-value or remaining sample) for each rarefaction level at each season sampling (Early, Mid or Late). If a sample had fewer sequences than the rarefaction level, it was removed by the pipeline.



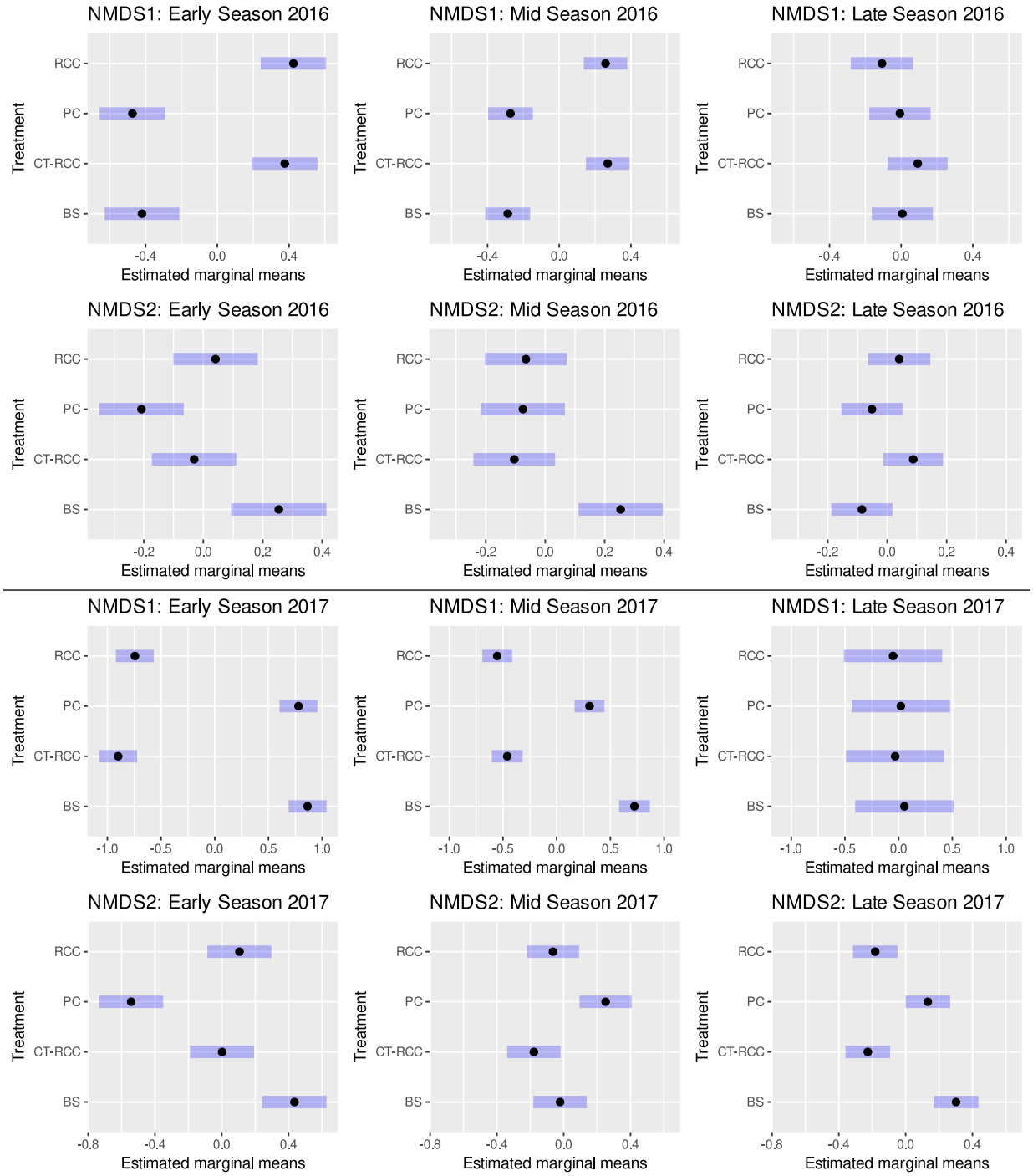
Supplemental Figure 1.7: Alpha diversity (Shannon index) for each treatment and sampling dates (Early, Mid or Late season) during the growing season of years 2016 and 2017. Horizontal red lines represent the mean value. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and BS (Bare Soil).



Supplemental Figure 1.8: Violin plot of ASV richness at each treatment and sampling date during the growing season of years 2016 and 2017. Horizontal red line represents the distribution mean. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and BS (Bare Soil).

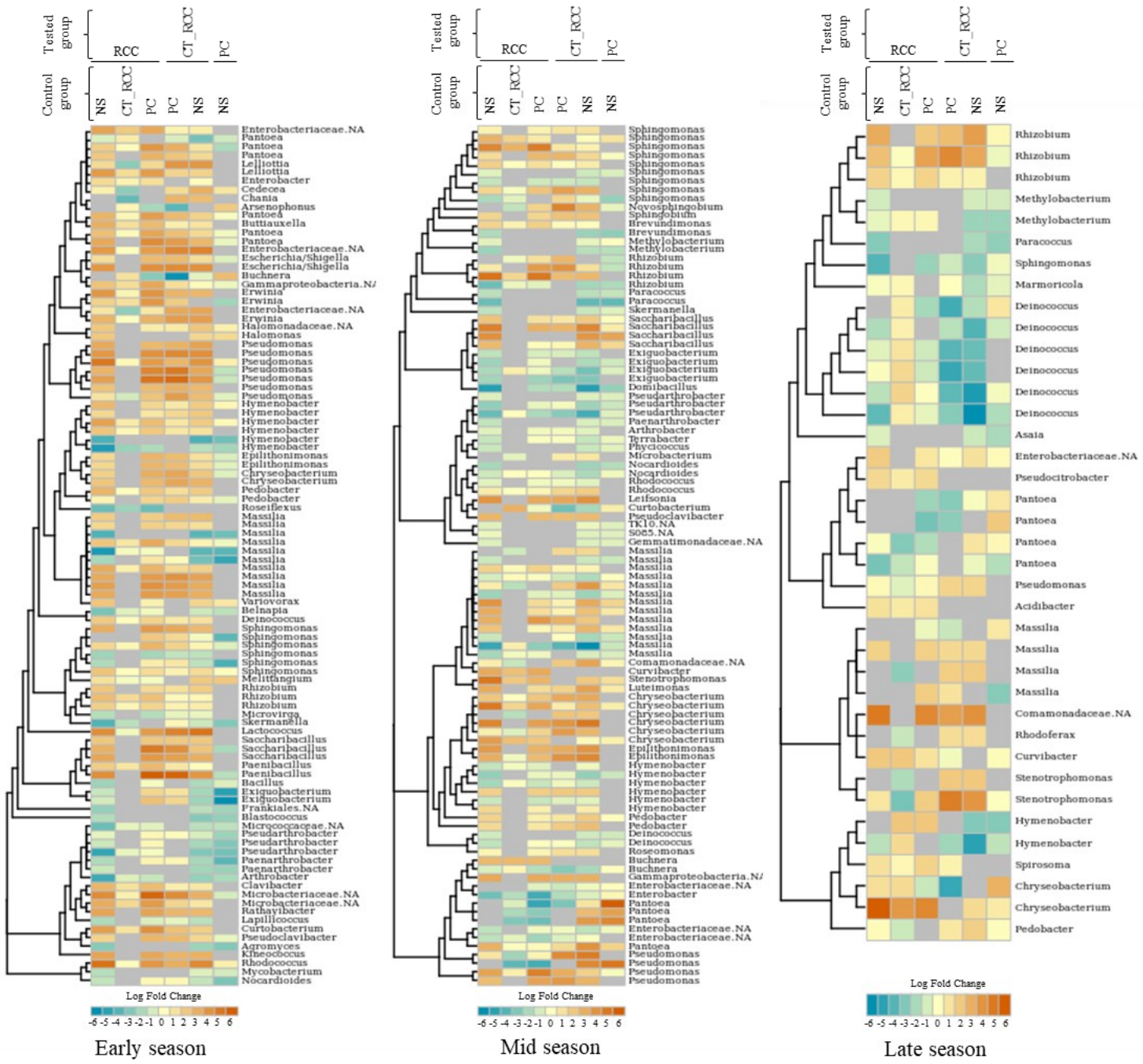


Supplemental Figure 1.9: Non-metric multidimensional scaling (NMDS) ordination of squash phyllosphere community during the growing season of 2016 and 2017 at the 3 sampling dates. Each point represents a phyllosphere community. Corresponding season sampling dates are as follows: Early=Date 1, Mid=Date 2, Late=Date 3.



