CYTOKININ-PRODUCING *Methylobacterium* AS BIOLOGICAL CONTROL AGENTS OF PHYTOPATHOGENS

A dissertation submitted to the Committee of Graduate Studies

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the Faculty of Arts and Science.

Trent University

Peterborough, Ontario, Canada

Environmental and Life Sciences Ph.D. Graduate Program

September 2025

ABSTRACT

CYTOKININ-PRODUCING *Methylobacterium* AS BIOLOGICAL CONTROL AGENTS OF PHYTOPATHOGENS

Daniel Michael Palberg

Methylobacterium spp., a dominant and functionally conserved group of plant-associated bacteria, have long been recognized for their roles in promoting host growth, stress tolerance, and phytohormone modulation. This body of work collectively repositions *Methylobacterium* not only as a plant growth-promoting genus but also as a promising agent of microbiome-mediated crop protection. Across several investigations, the ecological, biochemical, and functional attributes that underpin this potential were examined, with specific focus on hormone production, compatibility with agrochemical inputs, and antifungal activity.

A comprehensive inventory of 46 *Methylobacterium* strains revealed widespread production of cytokinins – including highly active forms such as *trans*-zeatin – and variable capacities to synthesize indole-3-acetic acid. Cytokinin output increased under carbon-limiting conditions, highlighting the genus's adaptive hormonal response. Parallel investigations demonstrated that commercial glyphosate-based herbicide formulations significantly inhibited the growth of most *Methylobacterium* strains, whereas pure glyphosate alone showed negligible toxicity. Key findings of experiments indicate that non-active formulation components participate in the disruption of beneficial bacteria by facilitating higher intracellular glyphosate concentrations and subsequent toxic effects.

This introduces a novel link between agrichemical formulation practices and the selective disruption of keystone microbial taxa.

Contrastingly, fungicide compatibility testing showed that *Methylobacterium* strains tolerate key fungicides such as azoxystrobin, fludioxonil, and metalaxyl-M, supporting their inclusion in integrated pest management frameworks. Subsequent functional antagonism assays further revealed that specific *Methylobacterium* isolates inhibit phytopathogenic *Fusarium* species *in vitro* and *in planta*. Notably, *M. organophilum* enhanced soybean seedling vigor and reduced disease severity when co-inoculated with *F. graminearum* by preserving the integrity of the seed coat, demonstrating protective activity with unique mechanics.

Finally, differential hormone profiling at the pathogen-antagonist interface revealed that biocontrol-effective *Methylobacterium* strains not only produce higher levels of auxin and salicylic acid but also induces jasmonic acid production – likely derived from *Fusarium* – suggesting complex cross-signalling and interference with fungal development and sensing pathways. Together, these findings advance our understanding of *Methylobacterium* as a keystone genus in the phytobiome, capable of contributing to both plant vigor and pathogen suppression and reinforce its relevance in the design of next-generation biocontrol strategies.

KEYWORDS: *Methylobacterium*, phytobiome, biological control, *Fusarium* antagonism, phytohormone signalling, cytokinin interference, agrochemical interactions.

PREFACE

This thesis is presented in manuscript form. Chapter 1 serves as an introduction to the central concepts underpinning the work. Content from Chapters 2 - 4 encompass published experimental work. Chapters 5 and 6 encompass experimental work that will be submitted for publication upon completion of this thesis. All co-authors and their associated contributions are listed in the preface of each chapter. Copyright authorization associated with published chapters are presented in the preface of each chapter. References across all chapters have been uniformly presented in the style of the American Phytopathological Society (APS), and a digital object identifier (DOI) or an international standard serial number (ISSN) has been provided when available.

Additional publications not included in this thesis:

Ngoc Nguyen, H., Butler, C., **Palberg, D.**, Kisiala, A. B., Emery, R. J. N. 2022. The tRNA-degradation pathway impacts the phenotype and metabolome of *Arabidopsis thaliana*: evidence from *atipt2* and *atipt9* knockout mutants. Plant Growth Regulation. https://doi.org/10.1007/s10725-023-00987-1

Kaszecki, E., **Palberg, D.**, Grant, M., Griffin, S., Dhanjal, C., Capperauld, M., Emery, R. J. N., Saville, B. J. S. 2023. *Euglena mutabilis* exists in a FAB consortium with microbes that enhance cadmium tolerance. Springer International Microbiology. https://doi.org/10.1007/s10123-023-00474-7

Capperauld, M., **Palberg, D.**, Emery, R. J. N. 2024. Pulp or Potential? Magneto-Priming as a Seed Priming Technique Has Little Effect on Seedling Performance but Results in Significant Alteration of *trans*-Zeatin and cis-Zeatin in Soybean (*Glycine max*). International Journal of Plant Biology. https://doi.org/10.3390/ijpb15040089

Grich, N., Huynh, T., Kisiala, A. B., **Palberg, D.**, Emery, R. J. N. 2024. The biosynthesis and impacts of cytokinins on growth of the oyster mushroom, *Pleurotus ostreatus.* Mycologia. https://doi.org/10.1080/00275514.2024.2401320

AUTHOR'S NOTE ON GENERATIVE AI USE AND RIGHTS

No generative artificial intelligence (AI) was used in the conceptualization and development of ideas including experimental design or methodology, nor in the original writing of this work. All intellectual contributions are solely those of the author and respective cited contributors or co-investigators, as applicable.

The author expressly prohibits the use of this work – whether in whole or in part – for training, fine-tuning, or otherwise optimization of generative AI models, including but not limited to language models capable of producing text in a similar style or voice. The author reserves all rights to license or withhold permission for the use of this work and its novel contributions in the development of AI technologies.

This document contains unique security features designed to protect against unauthorized reproduction, distribution, or use, including imbedded markers which enable identification even in derivative or modified forms.

ACKNOWLEDGEMENTS

To all those who have graciously lent their guidance, insight, and support and have in some way aided in the preparation of this work, I owe my profound thanks.

To my advisor, Dr. Neil Emery, whose intellectual curiosity, rigorous standards, and patient mentorship have shaped every chapter of this work. Your leadership – equal parts incisive and encouraging – pushed me to think critically, write clearly, and to aim higher than I imagined possible. Beyond academia, your steady confidence in my potential provided a foundation during both the most challenging and rewarding moments of this journey. Your deep commitment as both a scholar and a mentor has left an indelible mark and will influence my approach to research in perpetuity. It has been an honor and a privilege to learn under your direction.

To Dr. Naomi Stock, whose guidance and presence have been a constant throughout nearly my entire decade at Trent University, I am equally indebted. Among all those who have influenced my academic journey, Naomi has unfailingly embraced the demanding – and often thankless – role of patiently engaging with each of my ideas, proposals, and ambitious schemes with sincere interest and thoughtful insight.

To Dr. Erin Morrison, for her thoughtful questions, her distinct ability to probe assumptions, and her ability to offer constructive suggestions. Your feedback has played a significant role in refining the broader conceptual framework and narrative arc of this dissertation, and for that I am especially grateful.

For over half a decade, I have had the privilege of working in the Emery Lab – an environment distinguished by its spirit of collaboration and shared purpose. For their advice, support, and camaraderie, I extend my sincere thanks to my colleagues:

Dr. Megan Aoki, Peter Andreas, Zeynab Azimychetabi, Kimberly Molina Bean, Cody Butler, Dr. Scott Farrow, Vedanti Ghatwala, Nourhene Grich, Stacy James, Dr. Anna Kisiala, Alex Kuhne, Dr. Ainsely Lewis, Dr. Shaojun Li, Hai Nguyen, Dr. Thien Nguyen, Imesha Perera, Mark Seegobin, Dr. Ewart Smith, and Dr. Zhiyong Zhang.

To the members of the Department of Biology, the Department of Chemistry, the Department of Environmental and Life Sciences, and the Department of Graduate Studies, and Science Facilities at Trent University, who have played a role in one way or another:

Smolly Coulson, Dr. Eric Keske, Dr. Shegufta Merchant, Debbie Lietz, Dr. Karla Newman, Dr. Steven Rafferty, Angela Sikma, Dr. Paul Szpak, Dr. Jayme Stabler, Dr. Karen Thompson, Dr. Matthew Teeter, and Chris Williams.

I also wish to express my profound gratitude to the late Professor David A. Ellis [2019], whose generosity of spirit and belief in young scholars left a lasting impression on me. He welcomed me as an undergraduate student with kindness and enthusiasm, offering his time and insight at a pivotal moment in my academic journey.

To my international colleagues:

in Germany – Dr. Alexandria Lanzinger, Daniela, Moni, Birgit, and Michael – and in the United States – Dr. Romina Gazis, Dr. Bruce Schaffer, Shelby, Carlos, Maria, Dr. Sara, and Dr. Monica Navia-Urrutia.

This research was also made possible by generous funding from

the Natural Sciences and Engineering Research Council of Canada (NSERC), the Ontario Graduate Scholarship (OGS) in Science and Technology, the School of Graduate Studies at Trent University, and Trent University's Environmental and Life Sciences Graduate Program. Their support allowed me to pursue ambitious experiments and present my findings at conferences around the world.

Beyond academia, I owe an immeasurable debt of gratitude to my family:

To my parents – who instilled in me the values of curiosity, perseverance, and integrity – for their unwavering confidence in my abilities, which have carried me through the inevitable challenges of this project. I am profoundly grateful for your steady presence, despite the distance between us and the many moments we missed together while I pursued my academic ambitions.

To my extended family – Aunt Elisabeth, Rebecca, Bernadette, Uncle Paul [2022], and David – thank you for embracing dinner-table lectures, patiently indulging my frequent interruptions "*Well, actually…*," and for always making my passions feel welcomed and valued. Your patience, humor, and wholehearted support have remained steadfast, even in times when my attention was drawn elsewhere.

To my cousins – Caleb, Luke, Joel, Alex, Lauren, Olivia, and Colton – who have filled the roles of siblings I never had: your encouragement, persistence, and belief in me have meant more than words can adequately capture. You've reminded me that my endeavors were never pursued in isolation, and that family, in all its forms, is a foundation I can always return to.

To my grandmothers, Wendy and Barbara [2024], whose strength, warmth, and wisdom have grounded me throughout my life: though time and distance have sometimes separated us, your reassurance has always reached and uplifted me. Barbara, whose absence in this final year was felt deeply, remains with me in every accomplishment.

To Dr. Aaron and Dr. Emily Slepkov – friends who provided perspective, laughter, offered advice, or simply asked how life was going – your kindness has mattered more than you know. And to William – my roommate for many years and companion in academic survival over the span of a decade – for enduring the suffering and the sleepless nights that are hallmarks of early academic life.

Finally, to my wife, Emma, for being my sounding board and a constant source of motivation, inspiration, and comfort; it is solely because of you that I am reminded that there is, in fact, a life to be had outside the laboratory – and for celebrating every milestone, no matter how seemingly insignificant.

While only my name may appear on the cover, this thesis is the product of a collective effort, impossible without a remarkable cast of characters. It is my hope that these pages in the very least, do justice to the support, guidance, and faith each of you has placed in me.

Nunc cognosco ex parte.

DEDICATION

For Mom and Dad.

TABLE OF CONTENTS

CHAPTER 7 – **General Discussion: *Methylobacterium* as candidates for the restoration and fortification of the phytobiome**

INDEX OF FIGURES

INDEX OF TABLES

INDEX OF ABBREVIATIONS

µg	microgram
µL	microliter
µm	micrometer
2-MeSiP	2-methylthio-N^6-isopentenyladenine
aa	amino acid
ADP	adenosine diphosphate
AGC	automatic gain control
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BA	*N*^{6}-benzyladenine
BC	biological control
BLAST	basic local alignment search tool
C	Celsius
CFU	colony forming units
CK	cytokinin
CKX	cytokinin oxidase
cZ	*cis*-zeatin
EC	extracellular
FB	free base
FS	full scan
g	gram
h	hour
HPLC	high-performance liquid chromatography
HPLC-(ESI+)	high-performance liquid chromatography-electrospray ionization (positive ion mode)
HRMS/MS	high resolution tandem mass spectrometry
HPLC-(HRAM)-FS-MS	high performance liquid chromatography-high resolution accurate mass-full scan mass spectrometry
HPLC-(ESI +)-HRMS/MS	high performance liquid chromatography-electrospray ionization (positive ion mode)- high resolution tandem mass spectrometry
IC	intracellular
iP	*N6*-isopentenyladenine
IPP	isopentenyl pyrophosphate
iPR	*N6*-isopentenyladenine-9-riboside
IPT	adenylate isopentenyltransferase
iptA	adenylate isopentenyltransferase gene

iptA	adenylate isopentenyltransferase protein
itpA	inosine triphosphate pyrophosphatase
m/z	mass-to-charge ratio
ms	millisecond
mm	millimeter
mM	micromolar
n.d.	not detected
NCE	normalized collision energy (eV)
ng	nanogram
nmol	nanomolar
NT	nucleotide
PBS	phosphate buffered saline
pmol	picomole
ppm	parts per million
PRM	parallel reaction monitoring
RCF	relative centrifugal force
SPE	solid phase extraction
TCA	tricarboxylic acid cycle
tRNA	transfer RNA
tRNA-IPT	tRNA-isopentenyltransferase

CHAPTER 1

1.1. PREFACE

Title: General Introduction

Author: Daniel Palberg

CHAPTER 1

GENERAL INTRODUCTION

1.2. THE GENUS *Methylobacterium*

The genus *Methylobacterium* (family *Methylobacteriaceae*, class *Alphaproteobacteria*) currently comprises 49 described species that are widely distributed in natural and agricultural ecosystems. These bacteria are frequently isolated from soil, water, air, and, most notably, the phyllosphere and endosphere of plants, where they form stable associations as part of the core phytobiome (Green, 2006; Kutschera, 2007). Most members of the genus are non-pathogenic and pose little health risk to humans and wildlife, save for those with immunocompromise (Gilardi and Faur, 1984; Sanders et al. 2000; Lai et al. 2011). As gram-negative, pink-pigmented, facultative methylotrophs, *Methylobacterium* spp. are capable of metabolizing single-carbon (C_1) compounds such as methanol, a trait that plays a key role in their ecological fitness and plant-associated lifestyle (Chistoserdova et al. 2009).

In plant-associated environments, certain strains of *Methylobacterium* function as plant growth-promoting bacteria (PGPB), a classification earned by their ability to produce and secrete phytohormones, which stimulate plant cell division, delay senescence, and enhance chlorophyll biosynthesis (Koenig et al. 2002; Zhao et al. 2024). By increasing the photosynthetic capacity and nutrient uptake efficiency of their host, CK-producing PGPB *Methylobacterium* can improve seed setting, biomass accumulation, and overall yield (Jameson, 2000; Holland, 1997). Recent studies have also explored the role of *Methylobacterium* in modulating plant stress responses and enhancing resilience to abiotic pressures such as drought and high salinity (Jorge et al. 2019; Lee et al. 2015). Interestingly, even foliar enrichment with CK-producing isolates

has been associated with increased antioxidant enzyme activity and an upregulation of stress-responsive genes in various crops (Madhaiyan et al. 2006; Ivanova et al. 2001).

Given their multifunctionality, the *Methylobacterium* are increasingly considered for applications in agriculture. As global trends shift toward a reduction in chemical inputs, interest in plant-associated symbionts has intensified (Compant et al. 2005; Glick, 2012). Several studies have demonstrated that inoculation with *Methylobacterium* can improve crop performance under optimal conditions and buoy their health during abiotic challenge. For instance, *M. oryzae* has been shown to increase plant height, biomass, and chlorophyll content in rice (Madhaiyan et al. 2004), while foliar application of *M. extorquens* similarly enhanced growth in tomato and mustard (Meena et al. 2017). In a more detailed mechanistic study, Jorge et al. (2019) demonstrated that the presence of *Methylobacterium* significantly increased seed yield in drought-stressed lentil (*Lens culinaris* Medik.) by modulating key hormonal pathways. Inoculated plants not only exhibited enhanced chlorophyll content and biomass accumulation but also elevated levels of cytokinins (including *trans*-zeatin), indole-3-acetic acid (IAA), abscisic acid (ABA), and salicylic acid (SA), alongside metabolic adjustments for enhanced osmoprotection (Jorge et al. 2019).

Beyond growth promotion, *Methylobacterium* spp. have shown potential as biocontrol agents through mechanisms that extend beyond nutrient competition or niche exclusion. Their capacity to secrete phytohormones along with other metabolites like volatile organic compounds (VOCs), suggests a broader role in priming plant immunity and disrupting pathogen development (Kutschera, 2007; Ehinmitan et al. 2024). The ability of *Methylobacterium* isolates to modulate host defense signalling pathways

through induced systemic resistance (ISR), is a characteristic increasingly exploited in bio-inoculant formulations (Pieterse et al. 2014; Audenaert et al. 2002).

Methylobacterium also exhibits traits favorable for commercial deployment, including environmental persistence, colonization efficiency, and safety. Their adaptability to a range of environmental conditions, particularly those with low nutrient density, provides a practical advantage in field applications where survivability of inoculum is a frequent challenge (Green, 2006; Sy et al. 2005). As the body of evidence concerning the agricultural utility of *Methylobacterium* grows, key isolates will be positioned not only as nutritional mutualists but as active participants in the structuring of the phytobiome and potentially serious candidates for biologically based crop protection strategies.

1.3. THE GENUS *Fusarium*

The genus *Fusarium* is a diverse and globally distributed group of filamentous ascomycete fungi encompassing more than 300 phylogenetically distinct species, many of which are well-known for their pathogenicity in plants (Dean et al. 2012; Geiser et al. 2013; O'Donnell et al. 2008, 2016; Zhang et al. 2006). Ubiquitous in soil and plant debris, *Fusarium* species can colonize a wide range of hosts, including virtually every economically important crop family ranging from cereals and vegetables to legumes and ornamentals (Leslie and Summerell, 2006). Phytopathogenic *Fusarium* species are considered among the most destructive fungal pathogens in agriculture, and are responsible for a vast spectrum of diseases, including vascular wilts, root rots, seedling blights, and head blights. Among the most prominent disease-causing species are *Fusarium oxysporum*, *F. graminearum*, *F. culmorum*, *F. fujikuroi*, and *F. solani*, each

with specialized pathogenic forms (*formae speciales*) adapted to specific hosts (Gordon, 2017; Dean et al. 2012).

The pathogenic success of *Fusarium* is largely driven by its remarkable genetic and biochemical plasticity. These fungi produce a wide range of virulence factors, including cell wall-degrading enzymes (CWDEs), effector proteins that manipulate host defenses, and secondary metabolites (SM) that impair host physiology (Brown et al. 2012; Ma et al. 2013). Many of these traits are encoded on lineage-specific chromosomes or supernumerary genomic regions that can be horizontally transferred between strains, enabling rapid adaptation and host range expansion (Ma et al. 2010; 2013).

Environmental persistence is another hallmark of *Fusarium* biology. Many species survive in soil or crop debris for extended periods as spores, and these propagules facilitate survival and transmission across growing seasons (Burgess, 1981). Some species, such as *F. oxysporum*, are particularly adept at colonizing vascular tissues, leading to quick systemic infection of the host – and persistence in even asymptomatic tissues (Fravel et al. 2003). As hemibiotrophs, *Fusarium* species are notorious for exhibiting both biotrophic and necrotrophic traits which aid greatly with the invasion and infection process – even participating in latent-phased infections – which owes to the overall complexity in diagnosis, treatment, and control of most diseases caused by *Fusarium*.

Efforts to manage *Fusarium* are complicated by the adaptability and enhanced tolerance of most species to changes in the environment, the limited efficacy of

fungicides across the species range, and the lack of durable host resistance. Conventional chemical control – unaided by mechanical, behavioural, or engineering controls – often fails to provide complete and lasting protection due to the endophytic nature of infection and the genetic diversity of *Fusarium* populations (Munkvold, 2003). As a result, there is growing interest in alternative control measures, including biological control (BC) using antagonistic microorganisms such as beneficial bacteria and fungi. These strategies aim to inhibit *Fusarium* growth, interfere with its signalling or virulence pathways, or prime plant defenses to reduce infection success (Harman et al. 2004; Junaid et al. 2013). However, the complexity of *Fusarium* biology and its interactions with both plants and other microbes necessitate a nuanced understanding of its molecular ecology to develop truly effective and sustainable solutions.

1.4. PHYTOHORMONES AND THE CYTOKININ FAMILY

Phytohormones are a group of naturally occurring small molecules that regulate every aspect of plant life, including growth, development, reproduction, and responses to environmental stimuli. Synthesized endogenously and active at exceedingly low concentrations (typically 1-10 nmol, but fmol in a number of systems), phytohormones coordinate cellular processes across different tissues and developmental stages, and function primarily as signalling molecules to orchestrate complex and highly regulated networks that allow plants to sense and integrate internal and external cues (Davies, 2010).

The classical categories of phytohormones include auxins, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA), and ethylene (ET). Over several decades, this list has expanded to include additional molecules and families including jasmonic acid

(JA), salicylic acid (SA), brassinosteroids (BR), and strigolactones (SL), which have distinct but frequently overlapping roles in plant physiology (Santner et al. 2009). These hormones interact through intricate networks of synergistic and antagonistic relationships, collectively referred to as hormonal crosstalk, and enable control of developmental programs and finely tuned stress responses.

Among the families, the CKs constitute a vital class of growth regulators which are primarily involved in promoting cell division, regulating shoot and root morphogenesis, and modulating nutrient signalling (Kieber and Schaller, 2018). Although the CKs are classically defined as plant hormones, their biosynthesis is not restricted to the plant kingdom. A diverse array of organisms – including plant-associated bacteria, fungi, and some insects – also produce CKs, which participate in the modulation of microbial metabolism, pathogen virulence, and interkingdom signalling within the plant holobiont (El-Showk et al. 2013; Spallek et al. 2018). Chemically, CKs are substituted derivatives of adenine that are sub-classified into several types based on their side chain structure. The naturally occurring isoprenoid cytokinins include *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), isopentenyladenine (iP), and dihydrozeatin (DZ), while aromatic cytokinins, such as benzylaminopurine (BAP) are also biologically active (Mok and Mok, 2001). In plants, CK biosynthesis primarily involves the enzyme isopentenyltransferase (IPT), which catalyzes the transfer of an isoprenoid side chain to an adenosine phosphate – the resulting cytokinin nucleotide (CK-NT) forms are then converted into active free-base (CK-FB) forms via additional modifications such as hydroxylation and dephosphorylation (Takei et al. 2001).

The transport and perception of CKs are highly organized processes. *trans*-zeatin-type (*t*Z) CKs are typically transported from roots to shoots via the xylem, whereas isopentenyl-type (iP) CKs are more commonly translocated through the phloem (Hirose et al. 2008). CK perception in plants involves a multistep phosphorelay system, analogous to bacterial two-component signalling, consisting of histidine kinase receptors (AHKs), histidine phosphotransfer proteins (AHPs), and type-A and type-B response regulators (ARRs) that control downstream gene expression (Kieber and Schaller, 2018; Hirose et al. 2008; Hwang and Sheen, 2001). Collectively, the CKs stimulate cell division in shoot and root meristems, promote shoot initiation and branching, delay leaf senescence by maintaining chloroplast function, and influence vascular patterning. They are also deeply involved in the regulation of reproductive development, by influencing nutrient allocation through modulation of source-sink dynamics and stimulating seed germination (Zwack and Rashotte, 2013; Sakakibara, 2006).

Beyond their endogenous roles, CKs also play significant roles in plant–microbe interactions. Certain plant-associated bacteria, including genera such as *Rhizobium*, *Azospirillum*, and *Methylobacterium*, produce CKs which stimulate plant growth as part of a larger nutrient-acquisition strategy (Koenig et al. 2002; Spaepen et al. 2007). Microbially-derived CKs – identical in structure and activity to forms endogenous to plants – enhance shoot biomass, increase chlorophyll content, and improve plant stress resilience by modulating endogenous hormone levels and gene expression in the host plant (Li et al. 2021; Akhtar et al. 2020; Mekureyaw et al. 2022). In fact, in symbiotic interactions, such as those involving *Methylobacterium*, bacterial CKs help establish beneficial colonization by promoting host vigor (Ivanova et al. 2001).

Contrastingly though, many phytopathogens exploit phytohormone signalling – including the CKs – to manipulate the host and establish infection. For instance, *Agrobacterium tumefaciens* transfers genes that induce CK overproduction in host tissues, leading to tumor formation, tissue hypertrophy, and hypervascularization – all of which drive nutrients to the site of rapid growth to the benefit of the invader (Akiyoshi et al. 1984). Another compelling example involves *Ustilago maydis* – a biotrophic pathogen and the causal agent of corn smut disease (*Zea mays*) which actively synthesizes a range of CKs to modulate host responses (Bruce et al. 2011). In a study by Morrison et al. (2015), biochemical profiling of infected cob tissues revealed striking alterations in the CK landscape including a significant accumulation of highly bioactive CK-FB forms, especially in infections caused by a more virulent strain of *U. maydis*. The accumulation of active CKs coincided with tumor development in cob tissues, consistent with the established role of CKs in promoting cell proliferation and nutrient redistribution (Morrison et al. 2015). Importantly, in a follow-up study, deletion of CK biosynthesis genes in *U. maydis* resulted in reduced phytohormone accumulation in host tissues and impaired fungal virulence – further demonstrating the role of fungal-derived CKs in disease proliferation (Morrison et al. 2017).