Supplemental Figure 1.10: Least square means plot of linear mixed model comparison between treatment and the 2 NMDS axis scores, at each sampling date of the growing season 2016 and 2017. Least squares comparisons have been performed with the emmeans v1.4.8 R package (Lenth et al., 2018) on a linear mixed model with NMDS axis scores as a function of treatment (fixed effect) with experimental block as random effect. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and BS (Bare Soil). Two overlapping horizontal bars indicating that the 2 treatments are

not significantly different. The more distant the 2 bars, the greater the differences between the 2 associated treatments.



Supplemental Figure 1.11: Log2-fold change (LFC) heatmap of most differentially abundant ASV from DeSeq2 analysis for each sampling date of the 2016 samples. For each panel, the left track is the phylogenetic tree from PyNAST alignment of ASVs sequence while the right track is the corresponding taxonomic name at the Genus rank. Each heatmap column is a different contrast between two treatments mentioned in the header as follows: above name is the "tested" treatment whereas the below one is the "control" treatment meaning that positive an LFC value represents an ASV more abundant for the tested

treatment. Grey color represents no LFC for the ASV. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and BS (Bare Soil).

Supplemental Analysis 1.1: Effect of treatments on overall community diversity

Cover cropping treatments influenced the diversity of the bacterial community on squash leaves as measured by the Shannon index of rarefied bacterial communities (supplemental Figure 3.6). During the growing season, the community diversity was marginally significantly higher for the rye treatment in 2016 (TukeyHSD post-hoc test performed on linear model of community diversity * treatment; p=0.0565) and significantly higher for the bare soil (TukeyHSD post-hoc test performed on linear mixed model of community diversity ~ treatment with block as the random effect p= 0.0332) in 2017 as compare to the other treatments whereas overall treatment effect was marginally significant (linear model of community diversity ~ treatment; 2016: p=0.086, 2017: p= 0.053). Moreover, community diversity was significantly different between sampling dates (linear model of community diversity ~ sampling date; 2016, p> 0.001, 2017: p= 0.003). While this diversity was lower for sampling date 2 as compare to sampling date 1 (TukeyHSD post-hoc test performed on the above-mentioned linear model; p= 0.031) and date 3 (p= 0.003) in year 2017 (no significant differences between date 1 and 3), it was higher for both sampling dates 2 (TukeyHSD post-hoc test performed on the above-mentioned linear mixed model; p= 0.045) and 3 (p< 0.001) than the sampling date 1 in year 2016 (no significant difference between date 2 and 3).

The total richness of ASVs in bacterial communities also differed among treatments and dates (supplemental Figure 3.7). No significant ASVs number differences between treatments were observed during the 2016 overall growing season (linear model of community richness ~ treatment; p=0.201), however it was statistically different in 2017 (p< 0.001) with more ASVs in the bare soil (p< 0.001) as compared to other treatments. Unlike the alpha diversity of 2016, the ASVs numbers were different between sampling dates (linear model of community richness ~ sampling date; p= 0.009) and significantly lower at date 2 (TukeyHSD post-hoc test performed on the above-mentioned linear model; p= 0.009). On the contrary, as the overall alpha diversity, the ASVs number was still different in the growing season in 2017, with less ASVs at sampling date 2 as compared to dates 1 (p< 0.001) and 3 (p= 0.067).

ANNEXE B

Matériel supplémentaire du Chapitre 2

Supplemental Table 2.1: Sequences count per sample. Counts have been retrieved after the first round of host decontamination with Bowtie 2 (see material and methods).

Sample ID	Number of uncontaminated sequences
r1t2	212,275,386
r1t3	56,808,122
r1t4	208,406,374
r2t1	115,001,904
r2t2	151,266,556
r2t3	87,552,492
r2t4	267,472,550
r3t1	189,813,768
r3t2	282,167,256
r3t3	171,870,796
r3t4	147,943,552
Total	1,890,578,756

Supplemental Table 2.2: Counts of number of sequences with taxonomic annotations at the domain level for phyllosphere microbial communities on squash.

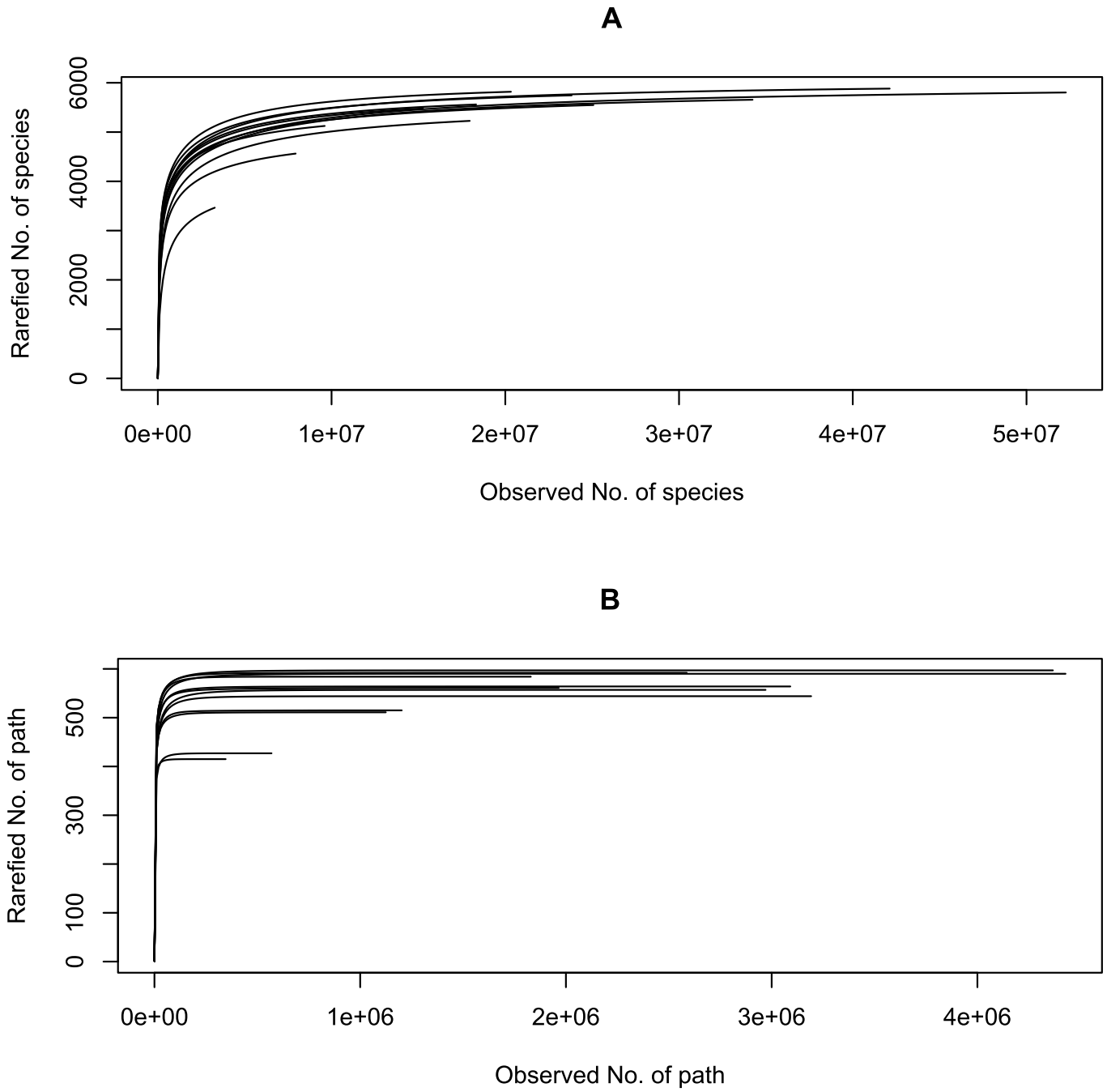
Domain	Abund	Abund (%)
Archaea	55,127	0.02
Fungi	251,961	0.09
Viruses	7,320	0.00
Bacteria	270,225,967	99.88
Total	270,540,375	100

Supplemental Table 2.3: Cover crop treatment effect over the entire squash phyllosphere community taxonomic composition based on rarefied count data. P-value and R^2 have been retrieved from PERManova perform with Euclidean distance matrix of Hellinger transformed taxonomic counts as a function of treatment.