Possibly an even more compelling example of CK-mediated pathogenesis can be found in the rice blast fungus, *Magnaporthe oryzae*. In a study by Chanclud et al. (2016), *M. oryzae* was shown to synthesize CKs via a tRNA-isopentenyl transferase encoded by the *CKS1* gene. Essential for CK production, deletion of *CKS1* resulted in CK-deficient mutants which featured markedly reduced virulence – despite having normal growth *in vitro* – elicited stronger host immune responses, and failed to maintain

typical levels of sugars and amino acids at the site of colonization. Crucially, exogenous application of CKs restored virulence to the mutant strains, again substantiating the role of phytohormones in pathogen virulence and the modulation of host immunity (Chanclud et al. 2016).

Hemibiotrophic pathogens, such as *Fusarium*, also produce hormone and hormone-mimicking compounds to offset hormone homeostasis in the host as part of immune silencing and to thwart physical barriers (Sørensen et al. 2018). In a recent study, Niehaus et al. (2016) investigated secondary metabolism across several species of *Fusarium*, ultimately identifying two biosynthetic routes for CK production: the canonical tRNA degradation pathway, and a novel, putative *de novo* cytokinin biosynthetic pathway encoded by discrete gene clusters (Niehaus et al. 2016). These clusters, which contained genes homologous to IPTLOG and cytochrome P450 monooxygenases – central to cytokinin biosynthesis in other systems – were found in several species, including *F. mangiferae*, *F. proliferatum*, *F. verticillioides*, and *F. fujikuroi*.

Biochemical analyses of culture filtrates showed that low levels of CK compounds – primarily iP and *c*Z – were produced under axenic culture conditions and were attributable to tRNA degradation (Niehaus et al. 2016). However, when the gene clusters (CK1 and CK2) were overexpressed in *F. fujikuroi*, the strains began to produce *t*Z – demonstrating the latent capacity of these clusters for functional CK biosynthesis. Transcriptomic data further indicated that while expression of the CK biosynthetic clusters was generally low in culture, *in planta* expression of the CK2 gene cluster was detected during infection of maize by *F. verticillioides* and *F. fujikuroi* – strongly

suggesting that the *de novo* pathway may be intentionally induced during host colonization and a resultant alteration of the phytohormone profile from axenic growth.

This study marks *Fusarium* as one of the few known fungal genera belonging to the Ascomycota (after *Claviceps*) with genomic, transcriptional, and biochemical evidence supporting dual CK biosynthetic capacities. While the functional role of CK production by *Fusarium* to overall virulence remains poorly understood, recent findings suggest that *Fusarium* spp. likely evolved pathways for hormone production as a method for facilitating host–pathogen interactions.

Contemporaneously, research has also shown that phytohormones, including CKs, may directly affect pathogens themselves. For example, exogenous application of synthetic CKs such as 6-benzylaminopurine (6-BAP) have been shown to disrupt the growth and development of the phytopathogen *F. oxysporum*, by impairing hyphal growth and spore production (Gupta et al. 2021). These findings suggest that CKs may potentially contribute to plant defense and be harnessed in emerging biological control (BC) strategies – a crop protection doctrine which aims to suppress the proliferation of plant diseases using natural antagonists, including beneficial microorganisms, insects, and viruses. As such, furthering our understanding of hormone dynamics of *Fusarium* within the broader framework of interkingdom signalling will continue to provide crucial insights into plant immunity, phytobiome ecology, and new molecular targets for crop protection.

1.5. BIOLOGICAL CONTROL OF PHYTOPATHOGENS

When optimized, use of BC is intended to offer a sustainable and reproduceable companion to conventional crop protection strategies, as part of an integrated pest management (IPM) system (Cook and Baker, 1983; Fravel, 2005). Microbial BC agents are of particular interest due to their adaptability, diverse modes of action – parasitism, competition, antibiosis, or priming of plant immune responses – and ease of cultivation, preservation, distribution, and formulation (Pal and McSpadden Gardener, 2006).

Bacteria have received significant attention as BC agents due primarily to their rapid growth, amenability to genetic manipulation, and compatibility with commercial equipment and infrastructure (Ayaz et al. 2023; Lee et al. 2023). To date, several genera, including *Agrobacterium*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Streptomyces*, and *Methylobacterium*, have demonstrated promising activity against a wide range of phytopathogens (Ayaz et al. 2023; Compant et al. 2005; Lugtenberg and Kamilova, 2009; Rios et al. 2019). Bacterial modes of action tend to also be multifaceted: many isolates produce antimicrobial metabolites, siderophores that sequester iron away from pathogens, or lytic enzymes (chitinase) that degrade fungal cell walls while their rapid proliferation applies nutrient limitation pressure to potential invaders (Raaijmakers et al. 2002; Haas and Défago, 2005). Bacterial antagonists also tend to be more resilient in diverse or hostile environments, further expanding the opportunity for wider applications including: seed coatings, foliar sprays, or soil amendments (Berg, 2009). Moreover, the use of bacteria from the genera *Methylobacterium*, *Bacillus*, and *Pseudomonas,* which are often naturally found predominating the phytobiome, have low invader potential and are less likely to disrupt

a preexisting healthy ecological balance when introduced in agricultural settings (Lugtenberg et al. 2001; Green, 2006).

Examples of success frequently involve strains from the *Bacillus* genus in particular, including *B. subtilis* and *B. amyloliquefaciens* – with select isolates notable for their broad-spectrum activity against several phytopathogens like *Botrytis cinerea*, *Rhizoctonia solani*, and *Fusarium* spp. (Collinge et al. 2022). The longest and arguably most well-studied, BC agents from the genus *Bacillus* are known to exert their pathogen-deterring effects through several complementary mechanisms: producing antimicrobial lipopeptides (such as surfactins, iturins, and fengycins), imparting aggressive competitive pressure for nutrients and space in phytobiome, and triggering inducible systemic resistance (ISR) in the host plant (Collinge et al. 2022). Several commercial formulations containing these bacteria have already received registration and adoption in both conventional and organic farming systems including, Serenade® (*B. subtilis*), RhizoVital® (*B. amyloliquefaciens*), and Taegro® (*B. subtilis*). Their stability under diverse environmental conditions, compatibility with common agricultural practices (such as seed coating and foliar sprays), and relatively long shelf life further contribute to their popularity (Collinge et al. 2022)

Nonetheless, the successful implementation of BC in the field remains challenged by a myriad of factors including inconsistent performance under variable environmental conditions, competition with native microbiota, untested compatibility with a broad range of food crops, and regulatory barriers. Contemporary research concerning BC focuses on the improvement of strain selection, enhancing formulations and adjuvants, and unraveling the molecular and biochemical interactions that govern

the antagonist-host-pathogen axis (Compant et al. 2019). Supported by the proliferation of omics technologies, a new epoch in the study of BC mechanics is rapidly emerging – one that will enable a more precise elucidation of the innate strategies employed by effective microbes and unlock an enhanced ability to select, optimize, or engineer BC organisms with tailored functionalities as precision tools in agriculture.

1.6. CORE RESEARCH OBJECTIVES

The overarching objective of this work was to investigate and redefine the role of *Methylobacterium* – a genus well-known for plant growth-promotion – as a critical player in plant defense and microbiome-mediated crop protection. This body of work was guided by a core ecological hypothesis that: *Methylobacterium*, a highly conserved and abundant symbiont within the phytobiome, may not only support plant vigor through hormone modulation, but also participate in the suppression of opportunistic pathogens such as *Fusarium* spp.

This hypothesis was first seeded by reports suggesting a link between the use of commercial pesticides and an increased incidence of fungal disease in field crops. Specifically, field-level associations between the use of glyphosate – a broad-spectrum herbicide – and increased *Fusarium* have been reported across multiple cropping systems, climactic regions, and geographies. In extensive multi-year surveys, researchers have shown that incidence of disease caused by *Fusarium* are increased with the use of glyphosate and glyphosate-based herbicides (GBH), including wheat, barley, and soy (Fernandez et al. 2005, 2007; Sanogo et al. 2000, 2001). In banana plantations, controlled studies revealed that application of glyphosate and glyphosate-based herbicides (GBH) accelerated colonization by *Fusarium oxysporum* during senescence

and increased soil inoculum density post-harvest (Anderson and Aitken, 2021). Similarly, in cotton systems, growers reported a resurgence of *Fusarium* wilt following the adoption of Roundup Ready® GR varieties, suggesting that glyphosate can reduce or offset in some way, previously effective varietal resistance (Harper, 2007; Johal and Huber, 2009). Even exceedingly low dosages of glyphosate were found to increase crown and root rot of tomato by *Fusarium* spp. under field conditions (Bramhall and Higgins, 1988), while application of GBHs simultaneous with *Fusarium* inoculation in controlled studies also significantly increased disease severity in maize seedlings relative to infected controls alone (Carranza et al. 2019).

Given the cosmopolitan distribution of *Methylobacterium* and the dominance of the genus in the phytobiome, its known contributions to phytohormone production, and its taxonomic and functional conservation across host plant species, the inciting question formed: could pesticide exposure selectively disrupt this key microbial partner, thereby weakening plant defenses and enabling pathogen establishment? From this central premise, a research program was structured, which aimed to evaluate three interconnected elements: (1) the species-level phytohormone secreting capabilities of *Methylobacterium*, (2) the compatibility of *Methylobacterium* isolates with key agrochemical inputs, and (3) the functional role of *Methylobacterium*, including hormone-secreting strains, in defending the plant host against fungal pathogens.

While previous work had only described isolated observations (Irvine et al. 2013), Chapter 2 of this work delivered the first comprehensive overview of phytohormone production across diverse *Methylobacterium* strains. Motivated by the potential vulnerability of this genus to agrochemical disturbance, Chapter 3 and 4

delivered the first species-level assessment of *Methylobacterium* sensitivity to several commercial pesticide products, including the broad-spectrum herbicide, glyphosate. This work confirmed that many *Methylobacterium* strains are highly susceptible to commercial formulations, but uniquely, proved that glyphosate was only non-toxic in isolation. These findings support a new mechanistic preposition for the reportable increases in fungal diseases following herbicide application – namely, that glyphosate in commercial formulation with surfactants, selectively removes protective microbial allies from the plant microbiome.

Chapter 5 demonstrated for the first time that strains of *Methylobacterium* inhibit the growth and pathogenicity of *Fusarium graminearum*, *F. oxysporum*, and *F. fujikuroi*. These effects were confirmed both *in vitro* and to a more limited extent, *in planta*, and substantiate *Methylobacterium* as a functional component of the phytobiome as a protective barrier and, in part, closes the loop between herbicide disturbance, microbiome disruption, and pathogen emergence.

Finally, Chapter 6 provides the first in-depth analysis of hormone dynamics in co-cultures of *Methylobacterium* and *Fusarium*, tracking both exogenous expression and intracellular content. Chapter 6 uniquely reveals that *Methylobacterium* appears to trigger a compensatory hormonal response in *Fusarium* that mimics the fungal reaction to plant immune activation. Taken together, the totality of this work repositions *Methylobacterium* as a keystone genus with significant implications for pathogen suppression, microbiome stability, and crop protection – both with respect to contemporary methods and future developments.

1.7. REFERENCES

Abanda-Nkpwatt, D., Müsch, M., Tschiersch, J., Boettner, M., and Schwab, W. 2006. Molecular interaction between *Methylobacterium extorquens* and seedlings: growth promotion, methanol consumption, and localization of the methanol emission site. Journal of Experimental Botany, 57(15): 4025–4032. https://doi.org/10.1093/jxb/erl173

Akhtar, S. S., Mekureyaw, M. F., Pandey, C., and Roitsch, T. 2020. Role of cytokinins for interactions of plants with microbial pathogens and pest insects. Frontiers in Plant Science. 10: 1777. https://doi.org/10.3389/fpls.2019.01777

Akiyoshi, D. E., Klee, H., Amasino, R., Nester, E. W., and Gordon, M. P. 1984. t-DNA of *agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proceedings of the National Academy of Sciences, 81(19): 5994–5998. https://doi.org/10.1073/pnas.81.19.5994

Anderson, J., and Aitken, E. 2021. Effect of in planta treatment of 'Cavendish' banana with herbicides and fungicides on the colonisation and sporulation by *Fusarium oxysporum* f. sp. *cubense* Subtropical Race 4. J. Fungi 7:184. https://doi.org/10.3390/jof7030184

Audenaert, K., Pattery, T., Cornelis, P., and Höfte, M. 2002. Induction of systemic resistance to botrytis cinerea in tomato by *Pseudomonas aeruginosa* 7nsk2: role of salicylic acid, pyochelin, and pyocyanin. Molecular Plant-Microbe Interactions, 15(11): 1147–1156. https://doi.org/10.1094/MPMI.2002.15.11.1147

Ayaz, M., Li, C. H., Ali, Q., Zhao, W., Chi, Y. K., Shafiq, M., Ali, F., Yu, X. Y., Yu, Q., Zhao, J. T., Yu, J. W., Qi, R. D., and Huang, W. K. 2023. Bacterial and fungal biocontrol agents for plant disease protection: journey from lab to field, current status, challenges, and global perspectives. Molecules. 28(18): 6735. https://doi.org/10.3390/molecules28186735

Berg, G. 2009. Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology, 84(1): 11–18. https://doi.org/10.1007/s00253-009-2092-7

Bramhall, R. A., Higgins, V. J. 1988. The effect of glyphosate on resistance of tomato to *Fusarium* crown and root rot disease and on the formation of host structural defensive barriers. Can. J. Bot. 66, 1547–1555. https://doi.org/10.1139/b88-213

Brown, N. A., Urban, M., and Hammond-Kosack, K. E. 2012. The infection biology of *fusarium graminearum*: defining the pathways of spikelet-to-spikelet colonisation in wheat ears. Fungal Biology Reviews, 26(1): 43–51. https://doi.org/10.1016/j.funbio.2010.04.006

Bruce, S. A., Saville, B. J., Emery, R. J. N. 2011. *Ustilago maydis* produces cytokinins and abscisic acid for potential regulation of tumor formation in maize. J Plant Growth Regul. 30: 51–63. https://doi.org/10.1007/s00344-010-9166-8

Burgess, L. W. 1981. General ecology of the *Fusarium* species. In P. E. Nelson, T. A. Toussoun, and R. J. Cook (Eds.), *Fusarium*: Diseases, Biology, and Taxonomy (pp. 225–235). Pennsylvania State University Press.

Carranza, C. S., Aluffi, M. E., Benito, N., Magnoli, K., Barberis, C. L., and Magnoli, C. E. 2019. Effect of in vitro glyphosate on *Fusarium* spp. growth and disease severity in maize. J. Sci. Food Agric. 99:5064–5072. https://doi.org/10.1002/jsfa.9749

Chanclud, E., Kisiala, A., Emery, R. J. N., Chalvon, V., Ducasse, A., Romiti-Michel, C., Gravot, A., Kroj, T., and Morel, J.-B. 2016. Cytokinin production by the rice blast fungus is a pivotal requirement for full virulence. PLoS Pathogens, 12(2): e1005457. https://doi.org/10.1371/journal.ppat.1005457

Chistoserdova, L., Kalyuzhnaya, M. G., and Lidstrom, M. E. 2009. The expanding world of methylotrophic metabolism. Annual Review of Microbiology. 63: 477–499. https://doi.org/10.1146/annurev.micro.091208.073600

Collinge, D. B., Jensen, D. F., Rabiey, M., Sarrocco, S., Shaw, M. W., and Shaw, R. H. 2022. Biological control of plant diseases: what has been achieved and what is the direction? Plant Pathol. 71:1024–1047. https://doi.org/10.1111/ppa.13555

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied and Environmental Microbiology. 71(9): 4951–4959. https://doi.org/10.1128/AEM.71.9.4951-4959.2005

Compant, S., Samad, A., Faist, H., and Sessitsch, A. 2019. A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. Journal of Advanced Research. 19: 29–37. https://doi.org/10.1016/j.jare.2019.03.004.

Cook, R. J., and Baker, K. F. 1983. The nature and practice of biological control of plant pathogens. American Phytopathological Society. ISBN: 978-0-89054-053-4

Davies, P. J. 2010. Plant hormones: biosynthesis, signal transduction, action! 3rd ed. Springer. https://doi.org/10.1007/978-1-4020-2686-7

Dean, R., Van Kan, J. A., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R., Ellis, J., Foster, G. D. 2012. The Top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol. 13(4): 414-30. https://doi.org/10.1111/j.1364-3703.2011.00783.x.

Desjardins, A. E. 2006. *Fusarium* mycotoxins: chemistry, genetics and biology. APS Press. ISBN: 0-89054-335-6.

Ehinmitan, E., Siamalube, B., Mamati, E., Ngumi, V., Juma, P., and Losenge, T. 2024. Evaluating Growth-Promotion and Drought Tolerance Properties of Endophytic *Methylobacterium* spp. from Semi-Arid Kenya Soil. *Scope*, *14*(3), 924-940.

El-Showk, S., Ruonala, R., Helariutta, Y. 2013. Crossing paths: cytokinin signalling and crosstalk. Development 140 (7): 1373–1383. https://doi.org/10.1242/dev.086371

Fernandez, M. R., Selles, F., Gehl, D., DePauw, R. M., Zentner, R. P. 2005. Crop production factors associated with *Fusarium* head blight in spring wheat in eastern Saskatchewan. Crop Sci. 45, 1908–1916. https://doi.org/10.2135/cropsci2004.0197

Fernandez, M. R., Zentner, R. P., DePauw, R. M., Gehl, D. T., Stevenson, F. C. 2007. Impacts of crop production factors on *Fusarium* head blight in barley in eastern Saskatchewan. Crop Sci. 47, 1574–1584. https://doi.org/10.2135/cropsci2006.09.0596

Fravel, D. R. 2005. Commercialization and implementation of biocontrol. Annual Review of Phytopathology, 43, 337–359. https://doi.org/10.1146/annurev.phyto.43.032904.092924

Fravel, D., Olivain, C., and Alabouvette, C. 2003. *Fusarium oxysporum* and its biocontrol. New Phytologist, 157(3), 493–502. https://doi.org/10.1046/j.1469-8137.2003.00700.x

Geiser, D. M., Aoki, T., Bacon, C. W., Baker, S. E., Bhattacharyya, M. K., Brandt, M. E., Brown, D. W., Burgess, L. W., Chulze, S., Coleman, J. J., Correll, J. C., Covert, S. F., Crous, P. W., Cuomo, C. A., De Hoog, G. S., Di Pietro, A., Elmer, W. H., Epstein, L., Frandsen, R. J., Freeman, S., Gagkaeva, T., Glenn, A. E., Gordon, T. R., Gregory, N. F., Hammond-Kosack, K. E., Hanson, L. E., Jímenez-Gasco Mdel, M., Kang, S., Kistler, H. C., Kuldau, G A., Leslie, J. F., Logrieco, A., Lu, G., Lysøe, E., Ma, L. J., McCormick, S. P., Migheli, Q., Moretti, A., Munaut, F., O'Donnell, K., Pfenning, L., Ploetz, R. C., Proctor, R. H., Rehner, S. A., Robert, V. A., Rooney, A. P., Bin Salleh, B., Scandiani, M. M., Scauflaire, J., Short, D. P., Steenkamp, E., Suga, H., Summerell, B. A., Sutton, D. A., Thrane, U., Trail, F., Van Diepeningen, A., Vanetten, H. D., Viljoen, A., Waalwijk, C., Ward, T. J., Wingfield, M. J., Xu, J. R., Yang, X. B., Yli-Mattila, T., Zhang, N. 2013. One fungus, one name: defining the genus *Fusarium* in a scientifically robust way that preserves longstanding use. Phytopathology. 103(5): 400-408. https://doi.org/10.1094/PHYTO-07-12-0150-LE.

Gilardi, G. L., and Faur, Y. C. 1984. Infections caused by *Methylobacterium* species. Journal of Clinical Microbiology. 20(6): 1047–1049.

Glick, B. R. 2012. Plant growth-promoting bacteria: mechanisms and applications. Scientifica. https://doi.org/10.6064/2012/963401.

Gordon, T. R. 2017. *Fusarium oxysporum* and the fusarium wilt syndrome. Annual Review of Phytopathology. 55: 23–39. https://doi.org/10.1146/annurev-phyto-080615-095919

Green, P. N. 2006. *Methylobacterium*. In D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (Eds.), Bergey's Manual of Systematic Bacteriology (2nd ed., Vol. 2, pp. 257–265). Springer. https://doi.org/10.1007/978-0-387-68572-4

Gupta R, Anand G, Pizarro L, Laor D, Kovetz N, Sela N, Yehuda T, Gazit E, Bar M. 2021. Cytokinin Inhibits Fungal Development and Virulence by Targeting the Cytoskeleton and Cellular Trafficking. mBio. https://doi.org/10.1128/mBio.03068-20.

Haas, D., and Défago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology. 3(4): 307–319. https://doi.org/10.1038/nrmicro1129

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. 2004. *Trichoderma* species—opportunistic, avirulent plant symbionts. Nature Reviews Microbiology. 2(1): 43–56. https://doi.org/10.1038/nrmicro797

Harper, M., 2007. The Review of the Moratorium on GM Canola. Australia. http://www.dpi.vic.gov.au/dpi/nrenfa.nsf/LinkView/5477226A88881F86CA2572E300074EEF89E6C67B4668BD2A7CA256FB70001BAB8.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., and Sakakibara, H. 2008. Regulation of cytokinin biosynthesis, compartmentalization and translocation. Journal of Experimental Botany. 59(1): 75–83. https://doi.org/10.1093/jxb/erm157.

Holland, M. A. 1997. Occam's razor applied to hormonology. Are cytokinins produced by plants? Plant Physiology. 115(3): 865–868. https://doi.org/10.1104/pp.115.3.865

Hwang, I., and Sheen, J. 2001. Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature. 413(6854): 383–389. https://doi.org/10.1038/35096500

Ivanova, E. G., Doronina, N. V., and Trotsenko, Y. A. 2001. Aerobic *Methylobacteria* are capable of synthesizing auxins. Microbiology. 70(4): 392–397. https://doi.org/10.1023/A:1010469708107

Jameson, P. E. 2000. Cytokinins and auxins in plant-pathogen interactions—an overview. Plant Growth Regulation. 32: 369–380. https://doi.org/10.1023/A:1010733617543

Johal, G. S., and Huber, D. M. 2009. Glyphosate effects on diseases of plants. European Journal of Agronomy. 31(3): 144–152. https://doi.org/10.1016/j.eja.2009.04.004

Junaid, J. M., Dar, N. A., Bhat, T. A., Bhat, A. H., and Bhat, M. A. 2013. Commercial biocontrol agents and their mechanism of action in the management of plant pathogens. International Journal of Modern Plant and Animal Sciences. 1(2): 39–57.

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. Development, 145(4). https://doi.org/10.1242/dev.149344

Koenig, R. L., Morris, R. O., and Polacco, J. C. 2002. tRNA is the source of low-level *trans*-zeatin production in *Methylobacterium* spp. Journal of Bacteriology. 184(7): 1832–1842. https://doi.org/10.1128/JB.184.7.1832-1842.2002

Kutschera, U. 2007. Plant-associated *Methylobacteria* as co-evolved phytosymbionts: a hypothesis. Plant Signalling and Behavior. 2(2): 74–78. https://doi.org/10.4161/psb.2.2.4073

Lai, C. C., Cheng, A., Liu, W. L., Tan, C. K., Huang, Y. T., Chung, K. P., Lee, M. R., Hsueh P. R. 2011. Infections caused by unusual *Methylobacterium* species. J Clin Microbiol. 49(9): 3329-3331. https://doi.org/10.1128/JCM.01241-11.

Lee, J., Kim, S., Jung, H., Koo, B., Han, J. A., and Lee, H. 2023. Exploiting Bacterial Genera as Biocontrol Agents: Mechanisms, Interactions and Applications in Sustainable Agriculture. J. Plant Biol. 66: 485–498. https://doi.org/10.1007/s12374-023-09404-6

Leslie, J. F., and Summerell, B. A. 2006. The *Fusarium* laboratory manual. Blackwell Publishing. ISBN-13: 978-0-8138-1919-8

Li, S. M., Zheng, H. X., Zhang, X. S., Sui, N. 2021. Cytokinins as central regulators during plant growth and stress response. Plant Cell Rep. 40(2):271-282. https://doi.org/10.1007/s00299-020-02612-1

Lugtenberg, B., and Kamilova, F. 2009. Plant-growth-promoting rhizobacteria. Annual Review of Microbiology. 63: 541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918

Lugtenberg, B., Chin-A-Woeng, T. F., and Bloemberg, G. V. 2001. Microbe–plant interactions: principles and mechanisms. Antonie van Leeuwenhoek. 81(1): 373–383. https://doi.org/10.1023/A:1020596903142

Ma, L. J., Geiser D. M., Proctor, R. H,, Rooney, A. P., O'Donnell, K., Trail, F., Gardiner, D. M., Manners, J. M., Kazan, K. 2013. *Fusarium* pathogenomics. Annu Rev Microbiol. 67:399-416. https://doi.org/10.1146/annurev-micro-092412-155650

Ma, L. J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M. J., Di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P. M., Kang, S., Shim, W. B., Woloshuk, C., Xie, X., Xu, J. R., Antoniw, J., Baker, S. E., Bluhm, B. H., Breakspear, A., Brown, D. W., Butchko, R. A., Chapman, S., Coulson, R., Coutinho, P. M., Danchin, E. G., Diener, A., Gale, L. R.,

Gardiner, D. M., Goff, S., Hammond-Kosack, K. E., Hilburn, K., Hua-Van, A., Jonkers W, Kazan K, Kodira, C. D., Koehrsen, M., Kumar, L., Lee, Y. H., Li, L., Manners, J. M., Miranda-Saavedra, D., Mukherjee, M., Park, G., Park, J., Park, S. Y., Proctor, R. H., Regev, A., Ruiz-Roldan, M. C., Sain, D., Sakthikumar, S., Sykes, S., Schwartz, D. C., Turgeon, B. G., Wapinski, I., Yoder, O., Young, S., Zeng, Q., Zhou, S., Galagan, J., Cuomo, C. A., Kistler, H. C., Rep, M. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature. 464(7287):367-73. https://doi.org/10.1038/nature08850.

Madhaiyan, M., Poonguzhali, S., Ryu, J., Sa, T. 2006. Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. Planta. 224(2): 268–278. https://doi.org/10.1007/s00425-005-0211-y

Madhaiyan, M., Poonguzhali, S., Senthilkumar, M., Seshadri, S., Chung, H., Jeyaram, K., and Sa, T. 2004. Growth promotion and induction of systemic resistance in rice cultivar co-47 (*Oryza sativa*) by *Methylobacterium oryzae*. Botanical Bulletin- Academia Sinica Taipei. 45(4): 315-324

McMullen, M., Bergstrom, G., De Wolf, E., Dill-Macky, R., Hershman, D., Shaner, G., and Van Sanford, D. 2012. A unified effort to fight an enemy of wheat and barley: *Fusarium* head blight. Plant Disease. 96(12): 1712–1728. https://doi.org/10.1094/PDIS-03-12-0291-FE.

Meena, K. K., Sorty, A. M., Bitla, U. M., Choudhary, K., Gupta, P., Pareek, A., Singh, D. P., Prabha, R., Sahu, P. K., Gupta, V. K., Singh, H. B., Krishanani, K. K., Minhas, P. S. 2017. Abiotic stress responses and microbe-mediated mitigation in plants: the omics strategies. Frontiers in Plant Science. 8: 172. https://doi.org/10.3389/fpls.2017.00172

Mekureyaw, M. F., Pandey, C., Hennessy, R. C., Nicolaisen, M. H., Liu, F., Nybroe, O., and Roitsch, T. 2022. The cytokinin-producing plant-beneficial bacterium *Pseudomonas fluorescens* G20-18 primes tomato (*Solanum lycopersicum*) for enhanced drought stress responses. Journal of Plant Physiology. 270: 153629. https://doi.org/10.1016/j.jplph.2022.153629

Mok, D. W. S., and Mok, M. C. 2001. Cytokinin metabolism and action. Annual Review of Plant Biology. 52: 89–118. https://doi.org/10.1146/annurev.arplant.52.1.89

Morrison, E. N., Emery, R. J. N., and Saville, B. J. 2015. Phytohormone involvement in the *Ustilago maydis–Zea mays* pathosystem: Relationships between abscisic acid and cytokinin levels and strain virulence in infected cob tissue. PLoS ONE. https://doi.org/10.1371/journal.pone.0130945

Morrison, E. N., Emery, R. J. N. and Saville, B.J. 2017. Fungal derived cytokinins are necessary for normal *Ustilago maydis* infection of maize. Plant Pathol. 66: 726-742. https://doi.org/10.1111/ppa.12629

Munkvold, G. P. 2003. Cultural and genetic approaches to managing mycotoxins in maize. Annual Review of Phytopathology. 41: 99–116. https://doi.org/10.1146/annurev.phyto.41.052002.095510

Niehaus, E.-M., Münsterkötter, M., Proctor, R. H., Brown, D. W., Sharon, A., Idan, Y., Oren-Young, L., Sieber, C. M., Novák, O., Pěnčík, A., Tarkowská, D., Hromadová, K., Freeman, S., Maymon, M., Elazar, M., Youssef, S. A., El-Shabrawy, E. S. M., Shalaby, A. B. A., Houterman, P., Brock, N. L., Burkhardt, I., Tsavkelova, E. A., Dickschat, J. S., Galuszka, P., Güldener, U., and Tudzynski, B. 2016. Comparative "omics" of the *Fusarium fujikuroi* species complex highlights differences in genetic potential and metabolite synthesis. Genome Biol. Evol. 8:3574–3599. https://doi.org/10.1093/gbe/evw259.

Ongena, M., and Jacques, P. 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology. 16(3): 115–125. https://doi.org/10.1016/j.tim.2007.12.009

O'Donnell, K., Sutton, D. A., Fothergill, A., McCarthy, D., Rinaldi, M. G., Brandt, M. E., Zhang, N., and Geiser, D. M. 2008. Molecular Phylogenetic Diversity, Multilocus Haplotype Nomenclature, and *In Vitro* Antifungal Resistance within the *Fusarium solani* Species Complex. Journal of Clinical Microbiology. 46(8): 2477–2490. https://doi.org/10.1128/JCM.02371-07

O'Donnell, K., Sutton, D. A., Wiederhold, N., Robert, V. A. R. G., Crous, P. W., and Geiser, D. M. 2016. Veterinary Fusarioses within the United States. Journal of Clinical Microbiology, 54(11), 2813–2819. https://doi.org/10.1128/JCM.01607-16

Pal, K. K. and McSpadden Gardener, B. B. 2006. Biological control of plant pathogens. The Plant Health Instructor. https://doi.org/10.1094/PHI-A-2006-1117-02.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. 2012. Hormonal modulation of plant immunity. Annual Review of Cell and Developmental Biology. 28: 489–521. https://doi.org/10.1146/annurev-cellbio-092910-154055

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., van Wees, S. C. M., and Bakker, P. A. H. M. 2014. Induced systemic resistance by beneficial microbes. Annual Review of Phytopathology. 52: 347–375. https://doi.org/10.1146/annurev-phyto-082712-102340

Raaijmakers, J. M., Vlami, M., and de Souza, J. T. 2002. Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek. 81(1): 537–547. https://doi.org/10.1023/A:1020501420831

Rios, M. M., Torres, L. F. C., Díaz, S. L., González, J. V., and Hernández, C. V. S. 2019. Effect of *Methylobacterium extorquens* on tomato development in the presence or absence of *Fusarium oxysporum*. Revista mexicana de ciencias agrícolas. 10(7): 1469-1479. https://doi.org/10.29312/remexca.v10i7.644

Sakakibara, H. 2006. Cytokinins: activity, biosynthesis, and translocation. Annual Review of Plant Biology. 57: 431–449. https://doi.org/10.1146/annurev.arplant.57.032905.105231

Sanders, J. W., Sanders, C. C., and Goering, R. V. 2000. *Methylobacterium infections*: a case series and review of the literature. Clinical Infectious Diseases. 30(6): 936–940.

Sanogo, S., Yang, X.B., Lundeen, P. 2001. Field response of glyphosate-tolerant soybean to herbicides and sudden death syndrome. Plant Dis. 85: 773–779. https://doi.org/10.1094/PDIS.2001.85.7.773

Sanogo, S., Yang, X.B., Scherm, H. 2000. Effects of herbicides on *Fusarium solani* f. sp. *glycines* and development of sudden death syndrome in glyphosate-tolerant soybean. Phytopathology. 90: 57–66. https://doi.org/10.1094/PHYTO.2000.90.1.57

Santner, A., Calderon-Villalobos, L. I., and Estelle, M. 2009. Plant hormones are versatile chemical regulators of plant growth. Nature Chemical Biology. 5(5): 301–307. https://doi.org/10.1038/nchembio.165

Sørensen, J. L., Benfield, A. H., Wollenberg, R. D., Westphal, K., Wimmer, R., Nielsen, M. R., Nielsen, K. F., Carere, J., Covarelli, L., Beccari, G., Powell, J., Yamashino, T., Kogler, H., Sondergaard, T. E., and Gardiner, D. M. 2018. The cereal pathogen *Fusarium pseudograminearum* produces a new class of active cytokinins during infection. Mol. Plant Pathol. 19:1140–1154. https://doi.org/10.1111/mpp.12593.

Spaepen, S., Vanderleyden, J., and Remans, R. 2007. Indole-3-acetic acid in microbial and microorganism–plant signalling. FEMS Microbiology Reviews. 31(4): 425–448. https://doi.org/10.1111/j.1574-6976.2007.00072.x

Spallek, T., Gan, P., Kadota, Y., Shirasu, K. 2018. Same tune, different song—cytokinins as virulence factors in plant–pathogen interactions? Current opinion in plant biology. (44): 82-87. https://doi.org/10.1016/j.pbi.2018.03.002.

Sy, A., Timmers, A. C., Knief, C. and Vorholt, J. A. 2005. Methylotrophic Metabolism is advantageous for *Methylobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. Environmental Microbiology. 7(11): 1875–1884. https://doi.org/10.1128/AEM.71.11.7245-7252.2005.

Takei, K., Sakakibara, H., and Sugiyama, T. 2001. Identification of genes encoding adenylate isopentenyltransferase, a key enzyme in cytokinin biosynthesis, in arabidopsis. The Plant Cell. 13(11): 2479–2491. https://doi.org/10.1074/jbc.M102130200

Weller, D. M., Raaijmakers, J. M., McSpadden Gardener, B. B., and Thomashow, L. S. 2002. Microbial populations responsible for specific soil suppressiveness to

plant pathogens. Annual Review of Phytopathology. 40(1): 309–348. https://doi.org/10.1146/annurev.phyto.40.030402.110010.

Zhang, N., O'Donnell, K., Sutton, D. A., Nalim, F. A., Summerbell, R. C., Padhye, A. A., Geiser, D. M. 2006. Members of the *Fusarium solani* Species Complex That Cause Infections in Both Humans and Plants Are Common in the Environment. Journal of Clinical Microbiology. 44(6): 2186–2190. https://doi.org/10.1128/JCM.00120-06

Zhao, J., Wang, J., Liu, J., Zhang, P., Kudoyarova, G., Liu, C. J., and Zhang, K. 2024. Spatially distributed cytokinins: Metabolism, signaling, and transport. Plant Communications. 5(7). https://doi.org/10.1016/j.xplc.2024.100936

Zwack, P. J., Rashotte, A. M. 2013. Cytokinin inhibition of leaf senescence. Plant Signalling and Behavior. 8(7). https://doi.org/10.4161/psb.24737

CHAPTER 2

2.1. PREFACE

Title: A survey of *Methylobacterium* species and strains reveals widespread production and varying profiles of cytokinin phytohormones.

Authors: Daniel Palberg, Anna Kisiała, Gabriel Lemes Jorge and R. J. Neil Emery

Reference: Published in *BMC Microbiology* (2022), 22: 49
doi: 10.1186/s12866-022-02454-9

Contributions: A.K. and R.J.N.E. contributed to the study conception and design. Experimentation and data collection were performed by A.K. and G.L.J. with assistance from D.P. Manuscript was written by D.P.

All authors read and approved the submitted work.

CHAPTER 2

A survey of *Methylobacterium* species and strains reveals widespread production and varying profiles of cytokinin phytohormones.

Published in: *BMC Microbiology* (2022), 22: 49

2.2. ABSTRACT

Symbiotic *Methylobacterium* strains comprise a significant part of plant microbiomes. Their presence enhances plant productivity and stress resistance, prompting classification of these strains as plant growth-promoting bacteria (PGPB). *Methylobacteria* can synthesize unusually high levels of plant hormones, called cytokinins (CKs), including the most active form, *trans*-Zeatin (tZ). This study provides a comprehensive inventory of 46 representatives of *Methylobacterium* genus with respect to phytohormone production in vitro, including 16 CK forms, abscisic acid (ABA) and indole-3-acetic acid (IAA).

High performance-liquid chromatography—tandem mass spectrometry (HPLC–MS/MS) analyses revealed varying abilities of *Methylobacterium* strains to secrete phytohormones that ranged from 5.09 to 191.47 pmol mL^{-1} for total CKs, and 0.46 to 82.16 pmol mL^{-1} for *tZ*. Results indicate that reduced methanol availability, the sole carbon source for bacteria in the medium, stimulates CK secretion by *Methylobacterium*. Additionally, select strains were able to transform L-tryptophan into IAA while no ABA production was detected.

KEYWORDS: 2-methylthio-Zeatin, HPLC–MS/MS, methanol, plant growth promoting bacteria, *trans*-Zeatin

2.3. INTRODUCTION

Most organisms exist in the presence of multiple microbes to the extent that it is essentially difficult to regard them as singular entities. The microbiome describes any collection of bacteria, viruses, protozoa, and fungi – both beneficial and pathogenic – which colonize a host organism. The unique and functional relationship between a host and the specific members of its microbiome forms discrete ecological units, called holobionts (Vandenkoornhuyse et al. 2015). Bacteria which colonize plants inhabiting the root zone (rhizobial), leaf surfaces (epiphytic), and living within tissues (endophytic), are numerous and diverse (Kutschera 2007). These plant-associated microbiota often play critical roles in plant health, development, and productivity (Arau et al. 2002; Kuklinsky-Sobral et al. 2004; Hardoim et al. 2008; Ryan et al. 2008; Dourado et al. 2015; White et al. 2019). Conserved and optimized through the evolution, the biochemical bases of the symbioses between plants and beneficial microbes may provide keys to further understanding and improving plant health in a sustainable manner.

Although the plant microbiome (phytobiome) can be taxonomically diverse, the *Methylobacterium* genus often comprises a significant part of the bacteria present. Generally, *Methylobacteria* are ubiquitous in nature and non-pathogenic to humans or wildlife. They are rod-shaped, obligately aerobic microbes that can thrive in a wide range of environments including: soil, air, water, and plants (Omer et al. 2004). Most *Methylobacterium* species stain gram-negative and exhibit polar growth, although some exceptions exist (e.g. *M. jeotgali*) (Green 2006a). The distinct pink pigmentation of many strains across the genus indicates the presence of specialized carotenoids which likely confer their tolerance to ultraviolet (UV) radiation (Jacobs et al. 2005; Gourion

et al. 2008; Yoshida et al. 2017; Kamo et al. 2018) and provide a basis for further classifying individuals as pink-pigmented facultative methylotrophs (PPFMs) (Corpe and Rheem 1989; Yurimoto et al. 2021). Many PPFMs, including *Methylobacterium* strains, are suspected to participate in the development of pigmentation within their host-plants by modulating flavonoid and carotenoid levels within host tissues (Gholizadeh 2012). As facultative methylotrophs, *Methylobacteria* can either use common carbon sources such as monosaccharides, or oxidize a range of single-carbon compounds including: methanol, methylamine, and formaldehyde (Patt et al. 1976; Sy et al. 2005; Kutschera 2007; Šmejkalová et al. 2010; Yurimoto et al. 2021).

Methylobacterium strains can thrive across a wide range of temperatures, salinity, and pH (Green 2006a), with certain individuals exhibiting considerable tolerance to chlorine (Hiraishi et al. 1995) and exceptional resistance to gamma irradiation (Ito and Iizuka 1971). *Methylobacterium* have been studied for their suitability in a wide range of biotechnologies including bioremediation of environmental toxins (Aken et al. 2004; Zhang et al. 2008; Ventorino et al. 2014). Most recently, three novel strains of *Methylobacterium* (IF7SW-B2T, IIF1SW-B5, and IIF4SW-B5) were isolated from the international space station (ISS) during flight experiments conducted between 2015-2016; seeming to have evaded decontamination measures and proving suitability even for an oligotrophic environment in microgravity (Bijlani et al. 2021).

Early studies showed that enrichment of the phytobiome with *Methylobacterium* encouraged plant growth and productivity (Holland 1997a, 1997b); leading to the classification of these strains as plant growth-promoting bacteria (PGPB). These first

series of discoveries formed the basis of the hypothesis that *Methylobacterium* may be a major source of plant growth hormones known as the cytokinins (CKs) (Holland 1997a). Cytokinins are N^6 substituted adenine derivatives, such as the zeatins (isoprenoid functionality) and topolins (aromatic functionality). As a group, CKs are involved in a wide array of biological functions in plants including: cell division, cell elongation, shoot growth, nutrient uptake, vascular development, and gametophyte development (Sakakibara 2006; Kieber and Schaller 2018; Streletskii et al. 2019; Gibb et al. 2020).

A unique trait of *Methylobacterium* genus that distinguishes it from other PGPB is their ability to biosynthesize unusually high levels of the most active CK forms which includes, in particular, *trans*-zeatin (tZ). By producing bioactive CKs that are identical in chemical structure and bioactivity to those endogenously produced by plants, symbiotic *Methylobacterium* can stimulate plant cell division and increase the release of methanol, a by-product of cell wall construction (Nemecek-Marshall et al. 1995; Fall and Benson 1996; Holland 1997b; Šmejkalová et al. 2010). Thus, while the plant is stimulated to grow, *Methylobacterium*, which are able to utilize single-carbon compounds like methanol, receive a stable carbon source that allows them to proliferate (Abanda-Nkpwatt et al. 2006). In this exchange between host and symbiont, *Methylobacterium* have a clear advantage as many other microorganisms in the phytobiome have more complex habitat requirements and rely on C_6 nutrient sources that are subject to higher competition (Sy et al. 2005). This attribute in itself may prove especially advantageous for *Methylobacteria* in the case of regions with particularly challenging climate and low soil fertility (Biswas et al. 2019), or environments with limited resource bandwidth (space farming).

The production of growth-promoting phytohormones by *Methylobacterium* has propagated new interest in uncovering their role as bioinoculants in sustainable crop production systems (Dourado et al. 2015). Recent studies have gone beyond just illustrating how enrichment of a plant microbiome with *Methylobacterium* increases growth rate, to also demonstrating an increase in tolerance of the host-plant to high salinity (Lee et al. 2015) and drought stress (Jorge et al. 2019). The use of microbes like *Methylobacterium* to support growth and development of crops would be more technically efficient and cost-effective compared to genetic engineering procedures. Moreover, the microbial delivery of growth-promoting phytohormones would be more widely accepted by public consumers relative the more controversial GMO approaches.

The development of a successful bioinoculant begins with the selection of a suitable microbial agent for the target host-plant. Thus far, however, available studies examining CK biosynthesis in *Methylobacterium* are limited in both the number of strains evaluated and the range of CK forms analyzed. Nevertheless, existing research indicates that understanding the types and quantities of the CK forms produced by *Methylobacterium* can help elucidate how select strains facilitate beneficial or supportive effect on the host-plant and provide a foundation for novel crop production technologies. This process includes consideration of nutrient and environmental needs and requires no pathogenicity and a limited impact on the microbial community native to the host species (Benizri et al. 2001; Esitken et al. 2010; Maneewan and Khonsarn 2017). Furthermore, against a background of a well-established understanding of the positive effects endogenous CKs have in plant systems, uncovering the CK profiles of *Methylobacterium* symbionts may provide an orthogonal variable in the selection of a bioinoculant active agent.

In this study, we expand the knowledge of PGPB potential by producing a comprehensive inventory of 47 *Methylobacterium* strains with respect to their ability to produce and release phytohormones during *in vitro* culture. The analysed strains were specifically selected to capture a wide range of biometric and phenotypic traits, as well as vast differences in sources of origin. Microbial profiles of CK phytohormones, as well as their precursors and derivatives were analysed using the highly sensitive and accurate method of high-performance liquid chromatography positive electrospray ionization tandem mass spectrometry (HPLC-(+ESI)-MS/MS). To add further phytohormonal context, the bacterial strains were analyzed for a common CK antagonist, abscisic acid (ABA) through mass spectrometry, and a frequent complementary compound, indole acetic acid (IAA) through a colorimetric method.

2.4. MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Freeze-dried cultures of 47 *Methylobacterium* spp. strains were obtained from five microbe collections: (1) the Agricultural Research Service (ARS) of the Northern Regional Research Laboratory (NRRL), (2) the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), (3) the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) ["German Collection of Microorganisms and Cell Cultures"], (4) the Japan Collection of Microorganisms (JCM), and (5) the National Institute of Technology and Evaluation's (NITE) Biological Resource Center (NBRC). The strain selection was divided as follows; (NRRL) (ARS) – United States of America (n = 5), BCCM/LMG - Belgium (n =12), DSMZ – Germany (n =8), JCM – Japan (n =10), and NITE (NBRC) – Japan (n =13). The strains were originally isolated from different biological sources including plant organs (phyllosphere, flowers, roots, moss tissue),

soil, water, air, and other materials. The detailed information on strain taxonomy, origin, known characteristics and applications is provided in Table 2.1. The freeze-dried strains were revived in nutrient-rich R2 broth (VWR, Mississauga, Canada) and maintained as 15% (vol/vol) glycerol stocks at -80 ºC. The analysed strains varied in their growth characteristics, such as the intensity of pellet pigmentation (white, orange, pale pink to intense pink), growth rate (5 – 50 mg pellet in 15 mL culture), or ability to aggregation and biofilm formation (Figure 1).

Screening for Bacterial Phytohormone Production

For phytohormone analyses, *Methylobacterium* stocks were streaked onto agar plates and liquid cultures were started by suspending the bacterial single colonies with a sterile disposable loop in 50 mL Falcon tubes containing 15 mL of selective minimal medium prepared according to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures) index #125, supplemented with 0.5% MeOH as a sole carbon source. Aseptic bacteria cultures were incubated in a rotary shaker (28 °C/250 RPM). Supernatant samples were harvested on 5-7 day of the culture, when *Methylobacterium* reached the late exponential/early stationary phase (OD_{600} = 0.6-1.2, depending on the strain). To determine cell concentration in the liquid culture, bacterial growth was monitored spectrophotometrically by measuring OD_{600} and confirmed by cell count using a standard serial dilution technique.

Extraction and Purification of Cytokinins (CKs) and Abscisic Acid (ABA)

A modified protocol for liquid culture medium was used for CK and ABA extraction and quantification (Kisiala et al. 2013). The levels of bacterial

phytohormones were measured in 12 mL of the cell-free supernatant obtained after centrifugation (Thermo Scientific, Sorvall ST16; 10 min, 4,696 RCF) of harvested *in vitro* cultures. Samples of culture medium that were not inoculated with *Methylobacterium* were subjected to analysis as a negative control, to determine that the CKs detected in the bacteria culture supernatant were exclusively of microbial origin.

The profiles of 28 CK metabolites and ABA were analyzed, including: CK free bases (CK-FBs), their riboside (CK-RBs) and CK-nucleotide (CK-NTs) derivatives, CK-glucoside (CK-GLUCs) and methylthiol conjugates (2MeS-CKs), and select aromatic CKs. The cell-free supernatant samples were mixed with 1 mL of cold (–20°C) modified Bieleski #2 extraction solvent (CH_3OH:H_2O:HCO_2H [15:4:1, vol/vol/vol]) using vortex. Internal standards were added to each sample to enable endogenous hormone quantification through the isotope dilution method. Samples were spiked with 144.7 ng of $^2H_4$-ABA (NRC-PBI, Saskatchewan, SK, Canada) and 10 ng of each of the deuterated internal standard CKs (Table 2.2.) obtained from OlChemIm Ltd. (Olomouc, Czech Republic). As deuterated standards of cis-Zeatin-type CKs were not commercially available at the time of this investigation, the levels of cis-compounds were quantified based on the recovery of the deuterated standards of the corresponding *trans*-compounds.