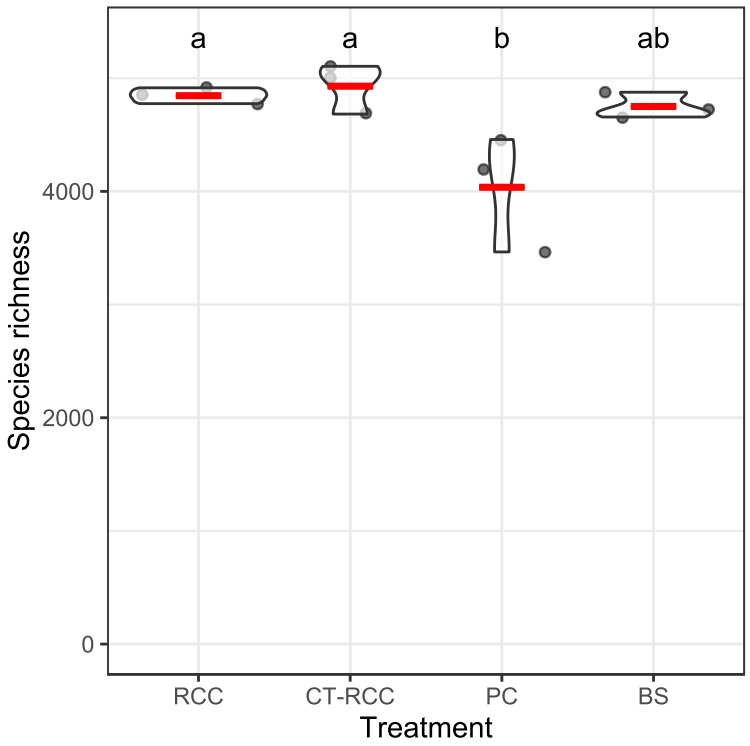
	phylum	class	order	family	genus	species
p value	0.005	0.001	0.006	0.019	0.002	0.003
R2	0.828	0.82	0.687	0.541	0.639	0.571

Supplemental Table 2.4: post-hoc test results performed on linear model with PCA axis scores as a function of treatments. Letters represent treatment grouping from TukeyHSD: different letters denote treatment differences significant at $p<0.05$

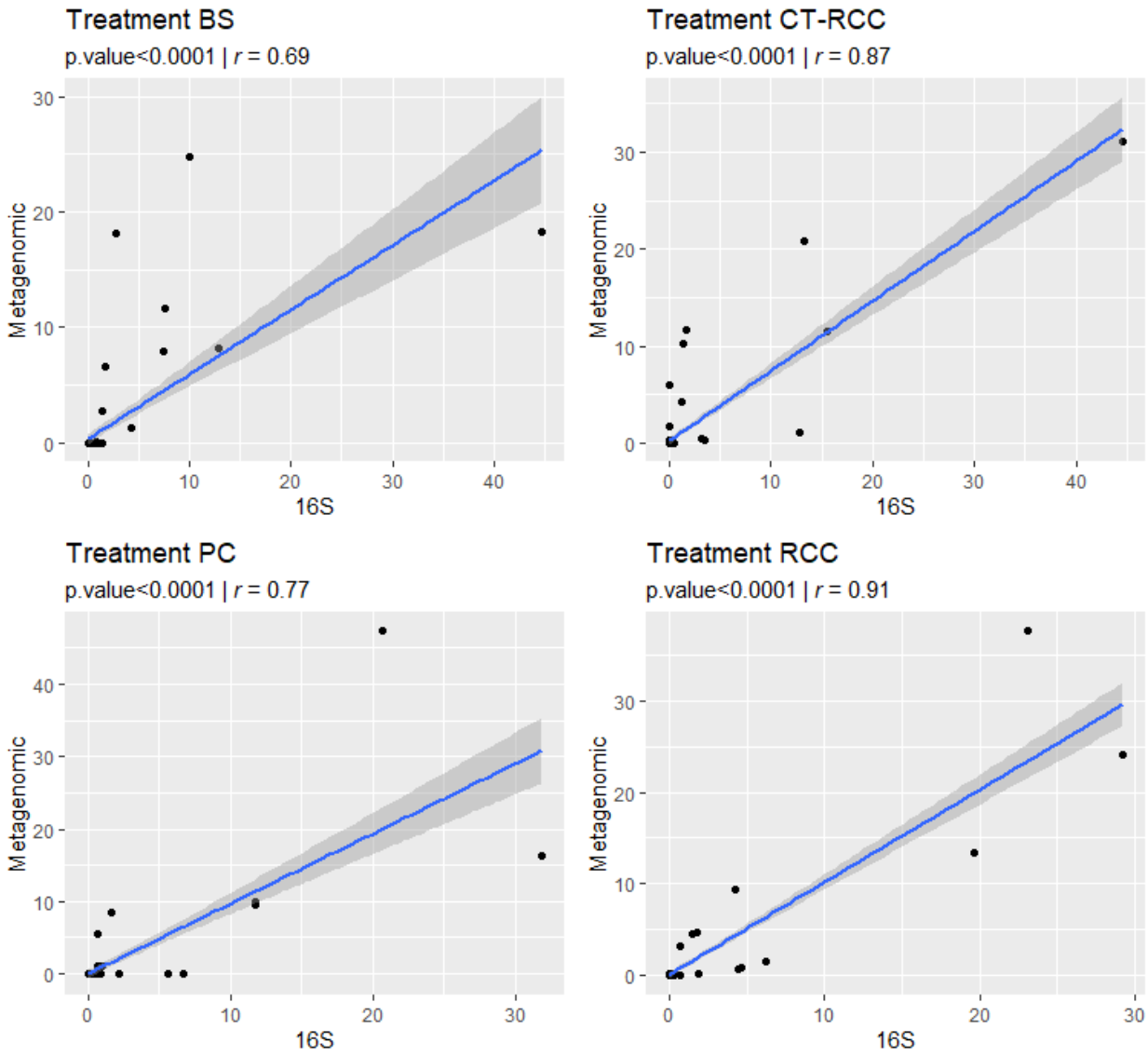
Treatment	PCA1	PCA2
RCC	a	a
CT-RCC	a	a
PC	b	a
BS	b	a