Samples were extracted overnight at –20 °C and evaporated to dryness at 35 °C under vacuum (Model SPD111V; Thermo Scientific, Ottawa, Canada). The extraction residues were reconstituted in 200 µL of 1 M HCO_2H (pH 1.4) for purification and concentration of metabolites using automated Gilson SPE 215 Solid Phase Extraction

System (Gilson Inc., Middleton, WI, USA). The hormone-containing fraction was purified and concentrated on a mixed mode, reverse-phase, cation-exchange SPE cartridges (Oasis MCX, Waters, Mississauga, Canada) as described by Dobrev and Kaminek (2002), with modifications (Farrow and Emery 2012). Cartridges were activated with high-performance liquid chromatography (HPLC) grade CH_3OH and were equilibrated using 1 M HCO_2H (pH 1.4). After equilibration, each sample was loaded and washed with 1 M HCO_2H (pH 1.4). Hormone groups were eluted based on the charge and hydrophobicity level. Abscisic acid was eluted first using CH_3OH. Cytokinin nucleotides (CK-NTs) were eluted next with 0.35 M NH_4OH. Cytokinin free bases, CK-RBs, 2MeS-CKs, CK-GLUCs and aromatic CKs were retained on the column based on charge and hydrophobic properties and, therefore, were lastly eluted, using 0.35 M NH_4OH in 60% [vol/vol] CH_3OH. Each collected elution was evaporated to dryness at 35 °C under vacuum.

Since intact CK-NTs cannot be analyzed directly with the present method, they were dephosphorylated by overnight incubation with 3 units of calf-intestinal alkaline phosphatase (New England Biolabs Ltd., Whitby, ON, Canada) in 1 mL of 0.1 M ethanolamine-HCl (pH 10.4) at 37 °C to form CK-RBs. This detection method of CK-NTs potentially reflects pooled contribution of mono, di- or triphosphates in that the isopentenyl or hydroxylated moiety may be transferred to AMP, ADP or ATP (Quesnelle and Emery 2007). The resulting CK-RBs were evaporated to dryness at 35 °C under vacuum. The samples were reconstituted in double-distilled water (dd-H_2O) and were further isolated on a reversed-phase C18 solid phase extraction cartridges (C18/14%, Canadian Life Science, Peterborough, ON, Canada), which were first activated with CH_3OH and equilibrated using dd-H_2O. Samples containing CK-RBs were loaded onto

the C18 cartridges and were allowed to pass through the column by gravity. The sorbent was washed with dd-H_2O. Resultant CK-RBs were eluted using 100% CH_3OH and eluents were evaporated to dryness at 35 °C under vacuum.

Prior to LC-MS/MS analysis, all phytohormone samples were dissolved in starting conditions solvent (CH_3OH:CH_3CO_2H:ddH_2O [5:0.08:94.92, vol/vol/vol] for ABA and CH_3CN:CH_3CO_2H:ddH_2O [5:0.08:94.92, vol/vol/vol] for CKs) and were centrifuged at 12,320 RCF for 10 min to remove any solid particles (Thermo Scientific). The supernatants were stored at –20 °C until further processing.

High performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-(ESI)-MS/MS) analysis

Levels of plant growth regulators were measured using high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-(ESI)-MS/MS; QTrap 5500; ABI Sciex Concord Ontario, Canada, coupled with Agilent 1100 series HPLC; Agilent, Mississauga, ON, Canada). An aliquot (40 µl) was injected on a Luna C18 reversed-phase column (3 µm, 150 × 2.0 mm; Phenomenex, Torrance, Canada). Abscisic acid was eluted using component A: H_2O with 0.08% CH_3CO_2H and component B: CH_3OH, both with 0.08% CH_3CO_2H, at a flow rate of 0.2 mL min^{-1}. Cytokinins were eluted with an increasing gradient of 0.08% CH_3CO_2H in CH_3CN (A) mixed with 0.08% CH_3CO_2H in ddH_2O (B), at a flow rate of 0.2 mL min^{-1}. For the ABA, the initial conditions of 50% B remained constant for 4 min, then changed linearly to 95% B over 6 minutes. This ratio was held constant for 1 minute before immediately returning to starting conditions and re-equilibrating for 20 minutes. The initial conditions for CK groups were 5% A and 95% B, changing linearly in 17 min to 95%

A and 5% B. Conditions remained constant for 5 min, and then, immediately returned to initial conditions for 18 min of re-equilibration.

Abscisic acid was analyzed in negative ionization mode and CKs were analyzed in positive ionization mode. Phytohormones were identified based on their analyte specific retention times and multiple reaction monitoring (MRM) channels. All data were analyzed with Analyst 1.6.2 software (AB SCIEX, Concord, ON, Canada). Hormone concentrations were established according to isotope dilution analysis upon direct comparison of the endogenous analyte peak area against the recovered internal standard (Farrow and Emery 2012). Quantification of *c*Z-type CKs was performed relative to the recovery of labeled *t*Z-types and retention time of unlabeled *c*Z standards.

Indole-3-Acetic Acid (IAA)

For the evaluation of the IAA production capacity, 47 strains were grown in the dark in R2 broth supplemented with L-tryptophan (2.5 mM) and in L-tryptophan-free medium. Seven-days-old cultures that were in late exponential phase, were subjected to quantitative screening for IAA production via colorimetric method using Salkowski's reagent (Gordon and Weber, 1951). Obtained by centrifugation (10 min, 4,696 × g), cell-free suspensions of each strain (1 vol) were incubated for 30 min (RT) with Salkowski reagent (1 mL 0.5M $FeCl_3$ in 50 mL of 35% $HClO_4$) (0.5 vol). IAA concentration was quantified spectrophotometrically at wavelength 540 nm. The spectrophotometer was calibrated against a blank media with L-tryptophan mixed with the Salkowski reagent. To determine the sensitivity and the operating range of IAA concentrations in bacterial cultures, a calibration curve was calculated based on dilution series of authentic IAA (0-100 μg mL^{-1}) (Sigma-Aldrich, Inc., St. Louis, MO, USA).

Statistical analysis

The experiments evaluating CKs, ABA, and IAA production by *Methylobacterium* were performed in three replicates for each of the tested strains. The levels of bacterial phytohormones were expressed as per pmol in mL of the culture supernatant ($n = 3 \pm SE$).

2.5. RESULTS

Cytokinin production by *Methylobacterium* strains

The collection of almost 50 *Methylobacterium* strains analysed in this study represented a wide range of growth habitats. Majority of the strains originated from plant organs and soil while some were derived from water, air and other sources (Tab. 1). All bacterial isolates were cultured in a developed for *Methylobacterium*, minimum medium supplemented with methanol as a sole carbon source (DSMZ-125). Use of a minimum medium was essential to ensuring that CKs detected using HPLC-MS/MS were strictly of bacterial origin and not background signal contribution from the constituents of nutrient-rich growth media (Aoki et al. 2021).

Total CK concentration in the cell-free supernatants of *Methylobacterium* strains ranged from 9.9 (*M. platani* JCM14648) to 191.5 pmol mL^{-1} (*M. oryzae* LMG23582) (Figure 1). Regarding the total CK levels secreted by bacteria, the three most productive strains (over 130 pmol mL^{-1} CKs) were isolated from plant organs - stem, leaf, and petiole (*M. oryzae* LMG23582(T), *M. phylosphaerae* LMG24361(T), and *M. organophillum* NBRC103119, respectively).

Of the 28 endogenous CKs monitored, 16 CKs forms were detected at different concentrations (Table S1). The HPLC-MS/MS analysis of CK levels in bacteria supernatants consistently revealed substantial levels of the most active CK-FB forms (mainly *t*Z, and *c*Z in a lower number of strains), as well as relatively high levels of the 2-methylthiol-Zeatin conjugate (MeSZ). In most bacteria strains analyzed, *t*Z (0.45 – 82.16 pmol mL^{-1}) and MeSZ (4.5 – 54.3 pmol mL^{-1}) were the main detected compounds, representing over 70% of the total detected CK content (Figure 2).

For most of the analysed strains, the main CK group secreted to bacterial supernatants were the most biologically active free bases (CK-FB), followed by methylthiols (2MeS-CKs), and lower levels of ribosides (CK-RB) and nucleotides (CK-NT) (Figure 2.3.). No considerable differences were observed between the strains regarding the distribution of two most abundant CK groups; however, whenever the ability to produce CK-FBs was more pronounced among the strains, the total 2MeS-CK concentration markedly decreased. Only in the case of *M. oryzae* strain LMG 23582(T), *c*Z was detected in higher quantity compared to *t*Z isomer in the cell-free supernatant (146.6 pmol mL^{-1} and 31.5 pmol mL^{-1}, respectively).

Across the inventory of bacteria strains, CK-RB and CK-NT derivatives of isopentenyladenine (iP) were identified at higher concentrations (0.64–6.26 pmol mL^{-1} for isopentenyladenosine (iPR), and 1.41 – 4.61 pmol mL^{-1} for isopentenyladenine nucleotide (iPNT)), relative to the CK-RB and CK-NT derivatives of any of Dihydrozeatin (DHZ), *t*Z, or *c*Z, which were detectable at minute levels in the supernatant of *Methylobacterium* strains. Likewise other forms such as DHZ, 2-methylthio-Zeatin riboside (MeSZR), and 2-Methylthio-isopentenyladenosine

(MeSiPR) were all only detected at very low levels. Glucoside derivatives and aromatic CKs were not present in the supernatants of any of the *Methylobacterium* strains analysed. No endogenous ABA was detected from any of the analysed strains.

Indole-3-acetic acid

The colorimetric method used in this study to quantify the levels of bacterial indole-3-acetic acid (IAA) revealed that out of the 47 analysed *Methylobacterium* strains, 30 were able to secrete IAA at varying concentrations (0.02-11.82 μg mL^{-1}; 0.11-67.5 nmol mL^{-1}) after incubation with an L-tryptophan precursor. Out of the 12 strains originally isolated from soil or rhizosphere, 8 secreted IAA in the concentration ranging from 0.55 to 11.82 μg mL^{-1} (Figure 2.4.). Supernatants of two *M. extorquens* strains (JCM2805 and JCM2806) had the highest IAA levels (over 10 μg mL^{-1}), which corresponded with one of the lowest total CK contents (below 20 pmol mL^{-1}). By contrast, the most active CK producer, *M. oryzae* (LMG 23582(T)) did not secrete detectable IAA levels to the culture supernatant, while two other strains characterised by high CK concentration in the culture supernatants, *M. phylosphaerae* (LMG 24361(T)) and *M. organophillum* (NBRC 103119) released only minute IAA levels (below 1 μg mL^{-1}). On the other hand, however, *M. thiyocyanatum* (NBRC 103122) and *M. radiotolerans* (LMG 6379) were associated with relatively high levels of both IAA conversion (over 10 μg mL^{-1}) and total CKs (over 70 pmol mL^{-1}).

Generally, the levels of IAA converted in *Methylobacterium* cultures from L-tryptophan supplement, were significantly higher compared to the secreted CK levels. However, no endogenous IAA was detected in any cultures that were untreated with L-tryptophan, a direct precursor of IAA biosynthesis.

2.6. DISCUSSION

Plant-microbe interactions are complex and variable depending on the relationship between different bacterial strains and plant genotypes. Prior to Holland's hypothesis (1997a) that any biosynthesis of plant CKs occurred exclusively by the microbial plant symbionts, the investigation of phytohormone production by microorganisms was largely focused on elucidating the mechanisms by which phytopathogens potentiate disease development. These original studies eventually led to important discoveries like IAA production by *Pseudomonas syringae* and its causal role in initiating tumour-like growths called galls (Akiyoshi et al. 1987; Iacobellis et al. 1994). More recently, the landscape surrounding phytohormone involvement in plant pathogenesis has shifted due to emerging evidence indicating their involvement in the intricate defence mechanisms, related to the host-plant responses to bacterial infections (Choi et al. 2010; Großkinsky et al. 2016) fungi infections (Jiang et al. 2013; Morrison et al. 2015), or attacks by insect predators (Dervinis et al. 2010). Interestingly, many taxa of non-pathogenic microorganisms also produce phytohormones, including bacteria (Shi et al. 2009: Kisiala et al. 2013) and fungi (You et al. 2013; Khan et al. 2015; Streletskii et al. 2019; Bean et al. 2021) The bacteria in question comprise many plant-associated strains of *Methylobacterium* (Ivanova et al. 2000, 2001; Koenig et al. 2002; Meena et al. 2012). Our results confirm that all *Methylobacteria* evaluated in this investigation can produce CKs, albeit at quite varying levels, which surpass levels of related hormones (e.g., ABA, IAA) even though they are demonstrably capable of making IAA.

Pink pigmented facultative methylotrophs (PPFMs) belonging to the *Methylobacterium* genus are a unique group of plant-associated microorganisms known

to be considerably beneficial to their hosts (Holland 1997b; Abanda-Nkpwatt et al. 2006; Green 2006b; Madhaiyan et al. 2006; Esitken et al. 2010; Meena et al. 2012; Jorge et al. 2019). They are ubiquitous in nature and reside in diverse environments such as leaf surfaces, plant tissues, soil, and in the air. Many plant endosymbiotic microbes from the *Methylobacterium* genus have been identified as important plant growth-promoting bacteria (PGPB) with natural capability of synthesizing high levels of phytohormones including CKs, which are highly beneficial to the host plant (Holland and Polacco 1994; Ivanova et al. 2000; Dourado et al. 2015). Until now, however, no comprehensive inventory of CK production across a wide breadth of *Methylobacterium* species has been conducted which couples a separation methodology as precise as high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS). It has been proposed that CKs are produced by *Methylobacterium* as downstream products of tRNA degradation (Koenig et al. 2002). Our work has formed a resource for further understanding the diversity and complexity of phytohormone biosynthesis across CK-producing *Methylobacterium* strains which have already been reported to enhance seed germination, seedling growth, and increase systemic resistance in many plant species (Elbeltagy et al. 2000; Omer et al. 2004; Madhaiyan et al. 2005; Abanda-Nkpwatt et al. 2006; Jorge et al. 2019).

The presence of CKs among various strains of *Methylobacterium* have been reported to date; however, these studies could only detect up to four CK forms at a time based on the dated hormone extraction techniques and detection limits (Phillips and Torrey 1972; Sturtevant and Taller 1989; Boiero et al. 2007). Previously, a comprehensive hormone extraction protocol enabled the screening of 25 CK compounds from rhizosphere bacteria with successful detection and identification of 11

forms among *Rhizobium* strains (Kisiala et al. 2013). Using a similar approach, in addition to detecting 16 different CK compounds within *Methylobacterium*, our study illustrates significant variation in the inventory of CK production by different *Methylobacterium* strains compared to other plant-associated microbes - specifically, with respect to the diversity of CKs produced and to the secretion of the highly active tZ free base and its derivatives.

trans-Zeatin (*t*Z), which is typically found in higher plants, can bind at least two plant CK receptors with high affinity and it was characterised as the most active CK in several bioassays (Romanov 2009). In plant systems, *t*Z participates in basic growth and development (Sakakibara 2006; Osugi et al. 2017), regulation of plant immunity (Großkinsky et al. 2013), or the alleviation of photoperiod stress (Frank et al. 2020). Within the microbial community, previous evaluation of the CK profiles in mycelial biomass of medicinal mushrooms revealed *t*Z as the most abundant CK form in the majority of species (Vedenicheva et al. 2018); yet, the regulatory functions of tZ in mushroom growth could not be fully elucidated. Contrastingly, an examination of CK production by temperate forest soil fungi indicated the complete absence of tZ across all 20 strains investigated (Morrison et al. 2015). In another study involving yeasts from different taxonomic groups and habitats, Z-type CKs were detected at high levels; however, the differentiation between discrete Z isomers was not established, nor was a clear conclusion drawn regarding a possible role of Z synthesis in the growth of yeasts (Streletskii et al. 2019).

Studies involving plant-associated bacteria, including rhizobia, have previously documented the production of mainly *c*Z- and iP-types of CKs, while our investigation

shows that *Methylobacteria* stand apart through their secretion of mostly the bioactive *t*Z. Comparison of phytohormone profiles of the 47 *Methylobacterium* strains revealed noticeable consistencies in the distribution and levels of CK groups and types, indicating there is a characteristic CK pattern among different species of this genus. In general, *t*Z was detected in higher abundance relative to *c*Z levels, with CK-FB forms found at higher concentrations than CK-NT and CK-RB forms. Though our work indicated greater presence of the highly active *t*Z among the representatives of *Methylobacterium* genus, its isomer *c*Z was also detectable.

Recent research suggests that *c*Z forms can have a great agricultural potential; *c*Z, beyond being involved in plant growth regulation, can also play a crucial role in intermediate responses to stress including infection and herbivory, among other environmental interactions (Schäfer et al. 2015) and the two isomer systems together are thought to offer a dual level of response, as either hard-hitting and brief, or with low activity and longer duration (Hluska et al. 2021). *trans*-Zeatin was found at high levels in some important experimental plants (i.e. Arabidopsis), while in legumes like chickpea and field pea, *c*Z was identified as the predominant CK form (Emery et al. 1998; Quesnelle and Emery 2007). Diversity in the relative abundance of CK types across different plant species is also an attribute mirrored in the CK profiles of the examined *Methylobacterium*. It can be suggested that selecting a bacterial strain with a CK production profile similar to that of the target plant can work to enhance the effects of beneficial phytohormones in the host through an additive mechanism. Conversely though, selecting a strain that has a CK profile opposite but complementary to the host plant, may in fact work to achieve positive outcomes through supplementation of hormones which have low endogenous expression with longer duration. Such beneficial

effects of supplementation may manifest as improvements in response and tolerance to certain biotic and abiotic stresses and may form the basis for the next chapter of research at the biochemical interface between PGPB and their hosts.

Methylthiol-CKs (2MeS-CKs) were the second most abundant fraction in *Methylobacterium* CK profiles. Methylthiol-CKs are the least characterized CK group as the breadth of their physiological effects and participation in signalling cascades have not yet been even partially elucidated (Gibb et al. 2020). This CK group is often suggested to be mainly of microbial origin, and a modification of the tRNA degradation pathway was proposed as a possible route for 2MeS-CK biosynthesis (Morrison et al. 2017; Gibb et al. 2020) including in *Methylobacterium* (Koenig et al. 2002). High levels of 2MeS-CKs were also detected in other studies involving bacterial symbionts such as *Rhizobia* (Phillips and Torrey 1972; Sturtevant and Taller 1989; Kisiala et al. 2013). Consequently, our findings align with earlier work by establishing a relatively high production rate of methylthiolated CKs among bacteria strains and consistency of the inverse relationship previously observed between 2MeS-CK production and that of more active forms such as *tZ* (Kisiala et al. 2013; Gibb et al. 2020).

Riboside (CK-RB) and nucleotide (CK-NT) forms are usually considered to be less biologically active, as CK-NTs are the stable precursors to more active CK forms (Sakakibara 2006; Romanov 2011), and CK-RBs are regarded as more suitable for transport within plants (Kudo et al. 2010; Osugi et al. 2017). On the other hand, Nguyen et al. (2021) contest that CK-RBs have roles beyond transport, including potential direct activity (Nguyen et al. 2021). In our study, both the CK-NTs and CK-RBs were less abundant than CK-FBs across the *Methylobacterium* analyzed. As the less bioactive

precursors, CK-NTs are more likely to be retained inside the bacterial cells while their secretion to the supernatant is not expected. Overall, only NT and RB derivatives of iP were detected at higher levels relative to other NT and RB forms evaluated. Microbial isopentenyladenine is synthesised via the isopentenyl-dependent mevalonate pathway (MVA; Fébort et al. 2011) and, thus, the presence of iP-type CKs suggests the presence of this pathway in *Methylobacterium*. Yet, the concentration of iP in *Methylobacterium* supernatants was several times lower than those of tZ, due in part because iP is less stable product of the pathway and it is frequently quickly hydroxylated to tZ by cytochrome P_{450} monooxygenases (Sakakibara 2006), or because of the dominant tZ production via tRNA modification (Koenig et al. 2002).

Glucoside-CK conjugates were not detected in any of *Methylobacterium* strains, and this is consistent with reported evolutionary patterns of CKs in prokaryotic organisms compared to higher plants in which glucosylation is exclusively found (Stirk and van Staden 2010; Kisiala et al. 2013). Indole-3-acetic acid (IAA) production from L-tryptophan by *Methylobacterium* strains was detected through use of the Salkowski reagent and quantified using a common spectrophotometric technique for measuring IAA production that is of microbial origin (Ivanova et al. 2001; Kim et al. 2017; Gang et al. 2019). Without L-tryptophan supplementation no endogenous IAA was detected; however, with L-tryptophan, the total IAA concentration ranged from 0 to 11.82 µg mL^{-1}. Among a range of biological functions that IAA participates in (response to light, gravity, ingression of pathogens), it can also stimulate root growth. Earlier studies with microorganisms showed IAA producers are often gram-negative microbes (Datta and Basu, 2000). In gram-negative rhizobacterial isolates from *Coleus* rhizosphere, IAA production was found at concentrations between 230 and 260 µg mL^{-1} through the use

of spectrophotometric methods including UV-HPLC and thin layer chromatography (Patel and Saraf 2017). Certain obligate and facultative methylotrophic bacteria, in the presence of L-tryptophan, were reported to produce IAA in the range of 5 – 14 μg mL^{-1} (Ivanova et al. 2001) which is comparable to the findings of this study.

In our investigation, out of 12 *Methylobacterium* strains of soil origin, 8 were found to have measurable levels of IAA when fed with L-tryptophan. While not all 12 soil-borne *Methylobacteria* could produce detectable levels of IAA, the biosynthesis of this compound does appear to be linked to the strains isolated from the phytobiome (leaves, roots) compared to those without a link to a host-plant (air, water, other sources). Furthermore, although the inverse relationship between CKs production and IAA conversion rate was not obvious for all the analysed *Methylobacteria*, the strains capable of secreting the most abundant CK levels (i.e., *M. oryzae* (LMG23582(T)) or *M. phylosphaerae* ((LMG24361(T)) were not converting IAA effectively, and high IAA producers were characterised by the low CK concertation in their culture supernatants. This may indicate high specialization and adaptation of *Methylobacterium* strains for their respective ecological niches.

2.7. CONCLUSION

Herein, we provide the most comprehensive to date, survey of CK production by PPFM of the *Methylobacterium* genus. We have established that *Methylobacterium* species are able to secrete a wide range of the most active CK form, *t*Z, at levels higher than any other PGPB which could indeed benefit plant hosts. Our data indicate that a detailed bacterial CK profile can be used as an attribute orthogonal to the criteria already existing for the selection of bacteria candidates to be investigated for purpose as plant growth promoters. For example, *M. oryzae* (LMG23582(T)) possesses valuable characteristics that contribute to its usefulness as a PGPB (Indiragandhi et al. 2008) and in conferring tolerance to high salinity stress in plants (Lee et al. 2015; Chanratana et al. 2017). Furthermore, another study supports the hypothesis that microbially-derived CKs play an important role in host-plant metabolism, including growth stimulation, enhancement of physiological parameters, and effective nutrient allocation and water management under drought exposure (Jorge et al. 2019). Retrospectively, the findings of these investigations align with the results presented herein where *M. oryzae* (LMG23582(T)) strain is likely mediating marked growth-promoting effects due to its uniquely high CK production. By contrast, other hormones scanned for (ABA, IAA) were not endogenously detected, unless a precursor was fed to the cells (IAA). In the latter case, IAA conversion capacity from L-tryptophan is inversely related to CK levels among many of the analysed strains.

The use of a multifarious selection strategy supported by knowledge of CK production could help reduce research costs associated with laboratory consumables, equipment, and labour associated with large scale greenhouse or field-trial experiments by streamlining the process of identifying the most potentially effective strains for

bioinoculant development. In future, other high tZ-producing strains of *Methylobacterium* genus should be investigated for growth-promotion and stress-alleviating effects in controlled experiments in plant systems to further elucidate their role in crop protection.

2.8. TABLES AND FIGURES

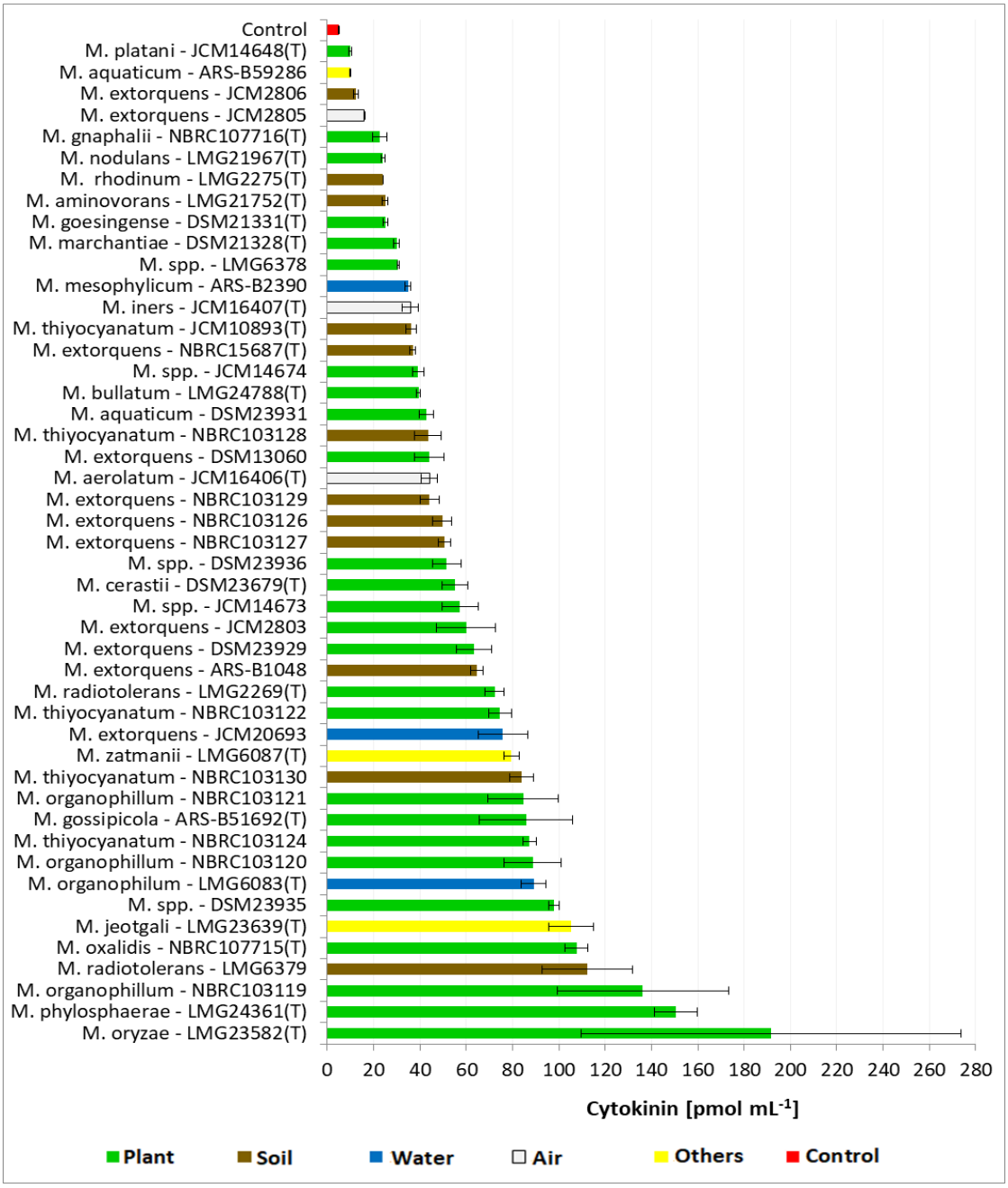

Figure 2.1: Total cytokinin (CK) concentration (pmol mL^{-1}) detected by HPLC-(ESI+)-MS/MS in the cell-free supernatants of 46 *Methylobacterium* strains cultured *in vitro* in the DSMZ-125 minimal medium. Cytokinin production was tested in the early stationary phase. Values are means ± SE of 3 replicates.

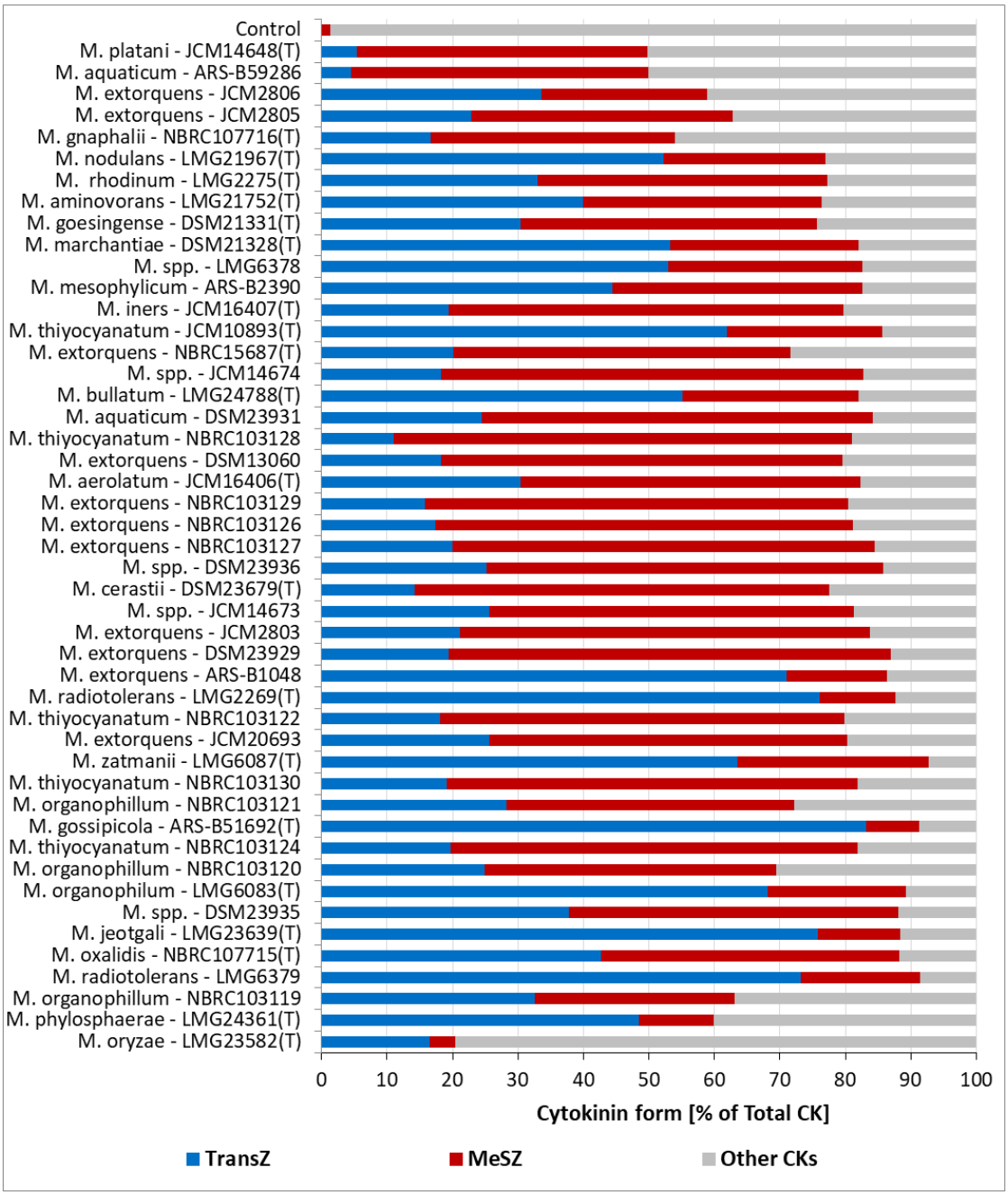

Figure 2.2: *trans*-Zeatin (transZ), 2-methylthio-Zeatin (MeSZ), and other cytokinin (CK) forms reported as percentage of total CK content in the cell-free supernatants of 46 *Methylobacterium* strains cultured *in vitro* in the 125DSM minimal medium. Cytokinin production was tested in the early stationary phase. Strains are ordered according to the increasing total CK production (n=3).

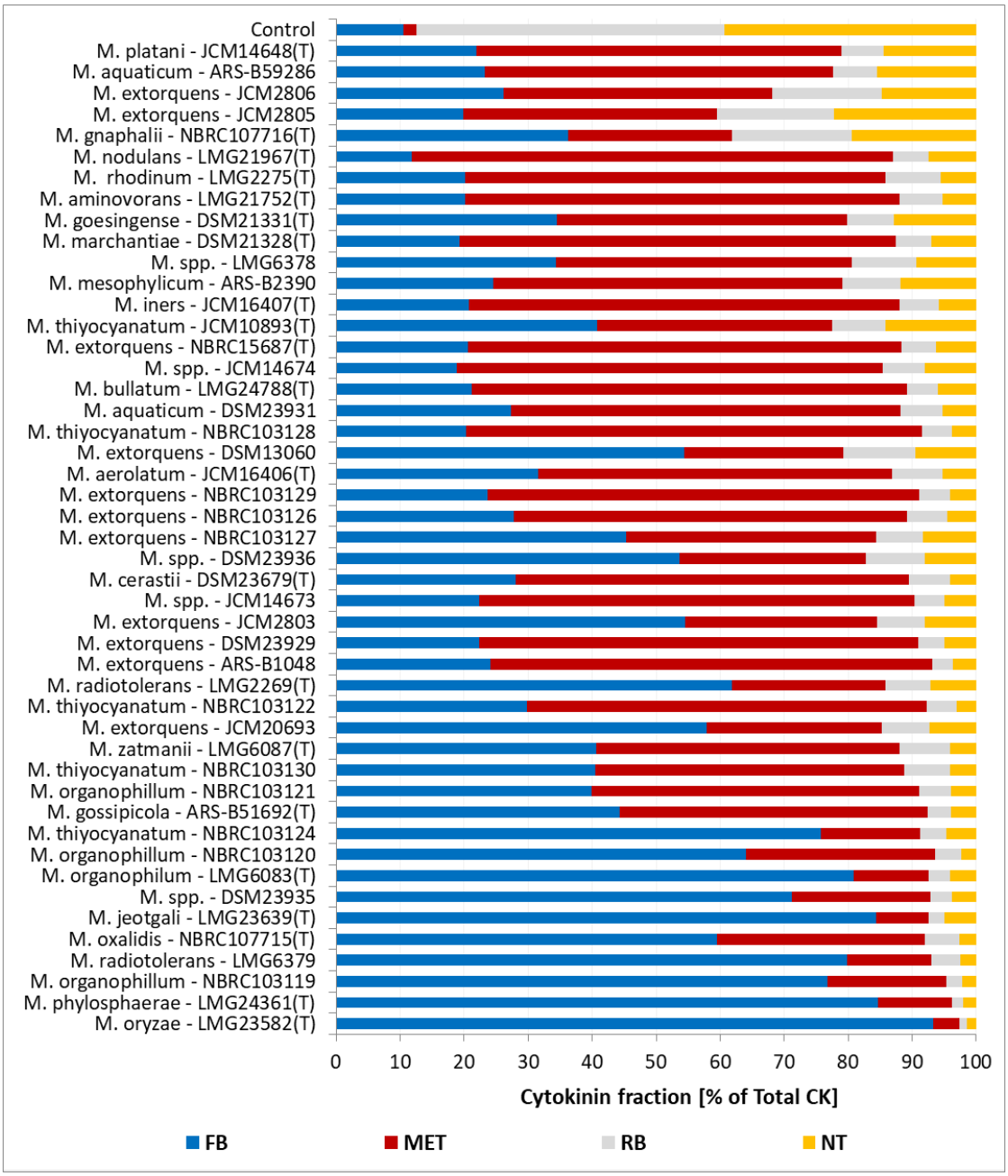

Figure 2.3: Cytokinin (CK) fractions: free bases (FB), methylthiols (2-MeS), ribosides (RB), and nucleotides (NT) reported as percentage of total CK content in the cell-free supernatants of 46 *Methylobacterium* strains cultured *in vitro* in the DSMZ-125 minimal medium. Cytokinin production was tested in the early stationary phase. Strains are ordered according to the increasing total CK production (n=3).

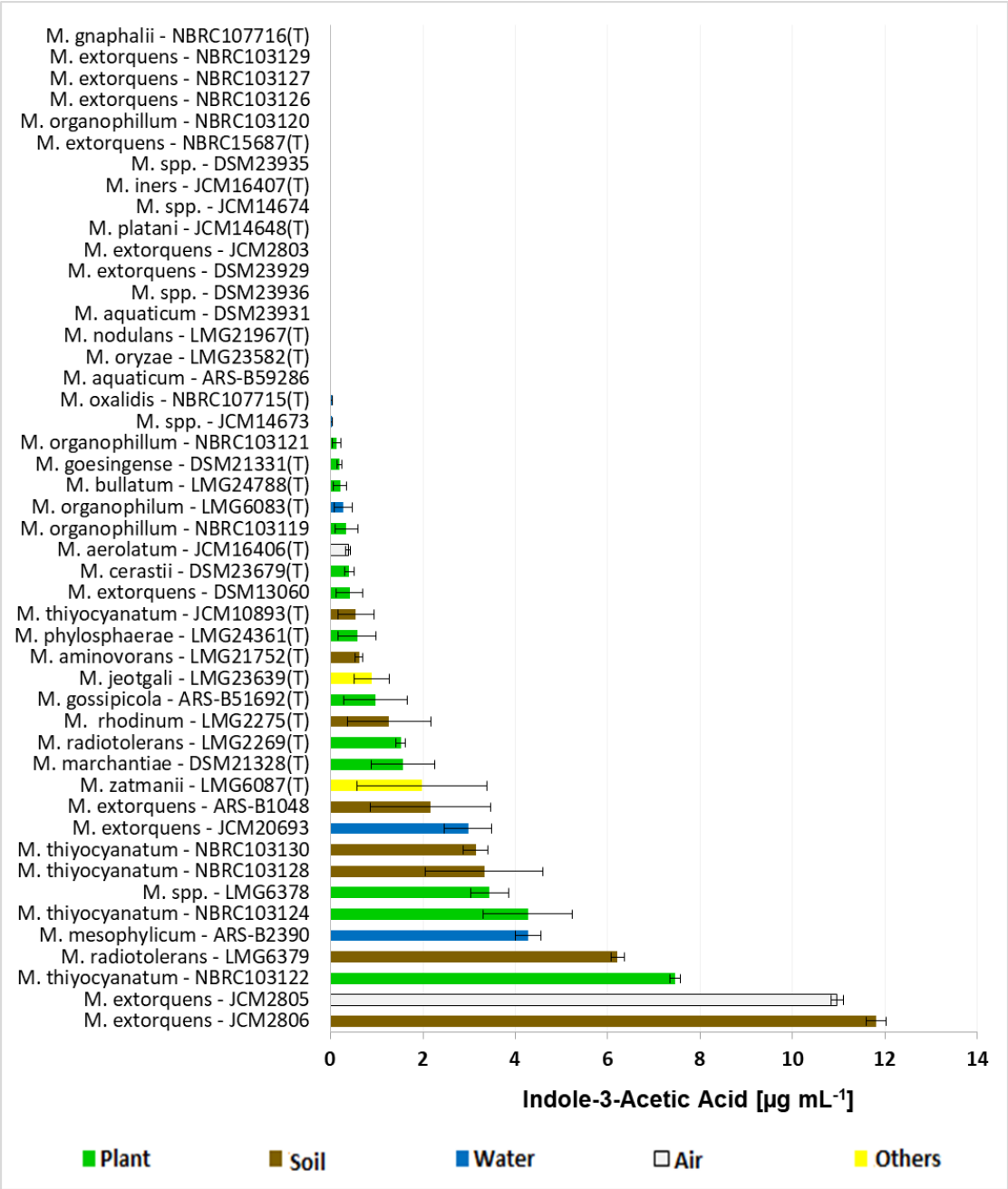

Figure 2.4: Total concentration of Indole-3-Acetic Acid (IAA (µg mL^{-1}) detected spectrophotometrically by a colorimetric method in cell-free supernatants of 46 *Methylobacterium* strains. Strains were cultured *in vitro* in R2 broth supplemented with 2.5 mM L-tryptophan. Indole-3-Acetic Acid concentration was measured in the early stationary phase. Values are means ± SE of 3 replicates.

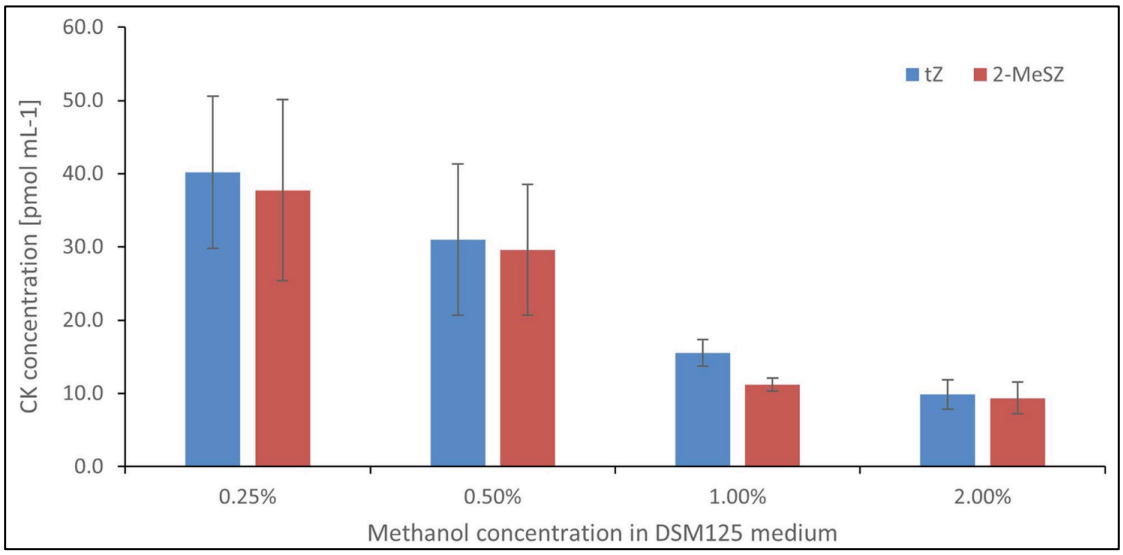

Figure 2.5: Cytokinin (CK) concentration (pmol mL^{-1}) in the cell-free supernatants of *Methylobacterium oryzae* - LMG23582(T), cultured under different concentrations of methanol (0.25-2.00%) in the DSMZ-125 growth medium. Cytokinin production was tested in the early stationary phase. Values are means ± SE of 3 replicates. (n=3).

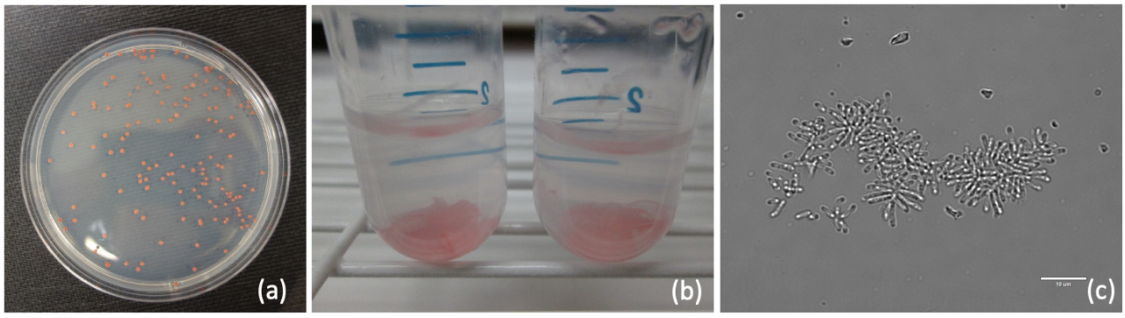

Figure 2.6: *Methylobacterium organophilum* growth on DSMZ-125 agar plate (a) and in DSMZ-125 liquid medium (b). Microscopic image of aggregate formation by *Methylobacterium organophilum* cells in liquid cultures (c).

Table 2.1. Inventory of *Methylobacterium* strains evaluated for phytohormone production.

Species	Strain	Isolation Source
M. aerolatum	JCM 16406 *(T)*	Air
M. aminovorans	LMG 21752 *(T)*	Soil
M. aquaticum	B-59286	Phoenix spacecraft surface
M. cerastii	DSM 23679 *(T)*	*Cerasium holosteoides* - phyllosphere
M. extorquens	B-1048	Garden soil enriched with sarcosine
	JCM 2805	Air
	NBRC 15687 *(T)*	Soil
	JCM 2806	Garden soil; slough
	DSM 13060	*Pinus sylvestris* - meristem tissue cultures
	DSM 23939	*Arabidopsis thaliana* - phyllosphere
	JCM 2803	*Psychotria mucronata* - phyllosphere
	JCM 20693	Mine water
	NBRC 103126	Soil-litter close to *Rumex* sp.
	NBRC 103127	Soil-litter close to *Arum* sp.
	NBRC 103129	Soil-litter close to *Eucalyptus* sp.
M. gnaphalii	NBRC 107716 *(T)*	*Gnaphalium spicatum* - phyllosphere
M. gossipiicola	B-51692 *(T)*	*Gossipium hirsutum* - phyllosphere
M. jeotgali	LMG-23639 *(T)*	Traditional fermented seafood (jeotgal)
M. mesophilicum	B-14246 *(T)*	*Lolium perenne* - phyllosphere
	B-2390	Household well water
M. nodulans	LMG-21967 *(T)*	*Crotalaria podocarpa* - phyllosphere
M. organophilum	LMG-6083 *(T)*	Lake water; sediment
M. organophilum	NBRC 103119	*Pelargonium zonale* - phyllosphere
	NBRC 103120	*Ficus elastica* - phyllosphere
	NBRC 103121	*Begonia* sp. - phyllosphere
M. oryzae	LMG-23582 *(T)*	*Oryzae sativa* cv. Nam-Pyeoung - surface-disinfected stem
M. oxalidis	NBRC 107715 *(T)*	*Oxalis corniculata* - phyllosphere
M. phyllosphaerae	LMG-24361 *(T)*	*Oryza sativa* cv. Dong-Jin - leaf tissues
M. platani	JCM 14648 *(T)*	*Platanus orientalis* - phyllosphere
M. radiotolerans	LMG-2269 *(T)*	Japanese unpolished (unhulled) aged commercial rice grain
	LMG-6379	Forest soil
M. rhodinum	LMG-2275 *(T)*	Alder (*Alnus*) - rhizosphere
M. thiocyanatum	JCM 10893 *(T)*	*Allium aflatuense* - rhizosphere soil
	NBRC 103122	*Bryophyllum* sp. - phyllosphere
M. thiocyanatum	NBRC 103128	Soil-litter close to *Mesenbryanthemum* sp.
	NBRC 103130	Soil-litter close to *Rumex* sp.
M. zatmanii	LMG-6087 *(T)*	
Methylobacterium spp.	DSM 23936	*Medicago truncatula* - phyllosphere
	JCM 14673	*Oryza sativa* SC-41 - phyllosphere
	JCM 14674	*Oryza rufipogon* W1964 - phyllosphere

(T) Indicates "type strain" cultures that were descended from a strain designated as the nomenclatural type.

Table 2.2. Cytokinins (CKs) scanned for using liquid chromatography-positive electrospray ionization tandem mass spectrometry in *Methylobacterium* supernatants.

Cytokinin (CK)	Labelled CK Standard
Nucleotides (mono- di- and triphosphates; CK-NTs)	
trans-Zeatin nucleotide (tZNT)	$[^2H_3]$DHZRMP
cis-Zeatin nucleotide (cZNT)	
Dihydrozeatin nucleotide (DHZNT)	
Isopentenyladenine nucleotide (iPNT)	$[^2H_6]$iPRMP
Ribosides (CK-RBs)	
trans-Zeatin riboside (tZR)	$[^2H_5]$ZR
cis-Zeatin riboside (cZR)	
Dihydrozeatin riboside (DHZR)	$[^2H_3]$DHZR
Isopentenyladenosine (iPR)	$[^2H_6]$iPR
Free Bases (CK-FBs)	
trans-Zeatin (tZ)	$[^2H_5]$Z
cis-Zeatin (cZ)	
Dihydrozeatin (DHZ)	$[^2H_3]$DHZ
Isopentenyladenine (iP)	$[^2H_6]$iP
Glucosides (CK-GLUCs)	
trans-Zeatin-O-glucoside (tZOG)	$[^2H_5]$ZOG
cis-Zeatin-O-glucoside (cZOG)	
Dihydrozeatin-O-glucoside (DHZOG)	$[^2H_7]$DHZOG
trans-Zeatin-O-glucoside riboside (tZROG)	$[^2H_5]$ZROG
cis-Zeatin-O-glucoside riboside (cZROG)	
Dihydrozeatin-O-glucoside riboside (DHZROG)	$[^2H_7]$DHZROG
trans-Zeatin-9-glucoside (tZ9G)	$[^2H_5]$Z9G
cis-Zeatin-9-glucoside (cZ9G)	
Dihydrozeatin-9-glucoside (DHZ9G)	$[^2H_5]$DHZ9G
Methylthiols (2MeS-CKs)	
2-Methylthio-Zeatin (2MeSZ)[a]	$[^2H_5]$2MeStZ
2-Methylthio-Zeatin riboside (2MeSZR)[a]	$[^2H_5]$2MeStZR
2-Methylthio-N^6-isopentenyladenine (2MeSiP)	$[^2H_6]$2MeSiP
2-Methylthio-N^6-isopentenyladenosine (2MeSiPR)	$[^2H_6]$2MeSiPR

[a] The analytical procedure used in this study does not facilitate separation of 2MeStZ and 2MeStZR from their corresponding cis-isomers, 2MeScZ and 2MeScZR. Therefore, the levels of the methylthiolated Z-type CKs were reported as total 2MeSZ and total 2MeSZR

2.9. REFERENCES

Abanda-Nkpwatt, D., Müsch, M., Tschiersch, J., Boettner, M., and Schwab, W. 2006. Molecular interaction between *Methylobacterium extorquens* and seedlings: growth promotion, methanol consumption, and localization of the methanol emission site. J. Exp. Bot. 57:4025–4032. https://doi.org/10.1093/jxb/erl173.