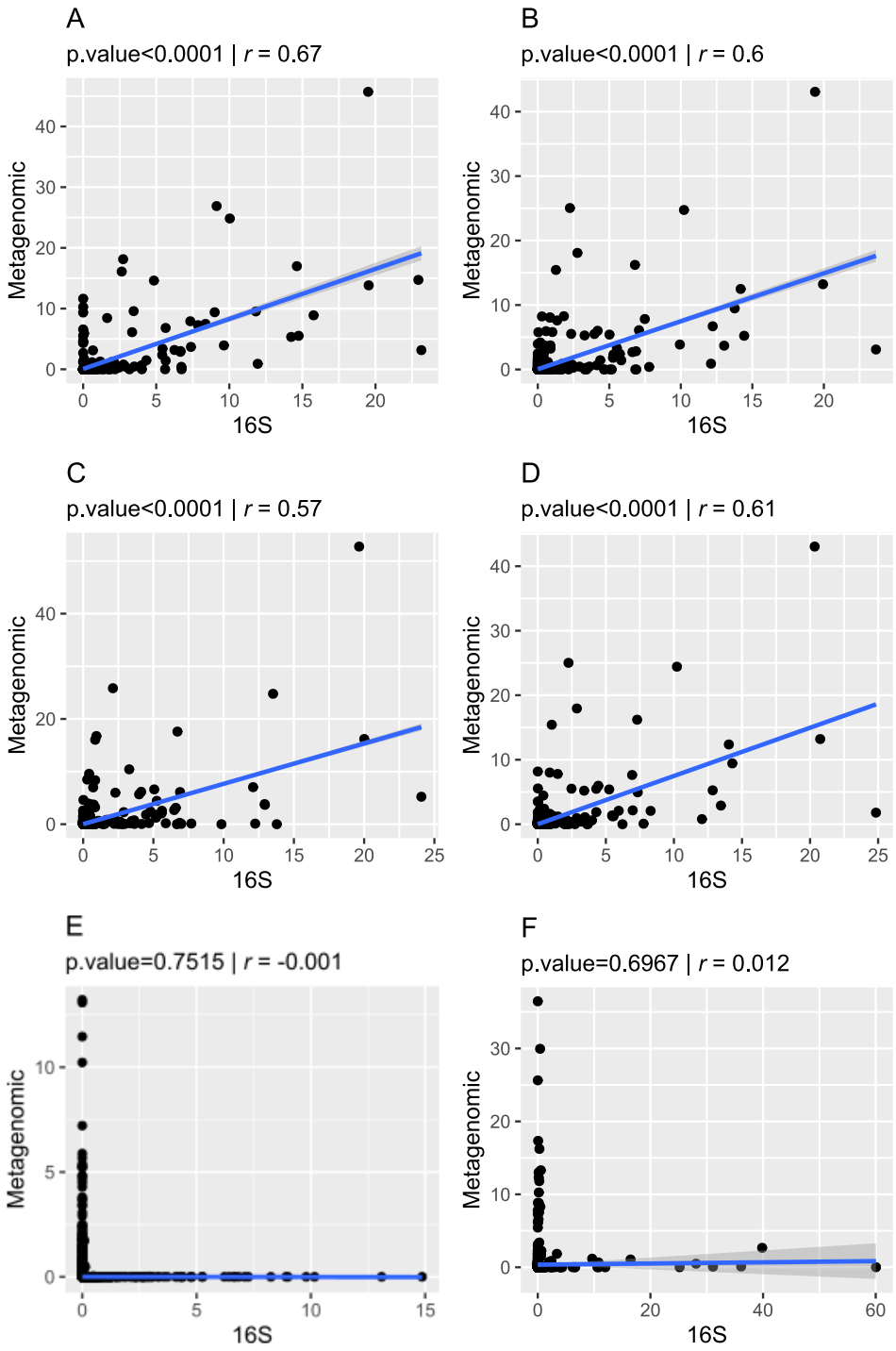
Supplemental Figure 2.1: rarefaction curves. Rarefaction curves for taxonomic (A) and pathway annotations (B). According to these rarefaction curves, subsequent rarefactions were performed with abundance of sample with the lowest total count, 3279280 and 346136 for taxonomic and pathway annotations respectively



Supplemental Figure 2.2: violin plots of the species number of bacterial communities present on the phyllosphere of squash between cropping treatments. The horizontal red line within each violin indicates the mean distribution. The letters above each violin represent post-hoc tests performed with the Tukey HSD analysis on the linear regression of species number as a function of treatment. Different letters indicate significant differences between treatment groups (p value < 0.05).

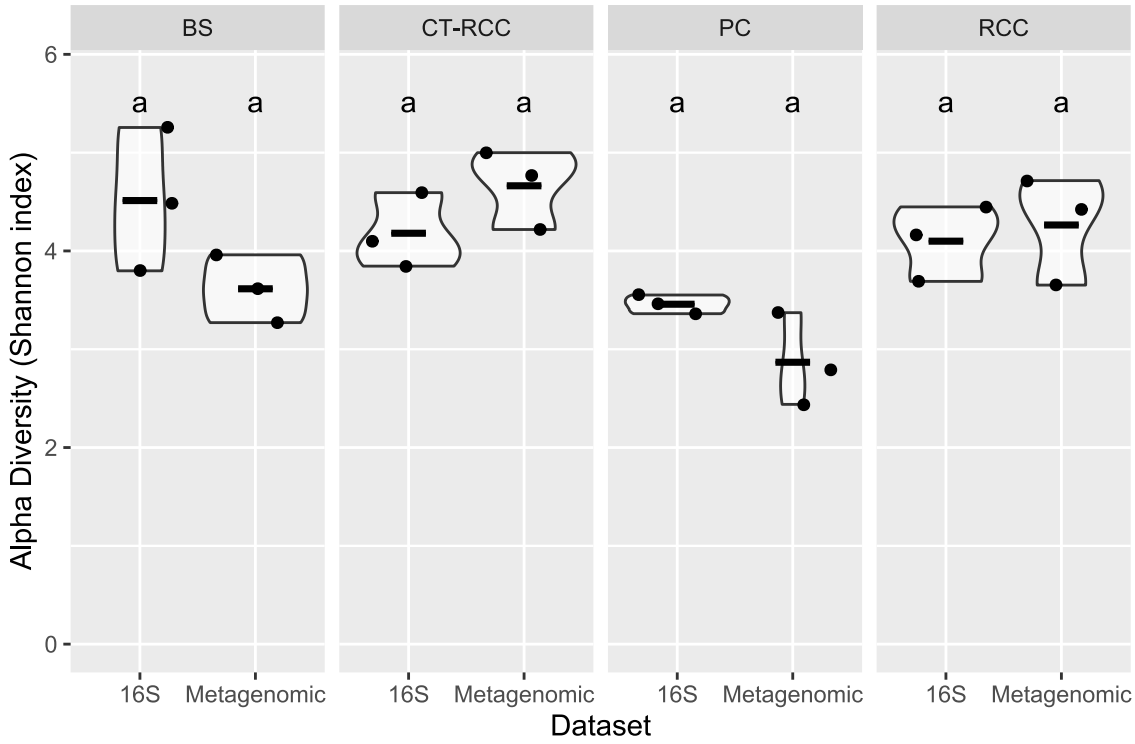


Supplemental Figure 2.3: Correlation of overall Phylum abundances between 16S rRNA gene amplicon sequencing and metagenomic *shotgun* sequencing methods for each treatment. Blue line: linear regression. Grey zone: 95% confidence interval. Pearson correlation test results (p-value and estimates) are shown in the title.

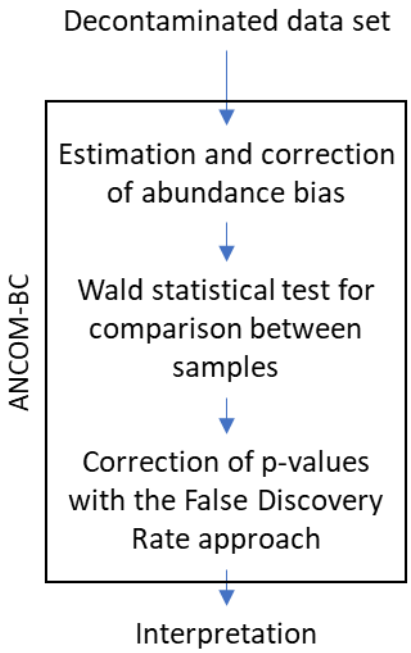


Supplemental Figure 2.5: Scatterplot of bacterial community relative abundance correlation between 16S rRNA gene amplicon sequencing and metagenomic *shotgun* sequencing at different taxonomic phylum rank. Correlation plot of overall taxonomic abundance between 16S and metagenomic methods for all treatment at rank: A: Order, B: Class, C: Family, D: Genus, E and G: Species. G: only the shared taxonomic annotations were kept for test correlation between sequencing methods.

Supplemental Figure 2.6: Venn diagram of shared taxonomic annotations at different phylogenetic rank between sequencing methods. Venn diagrams have been plotted for each taxonomic rank (rank specified in each plot title) for both sequencing methods: Blue: untargeted ("Metagenomic"); Yellow: targeted ("16S"). Each diagram has a raw taxonomic annotation count value and its relative count below shown as a percentage.



Supplemental Figure 2.7: violin plots of the alpha diversity of bacterial communities present on the phyllosphere of squash between different sequencing approach. The horizontal line within each violin indicates the mean distribution. The letters above each violin represent post-hoc tests performed with the Tukey HSD analysis on the linear regression of alpha diversity as a function of treatment. Different letters indicate significant differences between treatment groups (p value < 0.5).