Aoki, M. M., Kisiala, A. B., Rahman, T., Morrison, E. N., and Emery, R. J. N. 2021. Cytokinins are pervasive among common *in vitro* culture media: An analysis of their forms, concentrations and potential sources. J. Biotechnol. 334:43–46. https://doi.org/10.1016/j.jbiotec.2021.05.005.

Araújo, W. L., Marcon, J., Maccheroni, W., Jr, Van Elsas, J. D., Van Vuurde, J. W. L., and Azevedo, J. L. 2002. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl. Environ. Microbiol. 68:4906–4914. https://doi.org/10.1128/AEM.68.10.4906-4914.2002.

Bijlani, S., Singh, N. K., Eedara, V. V. R., Podile, A. R., Mason, C. E., Wang, C. C. C., and Venkateswaran, K. 2021. *Methylobacterium ajmalii* sp. nov., Isolated From the International Space Station. Front. Microbiol. 12:639396. https://doi.org/10.3389/fmicb.2021.639396.

Biswas, J. C., Kalra, N., Maniruzzaman, M., Haque, M. M., Naher, U. A., Ali, M. H., Kabir, W., and Rahnamayan, S. 2019. Soil fertility levels in Bangladesh for rice cultivation. Asian J. Soil Sci. Plant Nutr. 1–11. https://doi.org/10.9734/ajsspn/2019/v4i430051.

Boiero, L., Perrig, D., Masciarelli, O., Penna, C., Cassán, F., and Luna, V. 2007. Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Appl. Microbiol. Biotechnol. 74:874–880. https://doi.org/10.1007/s00253-006-0731-9.

Chanratana, M., Han, G. H., Roy Choudhury, A., Sundaram, S., Halim, M. A., Krishnamoorthy, R., Kang, Y., and Sa, T. 2017. Assessment of *Methylobacterium oryzae* CBMB20 aggregates for salt tolerance and plant growth promoting characteristics for bio-inoculant development. AMB Express 7. https://doi.org/10.1186/s13568-017-0518-7.

Corpe, W. A., and Rheem, S. 1989. Ecology of the methylotrophic bacteria on living leaf surfaces. FEMS Microbiol. Lett. 62:243–249. https://doi.org/10.1111/j.1574-6968.1989.tb03698.x.

Datta, C., and Basu, P. S. 2000. Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, Cajanus cajan. Microbiol. Res. 155:123–127. https://doi.org/10.1016/S0944-5013(00)80047-6.

Dobrev, P. I., and Kamínek, M. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. J. Chromatogr. A 950:21–29. https://doi.org/10.1016/s0021-9673(02)00024-9.

Dourado, M. N., Camargo Neves, A. A., Santos, D. S., and Araújo, W. L. 2015. Biotechnological and agronomic potential of endophytic pink-pigmented methylotrophic *Methylobacterium* spp. Biomed Res. Int. 2015:909016. https://doi.org/10.1155/2015/909016.

Elbeltagy, A., Nishioka, K., Suzuki, H., Sato, T., Sato, Y.-I., Morisaki, H., Mitsui, H., and Minamisawa, K. 2000. Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. Soil Sci. Plant Nutr. 46:617–629. https://doi.org/10.1080/00380768.2000.10409127.

Emery, R. J., Leport, L., Barton, J. E., Turner, N. C., and Atkins, C. A. 1998. Cis-isomers of cytokinins predominate in chickpea seeds throughout their development. Plant Physiol. 117:1515–1523. https://doi.org/10.1104/pp.117.4.1515.

Esitken, A., Yildiz, H. E., Ercisli, S., Figen Donmez, M., Turan, M., and Gunes, A. 2010. Effects of plant growth promoting bacteria (PGPB) on yield, growth and nutrient contents of organically grown strawberry. Sci. Hortic. (Amsterdam) 124:62–66. https://doi.org/10.1016/j.scienta.2009.12.012.

Fall R, Benson AA. Leaf methanol—the simplest natural product from plants. Trends Plant Sci. 1996;1(9):296–301. https://doi.org/10.1016/S1360-1385(96)88175-0

Farrow, S. C., Emery, R. J. N. 2012. Concurrent profiling of indole-3-acetic acid, abscisic acid, and cytokinins and structurally related purines by high-performance-liquid-chromatography tandem electrospray mass spectrometry. Plant Meth. 8(1): 1–8. https://doi.org/10.1186/1746-4811-8-42

Frank, M., Cortleven, A., Novák, O., and Schmülling, T. 2020. Root-derived trans-zeatin cytokinin protects *Arabidopsis* plants against photoperiod stress. Plant Cell Environ. 43:2637–2649. https://doi.org/10.1111/pce.13860.

Frébort, I., Kowalska, M., Hluska, T., Frébortová, J., and Galuszka, P. 2011. Evolution of cytokinin biosynthesis and degradation. J. Exp. Bot. 62:2431–2452. https://doi.org/10.1093/jxb/err004.

Gang, S., Sharma, S., Saraf, M., Buck, M., and Schumacher, J. 2019. Analysis of Indole-3-acetic acid (IAA) production in *Klebsiella* by LC-MS/MS and the Salkowski method. Bio Protoc. 9:e3230. https://doi.org/10.21769/BioProtoc.3230.

Gholizadeh, A. 2012. Molecular evidence for the contribution of *Methylobacteria* to the pink-pigmentation process in pink-colored plants. J. Plant Interact. 7:316–321. https://doi.org/10.1080/17429145.2012.693208.

Gibb, M., Kisiala, A. B., Morrison, E. N., and Emery, R. J. N. 2020. The origins and roles of methylthiolated cytokinins: Evidence from among life kingdoms. Front. Cell Dev. Biol. 8:605672. https://doi.org/10.3389/fcell.2020.605672.

Gordon, S. A., and Weber, R. P. 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26:192–195. https://doi.org/10.1104/pp.26.1.192.

Green, P. N. 2006. *Methylobacterium*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K, Stackebrandt E, editors. The prokaryotes–a handbook on the biology of bacteria. Springer. p. 257–65. https://doi.org/10.1007/0-387-30745-1

Großkinsky, D. K., Edelsbrunner, K., Pfeifhofer, H., van der Graaff, E., and Roitsch, T. 2013. Cis- and trans-zeatin differentially modulate plant immunity. Plant Signal. Behav. 8:e24798. https://doi.org/10.4161/psb.24798.

Hardoim, P. R., van Overbeek, L. S., and van Elsas, J. D. 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. 16:463–471. https://doi.org/10.1016/j.tim.2008.07.008.

Hiraishi, A., Furuhata, K., Matsumoto, A., Koike, K. A., Fukuyama, M., and Tabuchi, K. 1995. Phenotypic and genetic diversity of chlorine-resistant *Methylobacterium* strains isolated from various environments. Appl. Environ. Microbiol. 61:2099–2107. https://doi.org/10.1128/aem.61.6.2099-2107.1995.

Hluska, T., Hlusková, L., and Emery, R. J. N. 2021. The hulks and the deadpools of the cytokinin universe: A dual strategy for cytokinin production, translocation, and signal transduction. Biomolecules 11:209. https://doi.org/10.3390/biom11020209.

Holland, M. A., and Polacco, J. C. 1994. PPFMs and other covert contaminants: Is there more to plant physiology than just plant? Annu. Rev. Plant Physiol. Plant Mol. Biol. 45:197–209. https://doi.org/10.1146/annurev.pp.45.060194.001213.

Holland, M. A. 1997a. *Methylobacterium* and plants. Rec Res Dev Plant Physiol. 1: 207–213.

Holland, M. A. 1997b. Occam's razor applied to hormonology (Are cytokinins produced by plants?). Plant Physiol. 115(3): 865–868. https://doi.org/10.1104/pp.115.3.865.

Indiragandhi, P., Anandham, R., Kim, K., Yim, W., Madhaiyan, M., and Sa, T. 2008. Induction of defense responses in tomato against *Pseudomonas syringae* pv. tomato by regulating the stress ethylene level with *Methylobacterium oryzae* CBMB20 containing 1-aminocyclopropane-1-carboxylate deaminase. World J. Microbiol. Biotechnol. 24:1037–1045. https://doi.org/10.1007/s11274-007-9572-7.

Ito, H., Iizuka, H. 1971. Taxonomic studies on a radio-resistant *Pseudomonas*: part XII. Studies on the microorganisms of cereal grain. Agric Biol Chem. 35(10): 1566–71.

Ivanova, E. G., Doronina, N. V., Shepelyakovskaya, A. O., Laman, A. G., Brovko, F. A., Trotsenko, Y. A. 2000. Facultative and obligate aerobic *Methylobacteria* synthesize cytokinins. Microbiology. 69(6): 646–51.

Ivanova, E. G., Doronina, N. V., and Trotsenko, Y. A. 2001. Aerobic *Methylobacteria* are capable of synthesizing auxins. Microbiology. 70(4): 392–7.

Jacobs, J. L., Carroll, T. L. and Sundin, G. W. 2005. The role of pigmentation, ultraviolet radiation tolerance, and leaf colonization strategies in the epiphytic survival of phyllosphere bacteria. Microb Ecol. 49(1): 104–13. https://doi.org/10.1007/s00248-003-1061-4

Jorge, G. L., Kisiala, A., Morrison, E., Aoki, M., Nogueira, A. P. O., and Emery, R. J. N. 2019. Endosymbiotic *Methylobacterium oryzae* mitigates the impact of limited water availability in lentil (*Lens culinaris* Medik.) by increasing plant cytokinin levels. Environ. Exp. Bot. 162:525–540. https://doi.org/10.1016/j.envexpbot.2019.03.028.

Kamo, T., Hiradate, S., Suzuki, K., Fujita, I., Yamaki, S., Yoneda, T., Koitabashi, M., and Yoshida, S. 2018. Methylobamine, a UVA-Absorbing Compound from the Plant-Associated Bacteria *Methylobacterium* sp. Nat. Prod. Commun. 13:1934578X1801300. https://doi.org/10.1177/1934578x1801300208.

Kieber, J. J., and Schaller, G. E. 2018. Cytokinin signaling in plant development. Development 145. https://doi.org/10.1242/dev.149344.

Kim, A.-Y., Shahzad, R., Kang, S.-M., Seo, C.-W., Park, Y.-G., Park, H.-J., and Lee, I.-J. 2017. IAA-producing *Klebsiella variicola* AY13 reprograms soybean growth during flooding stress. J. Crop Sci. Biotechnol. 20:235–242. https://doi.org/10.1007/s12892-017-0041-0.

Kisiala, A., Laffont, C., Emery, R. J. N., and Frugier, F. 2013. Bioactive cytokinins are selectively secreted by *Sinorhizobium meliloti* nodulating and nonnodulating strains. Mol. Plant. Microbe. Interact. 26:1225–1231. https://doi.org/10.1094/MPMI-02-13-0054-R.

Koenig, R. L., Morris, R. O., and Polacco, J. C. 2002. tRNA is the source of low-level trans-zeatin production in *Methylobacterium* spp. J. Bacteriol. 184:1832–1842. https://doi.org/10.1128/JB.184.7.1832-1842.2002.

Kudo, T., Kiba, T., and Sakakibara, H. 2010. Metabolism and long-distance translocation of cytokinins. J. Integr. Plant Biol. 52:53–60. https://doi.org/10.1111/j.1744-7909.2010.00898.x.

Kuklinsky-Sobral, J., Araújo, W. L., Mendes, R., Geraldi, I. O., Pizzirani-Kleiner, A. A., and Azevedo, J. L. 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ. Microbiol. 6:1244–1251. https://doi.org/10.1111/j.1462-2920.2004.00658.x.

Kutschera, U. 2007. Plant-associated methylobacteria as co-evolved phytosymbionts: A hypothesis. Plant Signal. Behav. 2:74–78. https://doi.org/10.4161/psb.2.2.4073.

Lee, Y., Krishnamoorthy, R., Selvakumar, G., Kim, K., and Sa, T. 2015. Alleviation of salt stress in maize plant by co-inoculation of arbuscular mycorrhizal fungi and *Methylobacterium oryzae* CBMB20. J Korean Soc Appl Biol Chem 58:533–540. https://doi.org/10.1007/s13765-015-0072-4.

Madhaiyan, M., Poonguzhali, S., Lee, H. S., Hari, K., Sundaram, S. P., and Sa, T. M. 2005. Pink-pigmented facultative methylotrophic bacteria accelerate germination, growth and yield of sugarcane clone Co86032 (*Saccharum officinarum* L.). Biol. Fertil. Soils 41:350–358. https://doi.org/10.1007/s00374-005-0838-7.

Madhaiyan, M., Suresh Reddy, B. V., Anandham, R., Senthilkumar, M., Poonguzhali, S., Sundaram, S. P., and Sa, T. 2006. Plant growth-promoting *Methylobacterium* induces defense responses in groundnut (*Arachis hypogaea* L.) compared with rot pathogens. Curr. Microbiol. 53:270–276. https://doi.org/10.1007/s00284-005-0452-9.

Maneewan, K., and Khonsarn, N. 2017. Selection of bioinoculants for tomato growth enhancement and pathogen resistance. Asia-Pacific Journal of Science and Technology, 22(3), APST–22. https://doi.org/10.14456/apst.2017.37

Meena, K. K., Kumar, M., Kalyuzhnaya, M. G., Yandigeri, M. S., Singh, D. P., Saxena, A. K., and Arora, D. K. 2012. Epiphytic pink-pigmented methylotrophic bacteria enhance germination and seedling growth of wheat (*Triticum aestivum*) by producing phytohormone. Antonie Van Leeuwenhoek 101:777–786. https://doi.org/10.1007/s10482-011-9692-9.

Morrison, E. N., Emery, R. J. N., and Saville, B. J. 2017. Fungal derived cytokinins are necessary for normal *Ustilago maydis* infection of maize. Plant Pathol. 66:726–742. https://doi.org/10.1111/ppa.12629.

Morrison, E. N., Knowles, S., Hayward, A., Thorn, R. G., Saville, B. J., and Emery, R. J. N. 2015. Detection of phytohormones in temperate forest fungi predicts consistent abscisic acid production and a common pathway for cytokinin biosynthesis. Mycologia 107:245–257. https://doi.org/10.3852/14-157.

Nguyen, H. N., Nguyen, T. Q., Kisiala, A. B., and Emery, R. J. N. 2021. Beyond transport: cytokinin ribosides are translocated and active in regulating the development and environmental responses of plants. Planta 254:45. https://doi.org/10.1007/s00425-021-03693-2.

Omer, Z. S., Tombolini, R., and Gerhardson, B. 2004. Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). FEMS Microbiol. Ecol. 47:319–326. https://doi.org/10.1016/S0168-6496(04)00003-0.

Osugi, A., Kojima, M., Takebayashi, Y., Ueda, N., Kiba, T., and Sakakibara, H. 2017. Systemic transport of trans-zeatin and its precursor have differing roles in Arabidopsis shoots. Nat. Plants 3:17112. https://doi.org/10.1038/nplants.2017.112.

Patel, T., and Saraf, M. 2017. Biosynthesis of phytohormones from novel rhizobacterial isolates and their *in vitro* plant growth-promoting efficacy. J. Plant Interact. 12:480–487. https://doi.org/10.1080/17429145.2017.1392625.

Patt, T. E., Cole, G. C., and Hanson, R. S. 1976. *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. Int. J. Syst. Bacteriol. 26:226–229. https://doi.org/10.1099/00207713-26-2-226.

Phillips, D. A., and Torrey, J. G. 1972. Studies on cytokinin production by *Rhizobium*. Plant Physiol. 49:11–15. https://doi.org/10.1104/pp.49.1.11.

Quesnelle, P. E., and Emery, R. J. N. 2007. cis-Cytokinins that predominate in *Pisum sativum* during early embryogenesis will accelerate embryo growth in vitro. Can. J. Bot. 85:91–103. https://doi.org/10.1139/b06-149.

Romanov, G. A. 2009. How do cytokinins affect the cell? Rus J Plant Physiol. 56(2): 268–90. https://doi.org/10.1134/S1021443709020174

Romanov, G. A. 2011. The discovery of cytokinin receptors and biosynthesis of cytokinins: a true story. Rus J Plant Physiol. 58(4): 743–7. https://doi.org/10.1134/S1021443711040121

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., Dowling, D. N. 2008. Bacterial endophytes: recent developments and applications. FEMS Microbiol Let. 278(1): 1–9. https://doi.org/10.1111/j.1574-6968.2007.00918.x

Sakakibara, H. 2006. Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol. 57: 431–49. https://doi.org/10.1146/annurev.arplant.57.032905.105231

Schaller, G. E., Bishopp, A. and Kieber, J. J. 2015. The yin-yang of hormones: cytokinin and auxin interactions in plant development. Plant Cell. 27(1): 44–63. https://doi.org/10.1105/tpc.114.133595

Schäfer, M., Brütting, C., Meza-Canales, I. D., Großkinsky, D. K., Vankova, R., Baldwin, I. T., Meldau, S. 2015. The role of *cis*-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. J ExpBot. 66(16): 4873–84. https://doi.org/10.1093/jxb/erv214.

Šmejkalová, H., Erb, T. J. and Fuchs, G. 2010. Methanol assimilation in *Methylobacterium extorquens* AM1: demonstration of all enzymes and their regulation. PLoSOne. 5(10): e13001. https://doi.org/10.1371/journal.pone.0013001

Stirk, W. A. and Van Staden, J. 2010. Flow of cytokinins through the environment. Plant Growth Regul. 62(2): 101–16. https://doi.org/10.1007/s10725-010-9481-x

Streletskii, R. A., Kachalkin, A. V., Glushakova, A. M., Yurkov, A. M., Demin, V. V. 2019. Yeasts producing zeatin. Peer J. 7:e6474. https://doi.org/10.7717/peerj.6474

Sturtevant, D. B., Taller, B. J. 1989. Cytokinin production by *Bradyrhizobium japonicum*. Plant Physiol. 89(4): 1247–52. https://doi.org/10.1104/pp.89.4.1247

Sy, A., Timmers, A. C., Knief, C., and Vorholt, J. A. 2005. Methylotrophic metabolism is advantageous for *Methylobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. Appl Environ Microbiol. 71(11): 7245–52. https://doi.org/10.1128/AEM.71.11.7245-7252.2005.

Tarkowski, P., Václavíková, K., Novák, O., Pertry, I., Hanuš, J., Whenham, R., Vereecke, D., Šebela, M. and Strnad, M. Analysis of 2-methylthio-derivatives of isoprenoid cytokinins by liquid chromatography–tandem mass spectrometry. Anal Chim Acta. 2010;680(1–2):86–91. https://doi.org/10.1016/j.aca.2010.09.020

van Aken, B., Yoon, J. M. and Schnoor, J. L. 2004. Biodegradation of nitro-substituted explosives 2, 4, 6-trinitrotoluene, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine, and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides×nigra* DN34). Appl Environ Microbiol. 70(1): 508–17. https://doi.org/10.1128/AEM.70.1.508-517.2004.

Vedenicheva, N. P., Al-Maali, G. A., Bisko, N. A., Shcherbatiuk, M. M., Lomberg, M. L., Mytropolska, N. Y., Mykchaylova, O. B. and Kosakivska, I. V. 2018. Comparative analysis of cytokinins in mycelial biomass of medicinal mushrooms. Int J Med Mushrooms. 20(9): 837–47. https://doi.org/10.1615/IntJMedMushrooms.2018027797

Ventorino, V., Sannino, F., Piccolo, A., Cafaro, V., Carotenuto, R. and Pepe, O. 2014. *Methylobacterium populi* VP2: plant growth-promoting bacterium isolated from a highly polluted environment for polycyclic aromatic hydrocarbon (PAH) biodegradation. Sci World J. https://doi.org/10. 1155/ 2014/931793.

White, J. F., Kingsley, K. L., Zhang, Q., Verma, R., Obi, N., Dvinskikh, S., Elmore, M. T., Verma, S. K., Gond, S. K. and Kowalski, K. P. 2019. Endophytic microbes and their potential applications in crop management. Pest Manag Sci. 75(10): 2558–65. https://doi.org/10.1002/ps.5527

Yoshida, S., Hiradate, S., Koitabashi, M., Kamo, T. and Tsushima, S. 2017. Phyllosphere *Methylobacterium* bacteria contain UVA-absorbing compounds. J Photochem Photobiol. 167: 168–75. https://doi.org/10.1016/j.jphotobiol.2016.12.019

Yurimoto, H., Shiraishi, K. and Sakai, Y. 2021. Physiology of methylotrophs living in the phyllosphere. Microorganisms. 9(4): 809–20. https://doi.org/10.3390/microorganisms9040809.

CHAPTER 3

3.1. PREFACE

Title: Impact of glyphosate and glyphosate-based herbicides on phyllospheric *Methylobacterium*.

Authors: Daniel Palberg, Emma Kaszecki, Chetan Dhanjal, Anna Kisiała, Erin N. Morrison, Naomi Stock and R. J. Neil Emery

Reference: Published in *BMC Plant Biology* (2024), 24: 119
doi: 10.1186/s12870-024-04818-x

Contributions: Conceptualization, investigation, methodology, formal analysis, data analysis, figure and table creation, and writing of the initial draft by D.P. Assistance with data collection and figure creation by C.D. Statistical analysis, and assistance with figure and table creation by E.K. Review of methodology and editing by A.K. Writing-review, editing, and technical assistance by E.N.M. and N.S. Assistance with conceptualization, supervision, and acquisition of funding by R.J.N.E.

All authors read and approved the final manuscript.

CHAPTER 3

Impact of glyphosate and glyphosate-based herbicides on phyllospheric *Methylobacterium*.

Published in: *BMC Plant Biology* (2024), 24: 119

3.2. ABSTRACT

Symbiotic *Methylobacterium* comprise a significant portion of the phyllospheric microbiome, and are known to benefit host plant growth, development, and confer tolerance to stress factors. The near ubiquitous use of the broad-spectrum herbicide, glyphosate, in farming operations globally has necessitated a more expansive evaluation of the impacts of the agent itself and formulations containing glyphosate on important components of the plant phyllosphere, including *Methylobacterium*. This study provides an investigation of the sensitivity of 18 strains of *Methylobacterium* to glyphosate and two commercially available glyphosate-based herbicides (GBH). Nearly all strains of *Methylobacterium* showed signs of sensitivity to the popular GBH formulations WeatherMax® and Transorb® in a modified Kirby Bauer experiment. However, exposure to pure forms of glyphosate did not show a significant effect on growth for any strain in both the Kirby Bauer test and in liquid broth, until polysorbate-20 (Tween20) was added as a surfactant. Artificially increasing membrane permeability through the introduction of polysorbate-20 caused a 78–84% reduction in bacterial cell biomass relative to controls containing glyphosate or high levels of surfactant only (0–9% and 6–37% reduction respectively). Concentrations of glyphosate as low as 0.05% w/v (500 µg/L) from both commercial formulations tested, inhibited the culturability of *Methylobacterium* on fresh nutrient-rich medium. To better understand the compatibility of important phyllospheric bacteria with commercial glyphosate-based

herbicides, this study endeavours to characterize sensitivity in multiple strains of *Methylobacterium* and explore possible mechanisms by which toxicity may be induced.

KEYWORDS: Agrichemicals, glyphosate, surfactant, plant growth promoting bacteria, Phyllosphere, WeatherMax®, Transorb®

3.3. INTRODUCTION

In contemporary farming, the removal of problematic weeds is critical to minimizing crop loss caused by resource competition and reduce contamination during harvest. In contrast to manual and mechanical weeding, the use of herbicides offers a highly cost effective and resource-efficient method of obtaining control over opportunistic vegetation. Some herbicidal agents introduced since the mid 1900s widely recognized for their performance include 2,4-dichlorophenoxyacetic acid (2,4-D), atrazine, pendimethalin, and dicamba (Gianessi, 2013). While highly effective, these compounds have been associated with a wide array of unfavourable characteristics. For example, atrazine contaminates ground water sources due to low soil binding affinity, and dicamba is considered carcinogenic to mammals and toxic to aquatic life (Gianessi, 2013). With an array of non-target effects observed in first generation herbicides, the market desire for an herbicidal agent with an improved toxicological profile was substantial. It would not be until 1970 that the herbicidal activity of glyphosate was characterized by John E. Franz, despite Henri Martin discovering the molecule in 1950. Chemically, glyphosate [N-(phosphonomethyl) glycine] is an organophosphate with the chemical formula $C_3H_8NO_5P$, a molecular weight of 169.073 $g \cdot mol^{-1}$. The compound is synthesized through the oxidative coupling of methylphosphonic acid and a glycine residue (Figure 3.1.).