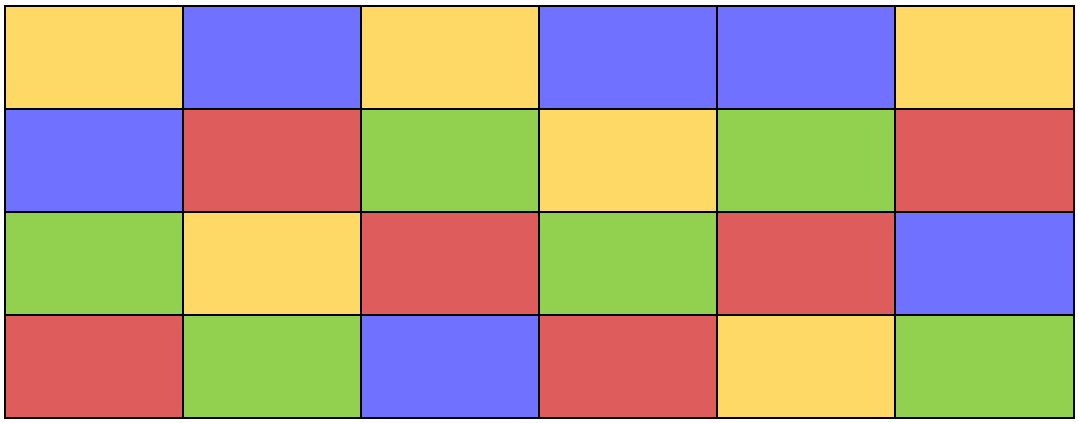
Supplemental Figure 2.8: schematic representation of differential abundance analysis overview performed with ANCOM-BC.

ANNEXE C

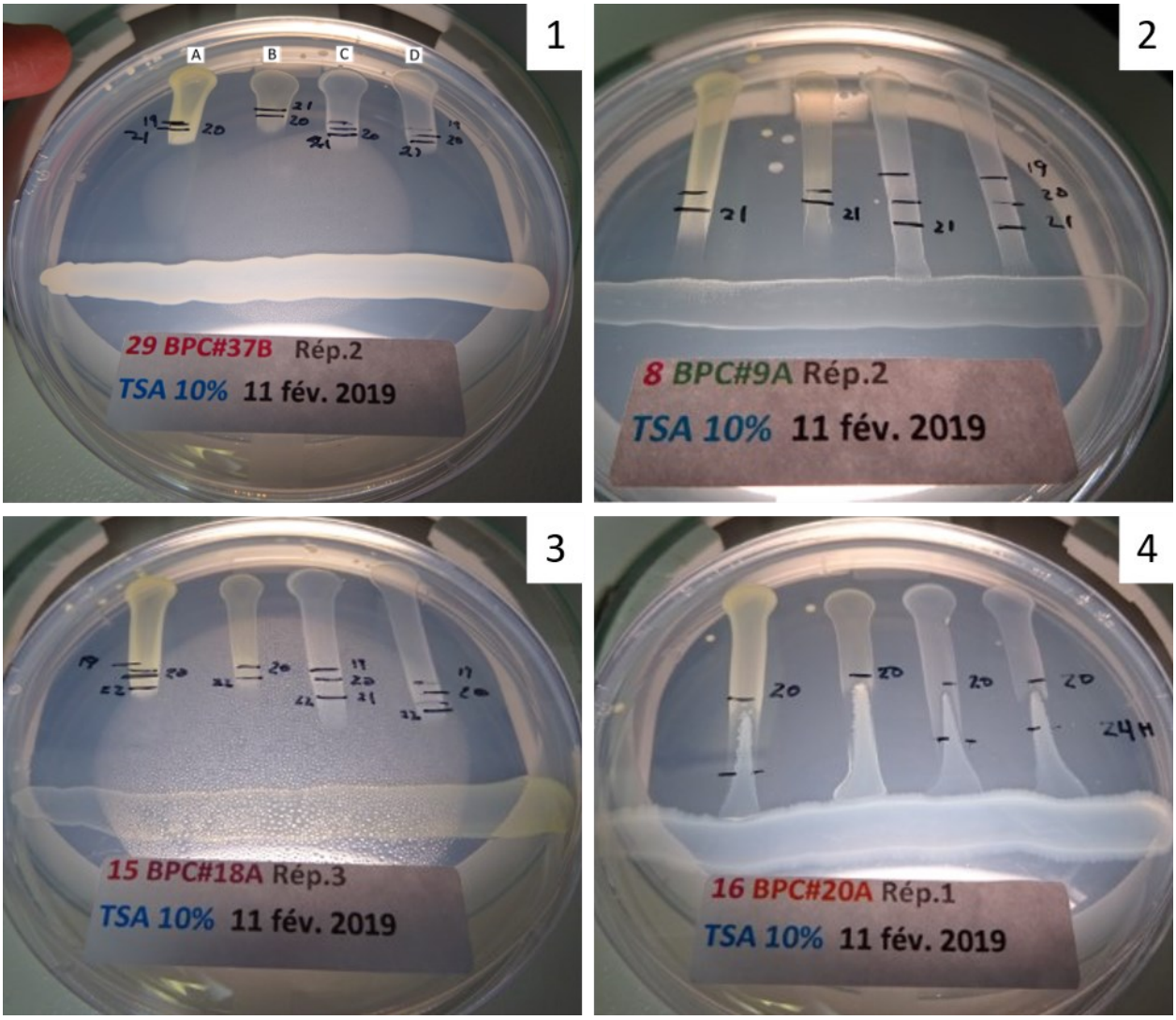
Matériel supplémentaire du Chapitre 3

Supplemental Methods 3.1: Microbial collection protocol description.

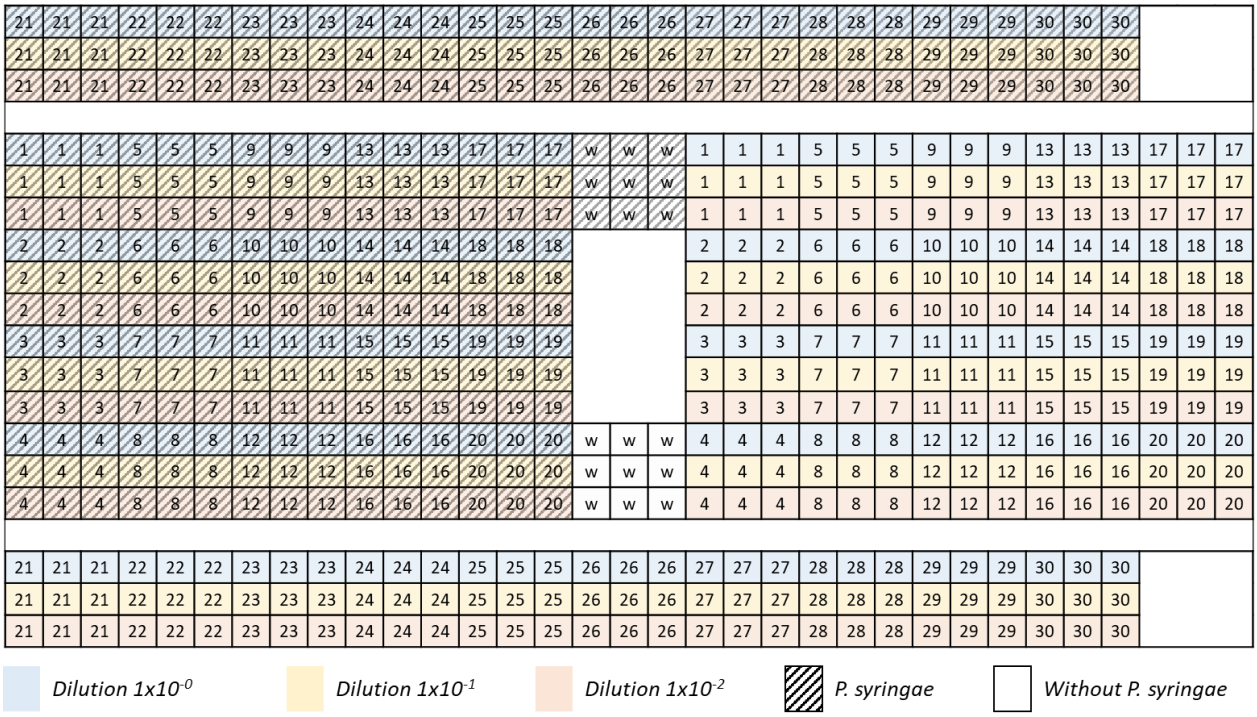
Each sample consisted of an average (± SD) of 16.8 ± 8.3 g and 20.7 ± 4.8 g of young and old leaves harvested from the squash canopy of an individual plant for the 2016 and 2017 seasons, respectively. Bacteria were also retrieved from rye cover crop material. Cover crop samples consisted of a mix of stems and leaves of the rye cover crops. Specifically, the material was collected in a 20 cm x 20 cm area, directly below the previously sampled squash phyllosphere. Three replicated samples of both squash leaves and rye cover crop material were collected per plot for a total of 72 samples (3 samples x 4 treatments x 6 replicates) per sampling date. Microbial cells were then retrieved by washing each sample with 110 mL of a sterile saline solution (0.85% NaCl). 1ml of the washed solution was used for microbial culture and the rest was frozen at -20°C.



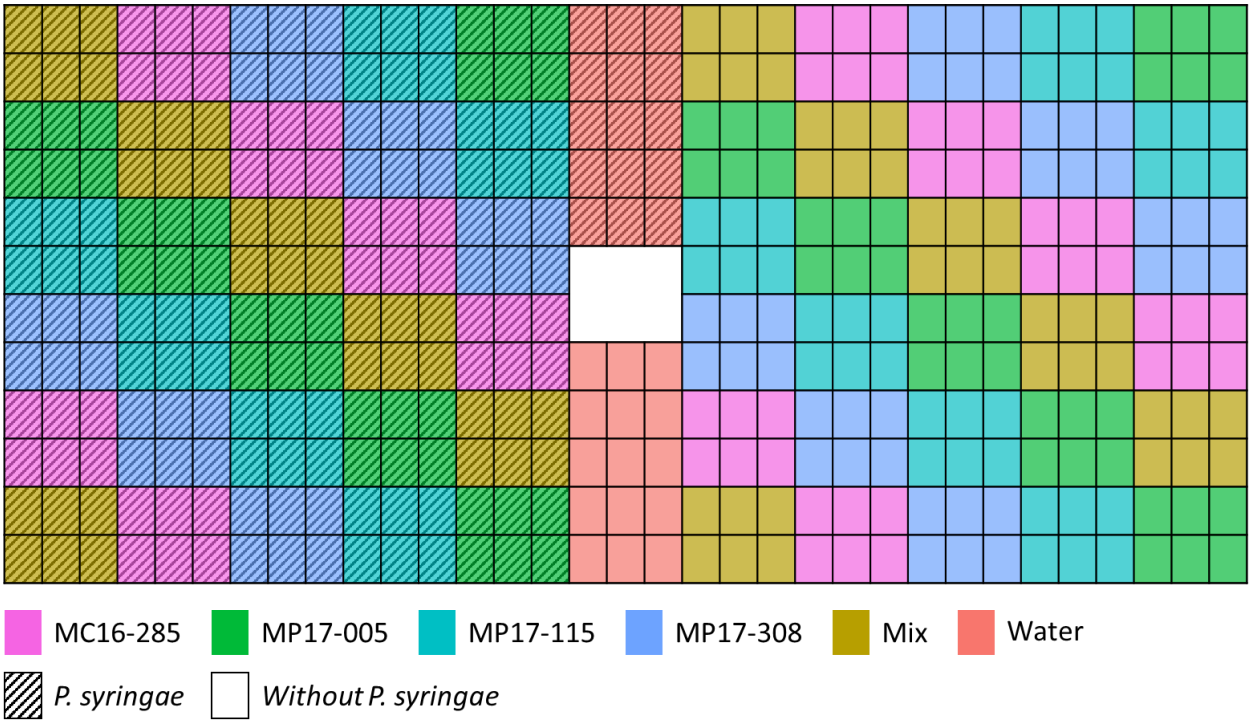
Supplemental Figure 3.1: Diagram of the field experiment layout. Cell colors correspond to treatments: yellow: Bare Soil (BS), blue: Plastic Cover (PC), green: Rye Cover Crop (RCC), red: Chemically Terminated Rye Cover Crop (CT-RCC).



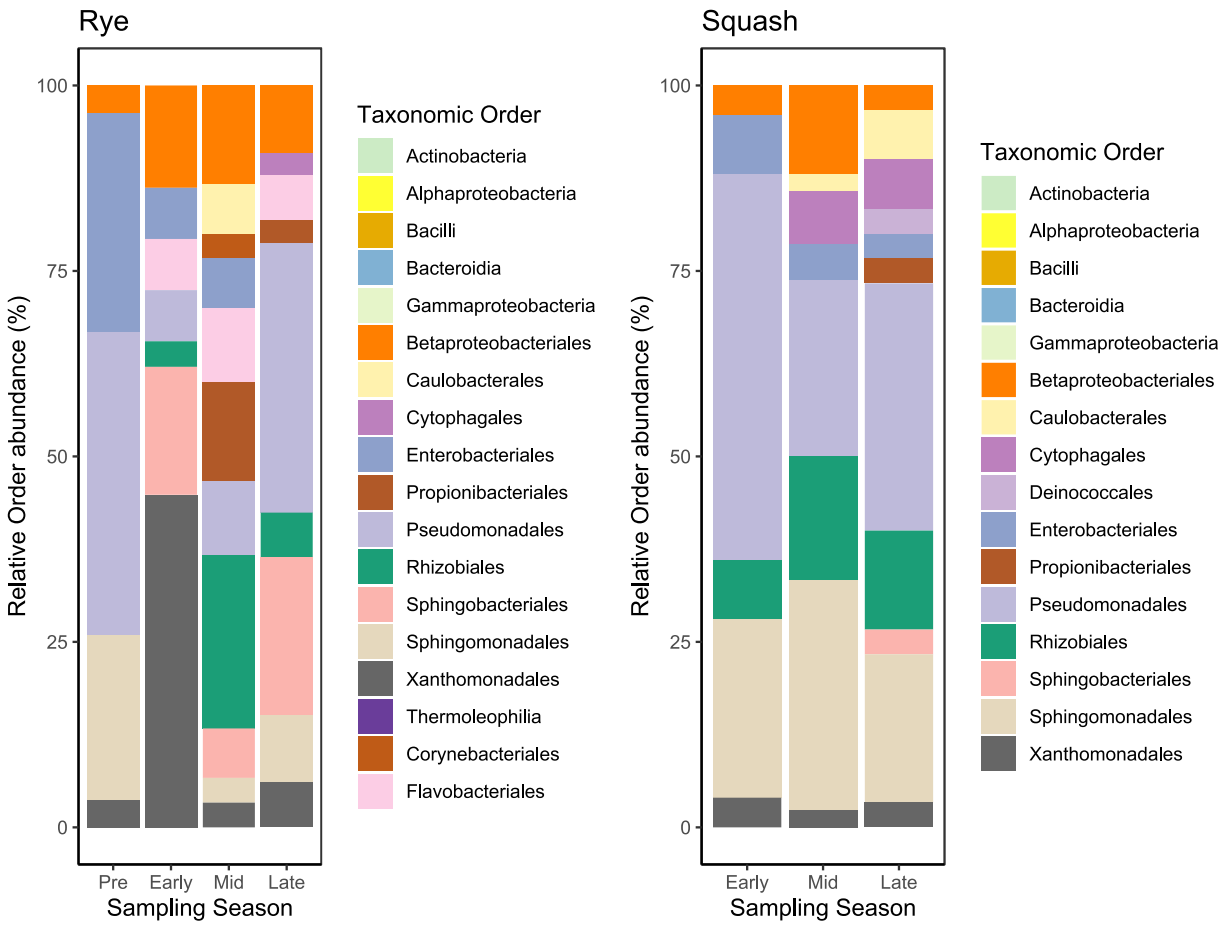
Supplemental Figure 3.2: Example of a pathogen growth inhibition assay on petri plate. Bacterial isolates were plated (stripe at bottom of plate) 4 days before testing against various pathogens (4 drops at top of plate)*:* A; *P. syringae* from *C. pepo*, B; *Xanthomonas campestris* from *Brassica oleracea,* C; *Xanthomonas hortorum* from *Lactuca sativa and* D; *P. syringae pv phaseolicola* from *Phaseolus vulgaris.* Pathogen growth inhibition by the isolate was assessed by measuring the distance between the edges of the pathogen growth (bottom edge) and the bacterial candidate growth line (top edge) using a ruler under a 60X binocular microscope, and expressing growth inhibition as a percentage of maximum possible inhibition. Numbers represent different inhibition profiles: 1: stable; 2-3: decreasing and 4: increasing.