By 1971, the Monsanto corporation patented glyphosate (U.S.A. Pat.No. 3,799,758) and marketed the compound in various formulations under the trade name RoundUp®. Shortly after RoundUp® products were released to market, glyphosate quickly became the leading herbicide applied by volume in the world, increasing from 1.4 million pounds in 1974 to 40 million pounds in 1995 (Richmond, 2018). Much of

the success of glyphosate is owed to its broad-spectrum herbicidal activity, rapid absorption through leaf tissues, and relatively high soil binding affinity (Kirkwood et al. 2000; Sharma and Singh, 2001; Reddy et al. 2004; Tong et al. 2017). Collectively, these attributes lower the required frequency of application and reduce the number of different products necessary to achieve adequate vegetation control. High binding affinity for soil also limits leachability of the active ingredient into groundwater and nearby aquatic ecosystems. In commercial formulations, additives for storage stability and adjuvants including surfactants, anti-foaming compounds, and buffering agents work in concert to enhance the activity of glyphosate by improving dispersal and persistence on, and permeability through, plant tissues. Once absorbed into a plant, glyphosate rapidly translocates to the roots, developing reproductive organs, and meristematic tissues which further heightens its potency (Pline et al. 2001).

The mechanism of action (MoA) of glyphosate is based in its ability to disrupt 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS is a monomeric enzyme belonging to the transferase family and is responsible for executing a critical step in the shikimate pathway. The shikimate pathway is the primary means for aromatic amino acid (AAA) production (tryptophan [Tyr]; tyrosine [Tyr]; and phenylalanine [Phe]) in plants, fungi, and bacteria, and acts as a biosynthetic shunt linking primary and secondary metabolism. There are currently two known isozymes of EPSPS: EPSPS class I, and EPSPS class II. Both forms catalyze the addition of phosphoenolpyruvate (PEP) to shikimate-3-phosphate and release 5-enolpyruvylshikimate-3-phosphate (EPSP) as the terminal product. Where EPSPS I (EPSPS: EC 2.5.1.19) is highly sensitive to glyphosate which inhibits binding of PEP, EPSPS II is not susceptible. Interestingly, while most plants, fungi, and gram-negative bacteria share EPSPS I, the

resistant CP4 EPSP synthase isozyme was first isolated from a unique strain of *Agrobacterium* found in a wastewater line at a glyphosate manufacturing site (Funke et al. 2006). Through the inhibition of the glyphosate-sensitive EPSPS I and subsequent inactivation of the shikimate pathway, AAA biosynthesis is impaired. In plants, the AAA are essential for the formation of structural components of the cell and participate in enzyme activation as they often facilitate protein folding. The AAA also serve as precursors to secondary metabolites (flavonoids, stilbenes, phenylpropanoids, alkaloids), and several important phytohormones (salicylate, auxin) (Steinrticken and Amrhein, 1980; Duke and Powles, 2008). AAA biosynthesis in bacteria fulfill similar structural and non-protein roles, including the development of antibiotic and antimycotic compounds (Suhadolnik and Chenoweth, 1958; Pojer et al. 2002; Bugg et al. 2006).

Lacking shikimate machinery should leave humans and wildlife generally unaffected by glyphosate. Despite a plethora of independent studies examining the toxic potential of glyphosate in a variety of models including rats (Dallegrave et al. 2003; Benedetti et al. 2004; Beuret et al. 2005; Hietanen et al. 1983), zebra fish (Schweizer et al. 2019; Lanzarin et al. 2021; Zhang et al. 2021; Liu et al. 2022), and fruit fly (Galin et al. 2019; Bednářová et al. 2020; Muller et al. 2021), investigation of the effects of glyphosate and GBH's on microorganisms is comparatively low, and this has remained especially so with respect to plant-associated bacteria. To date, investigations regarding the effects of glyphosate on the phyllosphere are mixed and focus greatly on rhizosphere bacteria over those in the shoot system (Busse et al. 2000; Kepler et al. 2020). This has likely been driven by research concerning the chemical behaviour of glyphosate in the soil in an effort to establish key physiochemical properties such as persistence,

bioaccumulation, migration, and leaching potential. However, this has resulted in little actually being known about how glyphosate may affect important microbial members of the phyllosphere.

Referring to the collection of microorganisms including bacteria, viruses, protozoa, archaea, and algae that inhabit plants, the plant microbiome plays crucial roles in the health and development of their host-plant. Similar to the human microbiome, microorganisms may establish a relationship with the host that varies on the scale between mutualistic to parasitic. To date, investigations regarding the effects of glyphosate on the plant microbiome are mixed and focus largely on the rhizosphere, leaving little understanding of the effects of glyphosate on the aerial plant microbiome (Busse et al. 2000; Haney et al. 2000; Irvine et al. 2013; Newman et al. 2016; Kepler et al. 2020).

The *Methylobacterium* is a genus of bacteria which often comprise a large part of the natural microbiota that inhabit plants (Korpe and Rheem, 1989) and are so ubiquitous in nature that they have also been isolated from a wide array of sources including soil, air, water, humans, food, and spacecraft (Hiraishi et al. 1995; Anesti et al. 2004;Aslam et al. 2007; Bijlani et al. 2021). In addition to theories suggesting that microbial colonization is motivated by methanol emissions produced from cell wall remodelling (Nemecek-Marshall et al. 1995; Fall and Benson, 1996; Abanda-Nkpwatt, 2006; Šmejkalová et al. 2010), *Methylobacterium* spp. also actively play important roles in plant growth promotion. Several strains have been characterized as plant growth-promoting bacteria (PGPB) based on their ability to synthesize high quantities of growth-enhancing phytohormones including cytokinins (CKs) (Ivanova et al. 2000;

Ivanova et al. 2001; Jorge et al. 2019; Palberg et al. 2022), while others uniquely facilitate nitrogen-fixation as is the case with *M. nodulans* (Sy et al. 2001; Renier et al. 2008). Significant tolerance to chlorine exposure (Hiraishi et al. 1995), unfavourable salinity, pH, and temperature (Ivanova et al. 2001) are also documented traits of several *Methylobacterium* species, along with their ability to utilize both common carbon sources like carbohydrates in addition to oxidizing several single-carbon molecules including methanol, methylamine, and formaldehyde (Anesti et al. 2004; Patt et al. 1976; Ito and Iizuka, 1971). *Methylobacterium* are aerobic, gram-negative, facultative methylotrophs that use single-carbon compounds to grow, although several species have adaptations that allow the use of C2 and C3 compounds as well (Šmejkalová et al. 2010). A distinct pink pigmentation is a frequently recognizable characteristic of *Methylobacterium*, however some exceptions to this have been established (*Methylobacterium jeotgali*) (Aslam et al. 2007). The presence of carotenoids have been suggested to cause the pink pigmentation which may in fact confer the ultraviolet (UV) and gamma radiation tolerance observed in earlier studies (Corpe and Rheem, 1989; Ito and Iizuka, 1971; Jacobs et al. 2005; Gourion et al. 2008; Gholizadeh, 2012; Yoshida et al. 2017; Kamo et al. 2018). Morphologically, *Methylobacterium* are rod-shaped, and exhibit polar growth.

While *Methylobacterium* spp. have been studied previously for suitability in a wide range of biotechnologies including bioremediation of environmental toxins (Zhang et al. 2008; Ventorino et al. 2014) and explosives (Van Aken et al. 2004), the activity of *Methylobacterium* within the plant microbiome, and subsequent influence on plant health has catalyzed interest for its agronomic potential (Dourado et al. 2015; Ryan et al. 2008; Zhang et al. 2021). Apart from improving the growth and yield of several

crop types, select strains of *Methylobacterium* have also been determined to increase host-resilience against abiotic stressors including high temperatures and severe drought (Aslam et al. 2007; Jorge et al. 2019; Balachandar et al. 2008; Holland, 1997; Madhaiyan et al. 2006; Esitken et al. 2010; Meena et al. 2012; Lee et al. 2015; Tani et al. 2015; Lipka and Panstruga, 2005). Based on the presence of the requisite cellular machinery, *Methylobacterium* spp. may also be capable of host-protection through use of enzyme classes like glycosidases, pectinases, and chitinase to mount direct counterattacks against pathogenic fungi (Dourado et al. 2015; Lipka and Panstruga, 2005; Pedrosa et al. 2011; Seo et al. 2012; Naznin et al. 2013). Studies examining resistance to UV (Kamo et al. 2018; Jacobs et al. 2005) and gamma radiation (Ito and Iizuka, 1971), have prompted theories which suggest that the distinct pink-pigmentation of the *Methylobacterium* may not only contribute to the colour of some plant organs but afford enhanced protection from ionization radiation as well (Gholizadeh, 2012).

However, in the development of sustainable biofertilizers and crop protection products, compatibility with existing application techniques, equipment, and contemporary agrichemicals remains an important consideration for performance and marketability. The popularity of herbicidal products containing glyphosate has risen steadily since the invention of glyphosate-resistant (GR) cultivars of popular cash crops. Today, GR crops available on the commercial market include soybean, corn, canola, cotton, grass seed, and alfalfa (Dill et al. 2008). Despite the availability of over 200 licensed varieties, over 60% of all soybeans planted each year belong to a GR cultivar (Dill et al. 2008). However, in the same way that glyphosate eradicates weeds by disabling AAA biosynthesis, it may also be capable of blocking this essential function in important members of the plant microbiome including *Methylobacterium* spp.

Moreover, little is known about the effects that *Methylobacterium* may have on the persistence, absorbability, and activity of glyphosate on target plants, should members across the genus tolerate or catabolize glyphosate.

Interestingly, in a 2010 patent filed by Monsanto Technology LLC (U.S.A. Pat.No. 7,771,736), glyphosate is rebranded as a highly capable agent for the prevention and therapy of infectious disease caused by microorganisms. As with studies involving animal models, the existing sphere of research concerning the sensitivity of bacteria to glyphosate has remained equivocal. To the knowledge of the authors, this study is the first comprehensive investigation focused on the sensitivity of *Methylobacterium* to commercial herbicide products containing glyphosate.

3.4. MATERIALS AND METHODS

Chemicals and materials

Two commercial formulations of glyphosate were used in this study, RoundUp WeatherMax® (Bayer Agrichemicals; 48.8% potassium salt of glyphosate composition [w/v], PCP Reg. No. 27,487, LOT #MYWF1108AJ) and RoundUp Transorb® (Bayer Agrichemicals; 48.8% potassium salt of glyphosate composition [w/v], PCP Reg. No. 28,198, LOT #MWBK0516AJ), in addition to HPLC-grade glyphosate as potassium salt (Fisher Scientific, CAS: 70901-12-1) sourced for use as a positive control. Due to the proprietary nature of the commercial formulations, exact ingredients including the surfactants used and their exact concentrations could not be determined. Internal standards (> 99% purity) of glyphosate, aminomethylphosphonic acid (AMPA), and sarcosine (N-methyl glycine) were sourced from Millipore Sigma.

The commercial formulations were selected based on their applicability to a wide range of crops and popularity in Canada. Analytical-grade formic acid (88%), and HPLC-grade acetonitrile (ACN), and methanol (MeOH) were obtained from Fisher Scientific. 50mL 0.2 μm conical filters used for filter sterilization of solutions were obtained from Fisher Scientific and ultra-pure water (18.2 $M\Omega^{-cm}$) was obtained from a Milli-Q system. Formaldehyde was sourced from Fisher Scientific as a 37% (w/w) stock solution, ammonium acetate reagent grade was obtained from Bio Basic Inc. at 98% purity, and anhydrous acetyl acetone (2,4-pentanedione) was sourced from Acros Organics at 99% purity. Sterile, blank paper disks (Oxoid, Fisher Scientific) of 6 mm diameter (< 1 mm thickness) were obtained to conduct a modified Kirby Bauer sensitivity assay. Polysorbate-20 (Tween20) was sourced from MiliporeSigma.

Selection of bacterial strains and culture conditions

Preparation of bacterial cultures were conducted aseptically in a biological safety cabinet (BSC). Freeze-dried cultures of *Methylobacterium* spp. were obtained from four microbe collections: the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) ["German Collection of Microorganisms and Cell Cultures"], the Japan Collection of Microorganisms (JCM), and the National Institute of Technology and Evaluation's (NITE) Biological Resource Center (NBRC). Strains were originally collected from different biological (living plants) and natural non-biological sources (soil, water, air). Information on strain taxonomy, origin, and known characteristics is provided in Table 1. The freeze-dried strains were revived in nutrient rich R2A broth (VWR, Mississauga, Canada) and cryogenically maintained as 15% (v/v) glycerol stocks at -80 °C. All nutrient-rich tryptic soy agar (TSA) plates were standardized to

1.5% (w/v) agar content, and 20 mL fill in sterile single-use petri dishes (Greiner bio-one, 94 × 16 mm).

Revival of cryogenic stocks

Methylobacterium strains were aseptically streaked on nutrient-rich tryptic soy agar (TSA) (Fisher Scientific) using a sterile loop. After 5 days of incubation at 27 ^{o}C, single colonies were extracted from each plate and used to inoculate 50 mL of TSB liquid growth media in 150 mL vented flasks (Fisher Scientific; 0.22 μm) and maintained in a rotary incubator for 5 days (27 ^{o}C and 110 RPM). When Methylobacterium cultures reached the late exponential/early stationary phase after approximately 6 days (OD600 = 0.6– 1.2, depending on strain, approximately 10^8 CFU / mL) (Genesys™ 10s Visible Spectrophotometer, Thermo Fisher Scientific) nutrient minimum agar plates were inoculated from the TSB liquid media using a sterile loop and streak method. Selective minimum nutrient medium was prepared in accordance with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) [“German Collection of Microorganisms and Cell Cultures”] recipe for Methylobacterium growth media (DSMZ Index 125): KNO_3—1.00 g; $MgSO_4$ × 7 H_2O—0.2 g; $CaCl_2$ × 2 H_2O—0.02 g; Na_2HPO_4—0.23 g; NaH_2PO4—0.07 g; $FeSO_4$ × 7 H_2O—1.00 mg; $CuSO_4$ × 5 H_2O – 5 μg; H_3BO_3—10 μg; $MnSO_4$ × 5 H_2O – 10 μg; $ZnSO_4$ × 7 H_2O—70 μg; MoO_3 – 10 μg; H_2O—1000 mL; CH_3OH—5 mL; pH 6.8). Transfer to a minimum nutrient medium ensures high selectivity for Methylobacterium and reduced potential for contamination during the initial transfer from cryogenically preserved stock solutions. After 5 days of incubation at 27 ^{o}C, single colonies of each bacterium were extracted from each plate and used to inoculate culture flasks, as necessary. Stock plates were sealed with parafilm and maintained at 4 ^{o}C.

Preparation of paper disks and optimization of loading volume

The Kirby Bauer sensitivity assay is a method for visually assessing sensitivity of microbial strains to specific compounds using impregnated paper disks and agar plates. In this study, sterile 6 mm paper disks (Oxoid, Fisher Scientific) were loaded with each herbicidal formulation and deposited on a TSB agar plate 5 days post inoculation. Prior to use on live cultures, optimization of the disk loading volume was required. This was accomplished by delivering 5 different volumes of water (5, 10, 20, 30, and 40 μL) to 5 paper disks in each volume cohort. After drying under ambient temperature in a closed petri dish (150 × 60 mm) for 60 min, the paper disks were examined for adherence to the surface of a tryptic soy agar (TSA) plate. It was determined that the optimal fluid loading volume was 20 μL.

Final preparations were filter sterilized (0.22 μm; Fisher Scientific) under vacuum in 50 mL fractions. The concentration of final solutions was achieved by serial dilution in 2 mL fractions using sterile Milli-Q water (18.2 MΩ-cm) as diluent. Final concentrations chosen were designed to approximate glyphosate exposure in field application conditions, based on the recommended application rate for soybean in Canada and assuming a successful stand rate of 135,000 plants per acre (OMAFRA). Glyphosate resistant (GR) soybean can normally tolerate elevated glyphosate concentrations of 1.35 L/acre (3.33 L/ha). Both acquired glyphosate preparations indicate 540 g of acid equivalent per litre of solution. Following the recommended 10-gallon (37.85 L) water dilution for field spray operations would bring the final tank concentration to 19.22 g of glyphosate per litre. Based on the application rate and stand density of soybean per acre, the maximum expected concentration of glyphosate to reach each plant would be 5.4 mg (0.28 mL/plant tank mix). Herbicide timing for

soybean is 24–30 days after planting, approximately at the third vegetative stage of growth (V3). Due to the relatively low amount of foliage at the time of herbicide application, a conservative estimate of 50% canopy coverage was used. This assessment further reduces the estimated glyphosate concentration to 2.7 mg per plant, and approximately 40–340 µg of glyphosate per leaf, assuming successful development of the unifoliate leaves, and the first set of trifoliate leaves.

A similar quantitative field assessment performed by Harvey and Crothers (1988) found the deposition of glyphosate spray on flax (*Linum usitatissimum*) is largely dependent on the density of planting in the field, as well as equipment settings. Depositions were found to range between 400 and 600 µg per plant, with a minimum exposure of approximately 40 µg per plant required to cause desiccation of the target (Harvey and Crothers, 1988). Sterile Milli-Q water was used to load a control disk.

Preparation of paper disks, including serial dilution of glyphosate products, and loading of each disk across all 5 treatment cohorts were carried out aseptically. Loading solutions were prepared by serial dilution and 20 µL of each solution was transferred to paper disks using a filtered pipette, achieving four different concentrations of glyphosate: 380, 190, 95, and 48 µg. Each petri dish was covered and allowed to dry for 1 h.

Modified Kirby Bauersensitivity assay

Since use of a nutrient-minimum agar (DSMZ 125) reduced the density and uniformity of *Methylobacterium,* a nutrient-rich TSA was used for Kirby Bauer sensitivity assays. Petri dishes containing uniform volumes of 20 mL TSA were divided

into quadrants using permanent marker on the outer surface of the base. 100 µL of a 5-day old (~10^8 CFU/mL) liquid culture (DSMZ-125) was deposited onto the surface of each separate test plate. The inoculum was evenly spread across the surface of the agar using an L-shaped spreader, then covered and allowed to dry for 1 hour in a BSC in darkness at approximately 20 °C. Using sterile forceps, dry paper disks loaded with each agrichemical (prepared earlier) were transferred to the test plates, in the center of the quadrant corresponding to the respective disk concentration. A paper disk loaded with sterile water was placed at the center of each petri dish, at the intersection of quadrant lines as a control. Plates were sealed with parafilm and stored inverted. The zone of inhibition surrounding each disk was assessed after 7 days of incubation at 27 °C in darkness. Zone of inhibition was determined by measuring the diameter of the region with observable absence of microbial growth, inclusive of the paper disk diameter.

Assessment of Methylobacterium sensitivity to glyphosate

Sensitivity of Methylobacterium spp. to the commercial formulations (WeatherMax® and Transorb®) was evaluated at two concentrations of the active ingredient (AI) glyphosate: 0.05% (0.5 mg/mL), and 0.1% (1.0 mg/mL) in 6-well plates (Fisherbrand Cat.No. FB012927); 2 wells contained the WeatherMax® product (0.05% and 0.1% glyphosate, respectively), 2 wells contained the Transorb® product (0.05% and 0.1% glyphosate, respectively), and the final 2 wells were used as a TSB growth media control. The maximum fill volume for each well across each multi-well plate was set at 5mL to prevent overflow between wells.

To each well, 250 µL of a 5-day old liquid culture (DSMZ-125) was introduced (~ 108 CFU/mL) and the nutrient content of wells containing TSB only, were

normalized by adding sterile water (Milli-Q, 18.2 MΩ-cm). After inoculation, parafilm was used along the perimeter of each multi-well plate to seal the lid to the base and prevent evaporation and contamination. Plates were then placed on a shaker table (Thermo Fisher MaxQ) and rotated at 80 RPM and 27 ºC for 6 days. Following the incubation period, 100 µL of culture was withdrawn from each well and transferred to 6 corresponding microfuge vials (2 mL) containing 900 µL of sterile isotonic saline solution (0.9% NaCl). The 1:10 mixes were resuspended using a sterile pipette, and then 50 µL was transferred to a TSA plate (20 mL). A sterile L-shaped spreader was used to disperse inoculant across the agar surface, and each plate was sealed using parafilm and incubated at 27 ºC for 7 days inverted. Afterwards, plates were evaluated for colony growth and scored according to a custom scale based on the presentation of colony forming units (CFU): (–) no CFU, (+) if < 30 CFU, (+ +) if > 30 CFU or partial lawn, and (+ + +) if complete lawn present and CFU count impossible.

To assess culture viability following exposure to pure forms of glyphosate, an identical set of replicates were carried out, also in 6-well plates (Fisherbrand Cat.No. FB012927); 2 wells contained pure glyphosate only (0.05% and 0.1%, respectively), 2 wells contained glyphosate at 0.05% and 0.1% with Tween20 (polysorbate-20) at 2% (v/v) in each, 1 well contained Tween20 (2% v/v), and the final well contained only TSB growth media (with nutrient content normalized using sterile water).

Determination of influence of membrane permeability on cytotoxicity

Determination of the impact of membrane permeability on growth rate was carried out using 3 strains of *Methylobacterium* (*M. organophilum* [NBRC 103119], *M.gnaphali* [NBRC 107716], and *M. jeotgali* [LMG 23639]). The species were selected

based on differences in (a) their source of isolation, (b) morphology and pigmentation, (c) rate of proliferation, and (d) strain-specific outcome of the modified Kirby Bauer sensitivity assay using commercial GBH's. *Methylobacterium* strains were cultured in 18 mL of TSB media in 50 mL conical tubes (FroggaBio; Cat.No. TL50-500B) after inoculation with 1 mL of a 5-day old 50 mL culture (~10^8 CFU/mL) grown previously in a 250 mL Erlenmeyer flask (TSB media). The volume in each conical tube was made up to 20 mL through the addition of 1 mL of various stock solutions to achieve the following final conditions: (a) WeatherMax® at 0.1% glyphosate, (b) Transorb® at 0.1% glyphosate, (c) pure glyphosate at 0.1%, and (d) pure glyphosate at 0.1% with Tween20 at 2% (v/v). Conical tubes were sealed with parafilm, stored horizontally on a tilt table (VWR Rocking Platform; Model 100), and incubated at 27 ^{o}C for 7 days in darkness. Due to the well-characterized propensity for several species of the *Methylobacterium* to form aggregates in solution after excessively long growth periods or when exposed to stress conditions (Omer et al. 2004), determination of cell density through optical density (OD) or direct cell counting with a haemocytometer would become unreliable. Therefore, microbial growth rate under each condition was assessed by measurement of pellet dry weight.

After 7 days incubation, each conical tube was subjected to centrifugation at 4,700 RCF (Thermo Scientific, Sorvall ST16) for 20 min. The supernatant of each tube was removed and discarded. The remaining pellet was then transferred to pre-weighed 2 mL microfuge tubes using isotonic solution (0.9% NaCl) and centrifuged for 10 min at 11,180 RCF. The supernatant in each tube was removed and the pellet was freeze dried for 24 h (Labconco Model: 7753020 at -56 ^{o}C and 0.28 mBar). Each tube was subsequently reweighed (Sartorius Practum® 224) to determine the dry weight of each

pellet. To further investigate the influence of Tween20 (polysorbate-20) on the sensitivity of Methylobacterium to glyphosate, the aforementioned experiment was carried out again in triplicate, with expanded treatment conditions which included a range of different surfactant dosages with a fixed concentration of pure glyphosate: (a) 0.5-4% (v/v) Tween20 in TSB, and (b) 0.5-4% (v/v) Tween20 with glyphosate 0.1% (w/v) in TSB. Student's t-test was used to assess differences in dry pellet weight under each growth condition.

Extraction of intracellular glyphosate and secondary metabolites after RoundUp® exposure

Methylobacterium strains (*M. organophilum* [NBRC 103119], *M. gnaphali* [NBRC 107716], and *M. jeotgali* [LMG 23639]) were cultured in 18 mL of TSB media inside 50 mL conical tubes (FroggaBio). Each tube was inoculated with 1 mL of a 5-day old 50 mL culture (~ 10^8 CFU/mL) grown previously in a 250 mL Erlenmeyer flask (TSB media). Conical tubes were sealed with parafilm, stored horizontally on a tilt table (VWR Rocking Platform), and incubated at 27 ^oC for 4 days in darkness and constant agitation. On the fourth day (96 h elapsed), the liquid volume of the control cohort for each strain was brought up to 20 mL using sterile water (Ultrapure Milli-Q: Merck Millipore, Toronto, Canada), while the treatment group received a dose of the *Transorb*® formulation (filter sterilized, 0.22 µm PVDF) from a pre-diluted stock solution to achieve two separate final glyphosate concentrations: (a) 0.05 mg/mL (0.005% w/v), and (b) 0.5 mg/mL (0.05% w/v).