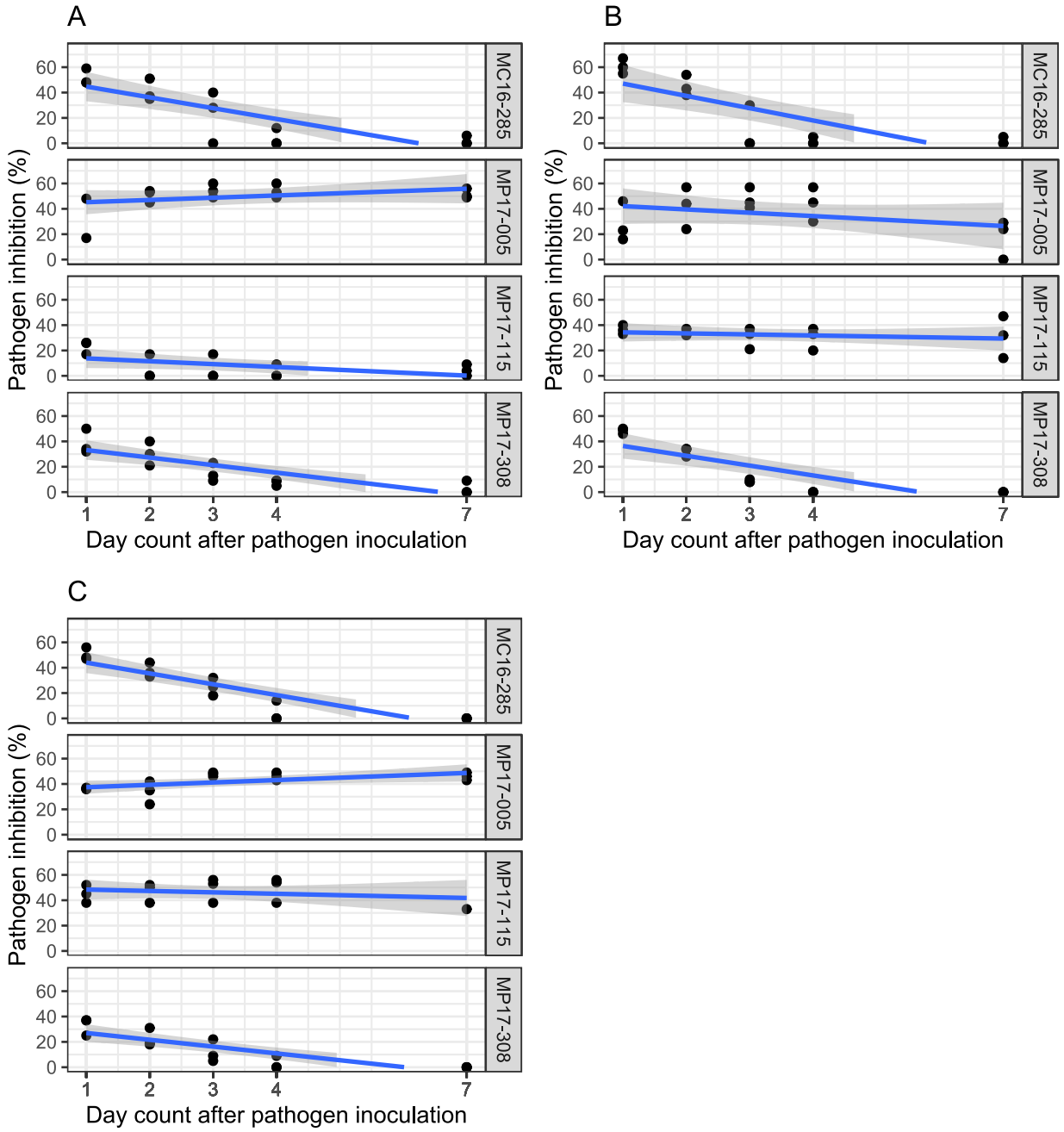
Supplemental Figure 3.3: Diagram of *In vivo* pre-selection assay in greenhouse. Each square represents a plant. 1-30 numbering represent the different antagonist candidates. Antagonist treatments were performed 4 days before the pathogen inoculation, by spraying the plant under a fume hood, at three different dilutions: blue; $1x10^{-0}$ (no dilution), yellow; $1x10^{-1}$, and pink; $1x10^{-2}$. Each pathogen inoculation condition (sterile water with or without *P. syringae*) is identified with a distinct pattern, as displayed in the legend at the bottom of the figure. 9 squash plants were grown per pot saucer for each antagonism candidate. Plant that did not receive pathogen during the inoculation were placed in a separate greenhouse room to be sprayed by sterile water. After one hour, all plants were returned to the original greenhouse room, at the same position, as displayed in this diagram. Since 468 squash plants were required for this preselection experiment, candidates 21 to 30 were grown in separate greenhouse sections. This greenhouse room was composed of 3 sections separated by 2 corridors (the two horizontal white space) used by workers.



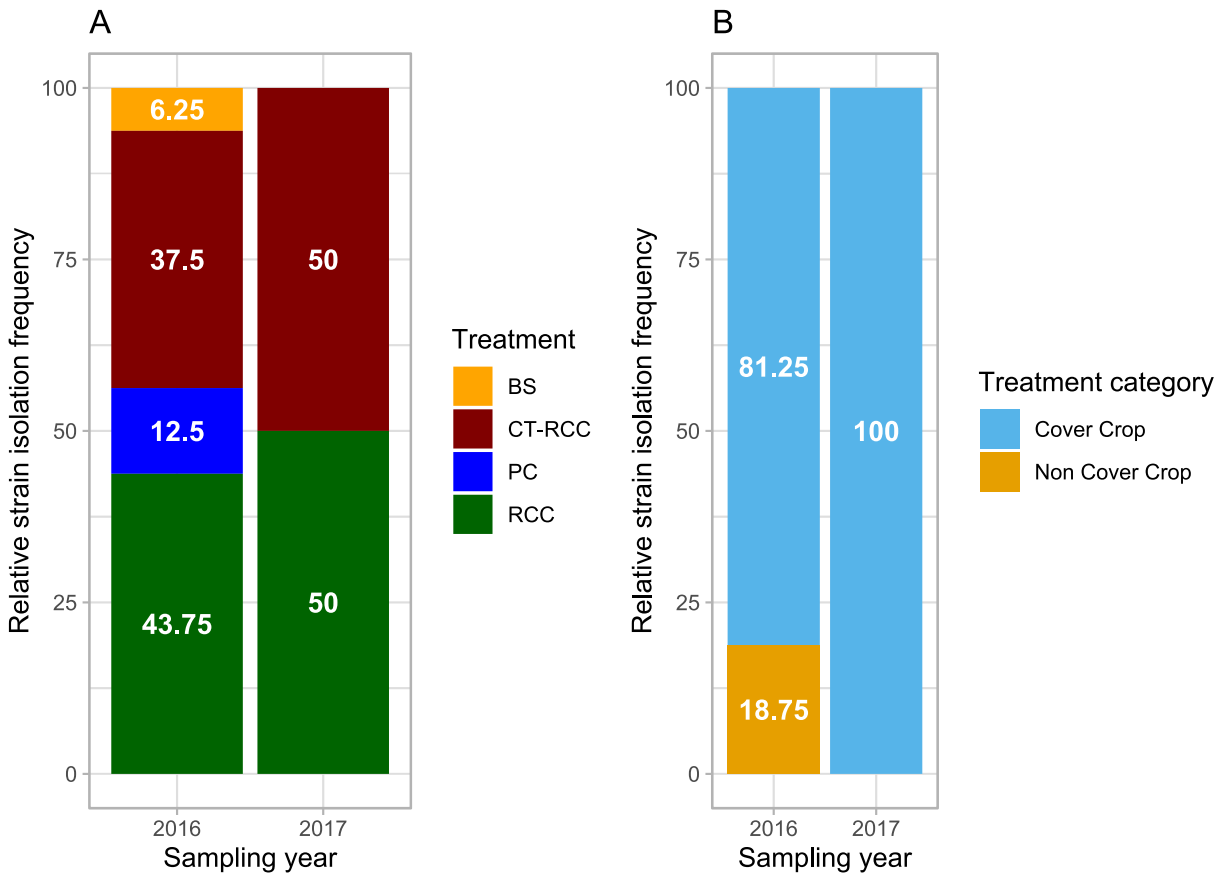
Supplemental Figure 3.4: Diagram of the *In vivo* greenhouse ALS disease symptom control experiment. Each square represents a squash plant. The antagonist treatments, performed 4 days before the pathogen inoculation, were randomized in a true Latin square. Each antagonism treatment is represented by a different color, and the two pathogen inoculation conditions (sterile water with or without *P. syringae*) are identified with a distinct pattern, as displayed in the legend at the bottom of the figure. Six squash plants were grown per greenhouse plot unit in the same pot tray (plant batch). Plants that did not receive pathogen during the inoculation were moved to a separate greenhouse room to be sprayed by sterile water. After one hour, all plants were returned to the original greenhouse room, at the same position, as displayed in this figure.



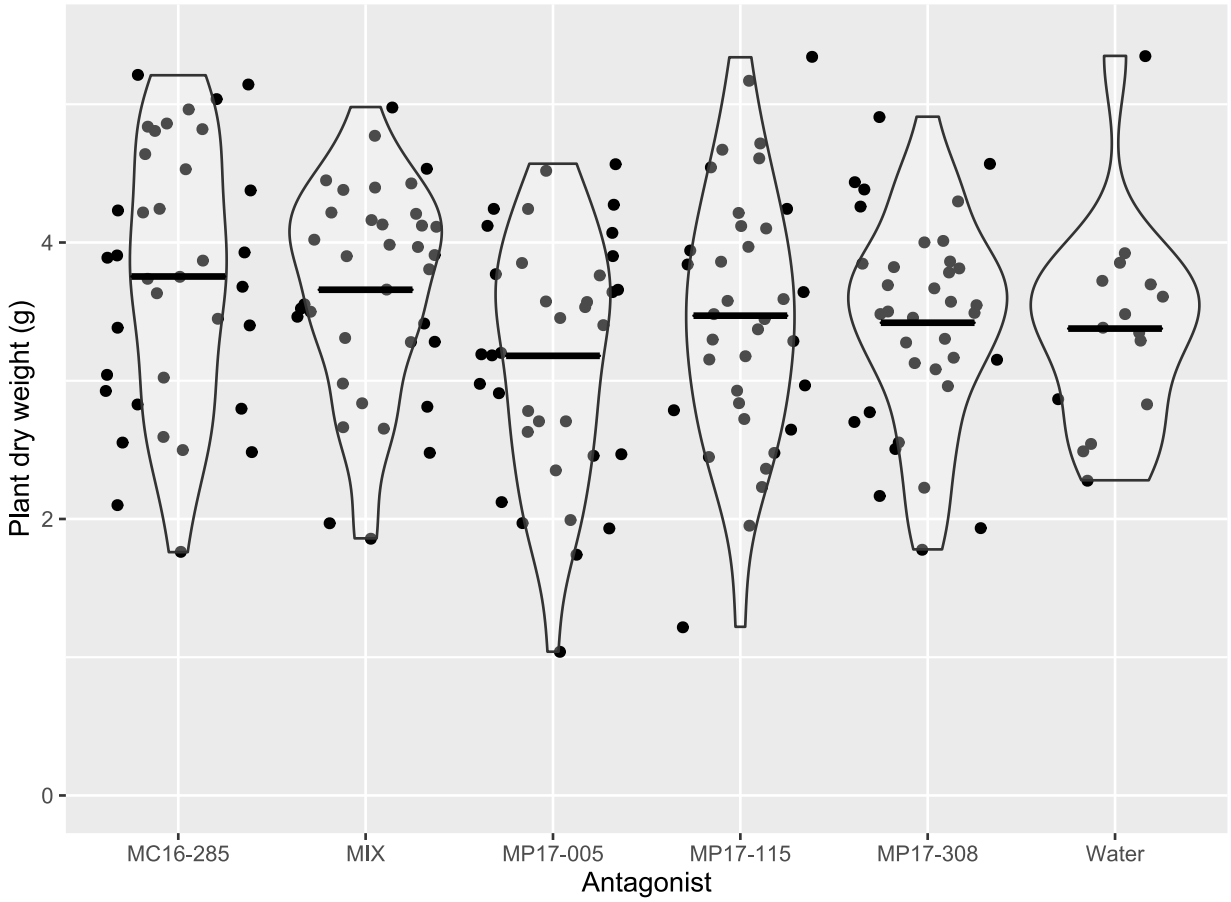
Supplemental Figure 3.5: Relative number of isolated strains belonging to different taxonomic orders. Annotation of bacteria at the taxonomic order rank, isolated from rye cover crop (left panel) or squash leaves (right panel) for each sampling date, *Pre, Early*, *Mid* or *Late.* Relative order abundance was computed based on the sum of sequence abundances per order across both growing seasons 2016 and 2017.



Supplemental Figure 3.6: Percentage of pathogen growth inhibition by antagonist candidates over time. This competition assay was conducted against three different pathogens: A; *Xanthomonas hortorum* from *Lactuca sativa*, B; *P. syringae pv. phaseolicola* from *Phaseolus vulgaris* and C; *Xanthomonas campestris* from *Brassica oleracea.*



Supplemental Figure 3.7: Relative frequencies per sampling year of the treatments from which antagonists of the *In vitro* test were isolated. A. Isolation frequency from each treatment: BS: Bare Soil; CT-RCC: Chemically Terminated Rye Cover Crops; PC: Plastic Cover; RCC: Rye Cover Crop. B. Isolation from Rye Cover Crop or Non-Rye Cover Crop treatment. In 2016, isolations of strain that showed antagonist effects against *P. syringae* during the *In vitro* test were significantly higher in both rye treatments as compared to BS and PC treatments according to a chi-square test (p=0.012). Moreover, all competent strains were isolated from a rye treatment in 2017. Finally, isolations events were not significantly different between rye treatments for both experimental years according to a Chi-squared test (2016; p=0.78 and 2017; p=1).



Supplemental Figure 3.8: Plant dry weight differences among antagonist treatments. After symptom counting in the *In vivo* competition assay, plants were harvested by cutting them at the soil surface. Then, plant dry weight was measured from the aboveground parts of the plant after 2 days in a dryer at 72°C. A linear mixed model with plant dry weight as a function of antagonist as a fixed effect and block as a random effect revealed that antagonists did not influence plant dry weight (p=0.07).

Supplemental Table 3.1: Mean daily temperature and humidity values at time of sampling during growing seasons 2016 and 2017. Values were extracted from the daily weather reports generated for the Acadie farm (see Methods section for details).

Year	Time of sampling	Temperature (°C)	Humidity (relative %)	Precipitation (mm)
2016	Pre (June 13)	14.5	71.5	2.7
	Early (July 12)	21.7	67.6	0
	Mid (August 1)	20.2	79.6	0
	Late (September 1)	18	78.2	1.4
2017	Pre (June 12)	12.4	70.6	0
	Early (July 13)	24.8	74.4	0.8
	Mid (August 1)	20.2	79.6	0
	Late (September 8)	23.1	86.5	0

Supplemental Table 3.2: Average ALS disease symptoms counts as a function of antagonist treatment for each assessed leaf. Differences among treatment were tested using Tukey's honestly significant difference (HSD) test, based on a generalized mixed model (Poisson distribution), were calculated with the emmeans R package. Treatments categorized by different letters were significantly different according to the Tukey HSD test ($p<0.05$), performed on the following model: *symptom_count ~ antagonist * leaf + (1|block row) + (1| block column).* Leaf A to Leaf D: from older leaves to younger leaves.

	Leaf A			**Leaf B**			**Leaf C**			**Leaf D**		
Antagonist	**Mean**	**SD**	**Tukey**	**Mean**	**SD**	**Tukey**	**Mean**	**SD**	**Tukey**	**Mean**	**SD**	**Tukey**
Water	4.3	4.0	a	5.3	5.0	a	2.6	3.4	a	1.5	1.9	a
MC16-285	1.6	2.4	cd	1.9	2.5	b	1.6	2.4	ab	0.4	0.8	b
MP17-005	1.3	1.6	d	1.5	2.7	b	1.1	1.6	b	0.6	1.1	b
MP17-115	3.1	6.7	ab	2.3	4.5	b	1.1	1.6	b	0.6	1.0	b
MP17-308	2.3	2.8	bc	1.8	2.3	b	1.7	2.6	ab	1.9	3.7	a
MIX	2.5	3.9	b	2.2	2.8	b	1.6	2.1	ab	0.6	1.6	b

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