After 6 days, each tube was centrifuged at 4,700 RCF for 25 min (Thermo Fisher Scientific; Sorvall ST16). Following centrifugation, the supernatant was discarded, and the pellet of each tube was resuspended with 5mL of isotonic 0.9% NaCl solution to

remove remnants of the TSB media containing glyphosate, then centrifuged again at 4,700 RCF for an additional 15 min. A repeat of the saline solution wash was conducted. After removal of the second saline wash, the pellet in each conical tube was transferred to 2 mL microfuge tubes using a small amount of fresh saline solution, centrifuged at 11,180 RCF for 15 min. The supernatant was transferred to a clean microfuge vial and the remaining pellet was flash frozen using liquid nitrogen (LN_2) and subjected to drying under high vacuum for 24 h (Labcono Model: 7753020 at -56ºC and 0.28 mBar).

The method of sample preparation was adopted as presented by Li and Kannan (2022) (Li and Kannan, 2022) with modifications made for available instrumentation and materials. Briefly, each lyophilized sample was reconstituted with 1.5 mL water:ACN mixture (95:5 *v/v*) containing 0.1% formic acid, and vortexed for 5 min. To tubes containing cell pellets, 2 zirconium oxide grinding beads (5 mm) were added, and vortexed for an additional 5 min 25 MHz using a ball mill (Retsch Mixer Mill MM 400). Cell fragments and particulate matter were settled through centrifugation at 10,000RPM for 10 min. Then 250 µL of supernatant from each tube were loaded onto an Oasis MCX (6 mL carrier, Mississauga, Ontario, Canada) cartridge that had already been preconditioned with 2 mL fractions of methanol and water. Eluent produced from the column were collected immediately as each sample was added to each respective column as only cationic contamination would be absorbed by the MCX cartridge and the target analyte, glyphosate, would flow through unimpeded. The cartridge was washed with an additional 2 mL fraction of water and added to the initial eluent volume. Each collection tube was vortexed for 2 min then 1.5 mL was transferred to a glass vial.

Liquid chromatography and high-resolution tandem mass spectrometry (LC-HRMS/MS)

Identification of glyphosate and target primary metabolites was performed using high performance liquid chromatography-electrospray ionization high-resolution mass spectrometry (HPLC-[ESI]-HRMS). Full scan data was acquired using a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose CA USA) coupled with a Dionex UltiMate 3000 HPLC (Thermo Fisher Scientific, San Jose CA USA). A 25 µL of each sample was injected onto a Thermo Scientific Acclaim™ 2.2 µm C18 column (150 mm × 3.0 mm; Canadian Life Science, Peterborough, Canada) using a flow rate of 0.35 mL min^{-1} with a mobile phase of ultra-pure water (Milli-Q) with 0.1% formic acid (A) and HPLC grade acetonitrile (Fisher Scientific, Ottawa, Canada) with 0.1% formic acid (B). Mobile phase B was held at 0% for 1.5 min, before increasing to 100% over 4 min. Solvent B was then held at 100% for 3 min before returning to 0% over 2 min, for column re-equilibration. The following conditions were used for heated electrospray ionization (HESI) probe: capillary temperature, 350 °C; sheath gas, 30 arbitrary units; auxiliary gas, 8 arbitrary units; probe heater temperature, 450 °C; S-Lens RF level, 60%; and capillary voltage, 3.9 kV. For HPLC-HRMS, each sample was analyzed in negative mode focusing on a mass range of *m/z* 50 − 700, and data were acquired at 35,000 resolution, with an automatic gain control (AGC) target of 2×10^6, and a maximum injection time (IT) of 540 ms.

For identification, the product ion spectrum of each target compound was obtained using parallel reaction monitoring (PRM). PRM parameters included: automatic gain control (AGC), 2×10^5; maximum injection time (IT), 100 ms; *m/z* 4.0 isolation window, normalized collision energy (NCE) of 30.0 eV, and a focused mass

range of *m/z* 50 − 200. All data was analyzed using Thermo Xcalibur (v 3.0.63) software (Thermo Scientific, San Jose, CA, USA), to integrate peak areas.

Assessment of intracellular formaldehyde

Assessment of intracellular formaldehyde concentration was carried out to determine whether the presence of glyphosate from commercial products would contribute to elevated formaldehyde loads. To examine this, 3 individual strains were cultured in 18 mL of TSB media contained within 50 mL conical tubes (FroggaBio). Each tube was inoculated with 1 mL of a 5-day old 50 mL culture (~ 10^8 CFU/mL) contained in a 250 mL Erlenmeyer flask, also in TSB media. A total of 4 conical flasks were inoculated per strain and incubated for 4 days at 27 ºC under constant agitation (VWR Rocking Platform; Model 100). On the fourth day, one of the conical tubes received 1 mL of sterile water (BPure,18 MΩ-cm), while the remaining tubes received a 1 mL aliquot from a series of prepared stocks of the *Transorb*® formulation (filter sterilized, 0.22 µm PVDF) to achieve three final glyphosate concentrations: 50, 250, and 500 µg/mL. After 48-hours, all the conical tubes were centrifuged at 4,700 RCF for 20 min. Following centrifugation, the supernatant was discarded, and the pellet of each tube was resuspended in 5 mL of an isotonic solution (0.9% NaCl). After vortex mixing for 1 min, the resuspended cells were subjected to centrifugation at 4,700 RCF for 10 min. The supernatant was again discarded, and the pellet was transferred to a 2 mL microfuge tube using 1.5 mL of fresh isotonic saline solution. Each microfuge tube was subsequently subjected to centrifugation at 10,000 RCF for 5 min. The supernatant was discarded, and a fresh 1.5 mL fraction of sterile water (BPure, 18 MΩ-cm, non-isotonic) was added to each tube. Two zirconium oxide grinding beads (5 mm) were added to each tube, and mechanical lysis was achieved through grinding for 10 min using a ball

mill (Retsch Mixer Mill MM 400) at high speed (25 MHz). Cell fragments and particulate matter were settled through centrifugation at 10,000 RCF for 10 min and the supernatant was collected in a clean glass test tube. The pellet was resuspended in an additional 1 mL of sterile water and subjected to a second iteration of mechanical disruption for 5 min. This process was repeated until the total volume transferred to the glass tube was 4.5 mL.

The presence of formaldehyde in spent media and cell pellet extract was determined by dispersive liquid-liquid microextraction ultraviolet visible light spectroscopy (DLLME-UV-Vis) according to Nassiri et al. with minor alterations made to accommodate for reduced sample volume (Nassiri et al. 2018). Briefly, acetyl acetone (2,4-pentanedione) and ammonium acetate were added to each solution type to reach final concentrations of 0.2 mol/L. Glass tubes were inverted to facilitate mixing, and subsequently placed into a hot water bath at 70 ^{o}C for 12 min. After incubation, tubes were allowed to stabilize at room temperature, undisturbed for 15 min. Then 500 µL of anhydrous ethanol and 300 µL of HPLC grade chloroform was added to each glass tube. Each tube was agitated violently to produce a turbid solution as the immiscible aqueous and organic fractions suspended. Separation was subsequently facilitated by moderate centrifugation at 3,000 RCF for 5 min (Thermo Fisher Scientific; Sorvall ST16). Then, 300 µL of the organic fraction was removed and dispensed into a quartz microcell with 300 µL of chloroform diluent, and absorbance was measured at 412 nm promptly.

Evaluation of the DLLME-UV-Vis method showed good linearity in a 5-point calibration curve between 1 and 500 µg/L of formaldehyde spiked in ultrapure water

(BPure). All results were normalized against media blank to account for trace formaldehyde in domestic water supply.

Confirmation of Potency

To prevent contamination of cell cultures, stock solutions containing glyphosate were filtered using 0.2 µm PVDF vacuum-assisted filtration system. While the filter pore size was not anticipated to interfere with the concentration of glyphosate present in final solutions, retention of glyphosate due to interaction with the filter material was unknown and previously untested. At low concentrations of glyphosate (< 1% v/v), filter binding could potentially cause loss of the herbicidal agent.

Twelve soybean plants (*Glycine max*) [non-GMO, Canada domestic white hilum variety] were cultivated from seed and allowed to mature to approximately the unifoliate stage (V_C). Plants were then transplanted into four 10” pots containing approximately 2 L of soil (Miracle-Gro™ potting mix, 0.21-0.11-0.16) and positioned approximately 2” apart. Plants were allowed to mature to the second trifoliate stage (V_2). Solutions containing 0.1% glyphosate (1 mg/mL) were then filter sterilized (0.2 µm, PVDF) and placed into 20 mL cosmetic spray bottles and dyed with non-toxic blue food colouring so that leaf saturation would be evident, and drift of any particles that did not contact the plant surfaces would be observable on contrasting surfaces.

3.5. RESULTS

Sensitivity of Methylobacterium to commercial GBH's

In nearly all cases, sensitivity to both the Weather-Max® and Transorb® formulations were observed in the Kirby Bauer assay when Methylobacterium strains were exposed to disks containing final doses of 95 µg glyphosate or higher. In this work, sensitivity to commercial formulations is defined as the formation of any zone of inhibition greater than the diameter of the paper disks (6 mm), subdivided into three levels; low (1 < > 9 mm), medium (10 < > 19 mm), and high (> 20 mm). Nearly always, a dose-dependent relationship was observed in relation to the zone of inhibition surrounding each paper disk. M. radiotolerans (LMG 6379) showed the highest degree of sensitivity to the WeatherMax® formulation, even at the lowest dose of 48 µg, and complete clearance of the quadrant at the maximum dose of 380 µg (Figure 3.2.). Trends between the magnitude of sensitivity and the isolation source of *Methylobacterium* or library of origin could not be established. Notably however, several strains were observed to have tolerance (< 9 mm inhibition zone) to both the WeatherMax® and Transorb® formulations even at the highest dose administered (Figure 3.2.); notably, *M. extorquens* (NBRC 103129), *M. organophilum* (NBRC 103119, NBRC 103121), *M. thiocyanatum* (NBRC 103128, JCM 10893), and *M. zatmanii* (LMG 6087). Interestingly however, several strains appeared to have increased sensitivity (> 5 mm inhibition zone) to the Transorb® formulation compared to identical experiments involving the WeatherMax® solution (NBRC 103119, LMG 23639), while others had greater sensitivity to the latter (DSM 23935, JCM 2806). As each formulation contains a proprietary blend of surfactants, the type and total surfactant content of either product cannot be used as a basis for the observed sensitivity. Replication of the modified Kirby Bauer method was conducted using pure formulations of glyphosate in the range of 48–

380 µg. Across all 18 strains of *Methylobacterium*, no measurable zone of inhibition was detected and, in several instances, *Methylobacterium* colonized glyphosate-impregnated disks. This apparent insensitivity to glyphosate in its pure form was consistent across all 3 replicates of the experiment. Sterile control disks, loaded with deionized water and included at the center of each test plate showed no indication of sensitivity in any strain, as expected (Figure 3.3.).

Assessment of permeability on cell viability

In experiments involving 6-well plates which used the WeatherMax® and Transorb® final formulations as the source of glyphosate (0.5 and 1.0 mg/mL glyphosate), culture growth was absent even after incubation for more than 7 days, and a measurable pellet could not be obtained. To discern whether the GBH formulations were lethal or bacteriostatic towards *Methylobacterium*, aliquots of 500 µL from each treatment were transferred to separate flasks of fresh media (50 mL TSB in 250 mL Erlenmeyer) and incubated for a further 7 days at 27 ^{o}C. In each case where inoculum had previously been exposed to commercial GBH's, growth in fresh media was not detectible. This phenomenon was further illustrated during viability tests where strains were unable to recover activity after transfer of a 50 µL aliquot from wells spiked with GBH's to GBH-free TSA (Table 3.2.). Direct exposure of Escherichia coli to the same concentrations of both the Transorb® and WeatherMax® GBH's, did not impact culture recovery and proliferation of the microorganism, producing a confluent GBH-free TSB plate each time. Interestingly, wells containing pure glyphosate at the same concentration (0.5 and 1.0 mg/mL) did produce viable cells that proliferated normally while in media spiked with glyphosate, and when transferred to glyphosate-free growth media (Table 3.3. and Figure 3.4.).

However, in the second iteration of the experiment which introduced custom mixes of pure glyphosate and the non-toxic surfactant Tween20 (polysorbate-20), deleterious effects to growth rate and viability were observed (Table 3.3.). Specifically, the lowest concentration of Tween20 required to induce negative impacts on growth was 0.5% (v/v). Importantly, the negative effects to *Methylobacterium* viability appeared to be a function of the surfactant concentration. In experiments involving uniform concentrations of pure glyphosate but varying surfactant content, the *Methylobacterium* strains tested, exhibited slower growth and a marked reduction in the number of viable colonies on recovery plates after 7 days in response to increasing surfactant (Tween20) concentrations (Figure 3.4.).

In culture growth experiments with three distinct *Methylobacterium* strains, conical tubes containing nutrient rich TSB media spiked with 0.1% pure glyphosate (1.0 mg/mL) resulted in statistical decreases in pellet weight only seen in *M. jeotgali* (LMG 23639). However, when the surfactant Tween20 is added to the growth media in combination with glyphosate, final pellet weights after 6 days were markedly reduced relative to controls despite receiving uniform inoculant (1 mL of 10^8 CFU/mL). While the pellet weight of *M. organophilum* (NBRC 103119) was significantly lower in the presence of Tween20 at 4% compared to TSB alone (7.70 ± 0.14 mg and 10.50 ± 0.07 mg, respectively), concomitant exposure of *M. organophilum* to glyphosate (1.0 mg/mL) and Tween20 also at 4%, caused significant decrease in pellet weight, especially when compared to glyphosate alone (1.83 ± 0.32 mg and 10.30 ± 0.13 mg, respectively, $p \cong 0.0002$) (Figure 3.5.). Over a 60% reduction in biomass was also observed in cultures of *M. gnaphali* (NBRC 17716) and *M. jeotgali* (LMG 23639) when exposed to glyphosate and a surfactant compared to glyphosate alone (Figure 3.5.). In

fact, statistical decreases in pellet weight were observed across all three strains of *Methylobacterium* grown in media with glyphosate and Tween20 at 4% when compared to TSB only and exclusively Tween20 at 4% (Figure 3.5.). Importantly, conical tubes spiked with either the WeatherMax® or Transorb® formulations at identical glyphosate concentrations to pure solutions tested (1.0 mg/mL and 0.5 mg/mL glyphosate) failed to produce any measurable biomass in any of the tested strains, in triplicate. Every attempt to inoculate media with *Methylobacterium* which already contained the WeatherMax® or Transorb® formulations failed to produce viable cultures, even when incubated beyond 12 days.

Assessment of intracellular formaldehyde

In experiments evaluating the intracellular concentration of formaldehyde, three morphologically distinct strains of *Methylobacterium* were included: *M. organophilum* (NBRC 103119), *M. gnaphali* (NBRC 107716), and *M. jeotgali* (LMG 23639). In all cases, increasing concentrations of glyphosate from the Transorb® formulation exposed to growing cultures, resulted in greater quantities of intracellular formaldehyde detectable by the DLLME-UV-Vis method, relative to controls. Because the spectrophotometric method is destructive, pre-exposure formaldehyde levels could only be obtained from a separate time-zero cohort; after 4 days of growth in TSB, intracellular formaldehyde concentrations for *M. organophilum* (NBRC 103119), *M. gnaphali* (NBRC 107716), and *M. jeotgali* (LMG 23639) were 10.26 ± 3.08 nM/mg (3.7 μg/L), 20.09 ± 4.81 nM/mg (7.2 μg/L), and 20.72 ± 5.00 nM/mg (7.5 μg/L), respectively (Figure 3.6.). The control cohort, which after 4 days of undisturbed growth were spiked with 1 mL of sterile isotonic solution (0.9% NaCl [w/v]) and were then harvested 2 days later, showed moderate increases to intracellular formaldehyde

content; 22.83 ± 1.00 nM/mg (8.2 µg/L), 36.94 ± 6.86 nM/mg (13.3 µg/L), and 37.85 ± 2.78 nM/mg (13.7 µg/L). Interestingly though, with the addition of the Transorb® product (glyphosate at 50 µg/mL) on the fourth day of growth, the average intracellular formaldehyde content of *M. organophilum* decreases approximately 27% (16.54 ± 5.35 nM/mg), while *M. gnaphali* and *M. jeotgali* experience an average increase of 91% (70.58 ± 4.81 nM/mg) and 20% (45.49 ± 1.35 nM/mg) relative to their control cohort, respectively. At 250 µg/mL of glyphosate in the growth media, average intracellular formaldehyde content peaks for *M. gnaphali* at 72.79 ± 2.55 nM/mg, then declines to 45.37 ± 0.47 nM/mg at the maximum exogenous glyphosate application (500 µg/mL). A similar trend is also observable for *M. jeotgali*, where exposure to the two higher glyphosate concentrations resulted in a decrease of average intracellular formaldehyde (34.46 ± 3.14 nM/mg and 28.60 ± 3.47 nM/mg). Only *M. organophilum* exhibited disproportional increases in average intracellular formaldehyde content with high concentrations of exogenous glyphosate (29.15 ± 4.46 nM/mg and 64.50 ± 4.42 nM/mg), suggesting formaldehyde tolerance may have a species-dependant component.

Measurement of intracellular glyphosate and secondary metabolites

Initially, severe matrix effects interfered with the reliable detection of glyphosate and suspected primary metabolites (AMPA and sarcosine) extracted from cell pellets of *Methylobacterium* exposed to Transorb® in the growth media and analyzed using HPLC-[ESI]-HRMS/MS. However, use of mixed-mode strong cation-exchange cartridges (Oasis MCX) as a cleanup method prior to analysis, enabled clear detection of all analytes. Specifically, the peak area of glyphosate detected in cell pellets was approximately 8-fold greater when MCX cartridges were used for the removal of cationic interference in the cell lysate when compared to the use of unprocessed extracts

(data not shown). Using the targeted parallel reaction monitoring (PRM) method, fragment ions of the target analyte, glyphosate, and two potential primary metabolites produced by different degradation pathways were used to quantify each compound (Tables 3.4. and 3.5.).

For example, degradation of glyphosate through an oxidoreductase pathway would be expected to yield aminomethylphosphonic acid (AMPA), while degradation via phosphatase would be expected to produce sarcosine as an intermediate metabolite (Figure 3.7.). Despite detection and confirmation of glyphosate and AMPA by comparison of fragmentation patterns against those produced by authentic standards, the well-characterized alternative glyphosate metabolite, sarcosine (N-methyl glycine), could not be detected in any strain, irrespective of Transorb® concentration. Interestingly though, method controls of the sarcosine internal standard (STD) were abundantly low compared to glyphosate and AMPA standards and were particularly masked by matrix effects (NL: 1.0E3) in QA/QC controls. Limit of detection (LOD) for sarcosine (50.0 pMol/L) standards were notably higher than the LOD for authentic standards of glyphosate and AMPA (11.9 and 17.8 pmol/L, respectively), which aligns with the findings of similar studies involving the detection of sarcosine. Although unconfirmed, it is also possible that the detectability of sarcosine may have been impacted by rapid oxidation to form glycine and formaldehyde, relegating the presence of sarcosine in the cytosol to be only transitory.

In each of the *Methylobacterium* strains analyzed, a near dose-dependent relationship between glyphosate exposure and the AMPA metabolite was observed. For example, in response to a tenfold increase to glyphosate applied extracellularly in the

form of the Transorb® commercial product, the average intracellular AMPA concentration in *M. organophilum* (NBRC 103119) rose by 11-fold and *M. gnaphali* by 8-fold (NBRC 107716). The full MS/MS spectra of glyphosate and one of the primary metabolites, AMPA, detected in a cell pellet of *M. gnaphali* (NBRC 107716) are shown in Figure 3.8.

These results confirm that exposure of *Methylobacterium* spp. to glyphosate in the Transorb® commercial formulation, do result in detectable levels of intracellular glyphosate and one of its known metabolites, AMPA. Cross-analysis of all purified standards did not result in detectable quantities of any other standards, eliminating the possibility of in-bottle degradation as a source of AMPA, and further confirming its presence in samples was as a result of metabolic activity of the *Methylobacterium* spp. tested.

3.6. DISCUSSION

Glyphosate cytotoxicity in Methylobacterium

Investigations of implications of the widespread use and reliance on glyphosate has existed for several decades and has involved contributions from a vast range of disciplines addressing ecological concerns, human health impacts, and the economics of the agri-food industry. However, a more thorough examination of the effects of glyphosate and GBH's on the phyllosphere have been largely absent in the relevant body of literature.

At present, several species of *Methylobacterium* have been reported to contain the glyphosate sensitive (GS) isoform of EPSP synthase (EC: 2.5.1.19) including M. aquaticum [MA-22 A], *M. phyllosphaerae* [CBMB-27], *M. radiotolerans* [JCM 2831], and *M. oryzae* [CBMB-20] (Tani et al. 2015; Marx et al. 2012; Kwak et al. 2014). While complete genomic sequencing of all known species of *Methylobacterium* has not yet been conducted, the reported presence of a sensitive EPSP synthase in several species is supportive of our findings of sensitivity. Uniquely however, our results suggest that the sensitivity of *Methylobacterium* to glyphosate may be a function of the quantity of glyphosate that is able to enter the cytosol. Across all growth experiments conducted, there was no statistical difference in growth rate or viability of *Methylobacterium* when exposed to pure forms of glyphosate. In experiments where permeability of the lipid bilayer was deliberately increased using Tween20 (polysorbate-20), cytotoxicity increased as a function of both the glyphosate dose and the content of surfactant in the growth media.

Commercial herbicide formulations are known to contain a wide range of additives including surfactants, which aid in product dispersibility and adherence to plant surfaces. GBH's specifically, often contain 1,4-dioxane as a preservative and polyoxyethyleneamine (POEA) as a surfactant. The toxicity of surfactants including POEA and its structural analogues has been investigated previously, with a majority of reports indicating that any observable toxicity in animals and tissue cultures exposed to GBH's are likely attributable to the surfactant itself (Bradberry et al. 2004; Lee and Guo, 2011). The use of Tween20 for the adjustment of membrane permeability has been documented extensively (Levy and Anello, 1969; Watson et al. 1980; Nakanishi et al. 1982; Dimitrijevic et al. 2010; Akhtar et al. 2011; Hua et al. 2018; Jiang et al. 2021). As a non-ionic surfactant, Tween20 effectively increases cell permeability without inducing the toxic effects exhibited with other surfactant classes. While the addition of Tween20 to cell cultures in the range of 0.1–10% (v/v) has previously been reported to cause disruptions to growth due to changes in membrane permeability and cell turgidity, our data indicates that *Methylobacterium* tolerate the presence and activity of Tween20 with moderate (< 30%) decreases in biomass relative to controls (Figure 3.5.).

Crucially, examination of the surfactant-only cohort, where growth rate was modestly decreased, revealed the cultures to remain viable beyond 10 days which would otherwise be impossible if the presence of Tween20 induced rupture of the lipid bilayer. Previously, Tween20 was examined in conjunction with glyphosate effectivity on several field-crops - including wheat, barley, oats, and rape - where the addition of Tween20 to tank mixes was found to enhance effectivity of glyphosate (O'sullivan and O'donovan, 1980). The results of the present work align with existing literature and indicate that the presence of surfactants enhances the effects of glyphosate likely as a

result of increasing membrane permeability and enabling greater cytosolic concentrations of glyphosate in bacterial cells.

As a gram negative bacteria, *Methylobacterium* have hardened defence systems that prevent biomolecules and synthetic chemicals from damaging critical cellular components. Most often, foreign molecules are repelled by the outer membrane (OM) – an asymmetric bilayer that is fully coated with lipopolysaccharides on its outer surface – without ever gaining entry to the cell (Zgurskaya et al. 2016). Should a foreign molecule pass through the OM by means of a porin protein, a second layer of protection within the periplasmic space (the fluid envelope existing between the outer and inner membranes) would likely activate to further protect the cell. In addition to efflux pumps – which work to move foreign matter back across the OM and into the extracellular matrix – the periplasmic space is known to contain a litany of defensive and detoxifying enzymes including phosphatases, proteases, and endonucleases in gram-negative bacteria (Nikaido, 1994; Miller and Salama, 2018; Zgurskaya and Rybenkov, 2020).

In the absence of a surfactant and because of the architecture of the cell wall shared amongst gram-negative bacteria, *Methylobacterium* may simply reject the molecule and process what small quantities of glyphosate pass through porin proteins in the periplasmic space. In all replicates of the modified Kirby Bauer assay, none of the tested strains showed signs of sensitivity to pure glyphosate; however, moderate sensitivity (10 > < 19 mm zone of inhibition) was clearly observable in tests involving commercial formulations containing glyphosate. Similarly, growth media spiked with pure glyphosate showed no statistical impact on growth rate or viability, whereas use of commercial formulations as the source of glyphosate produced non-viable cultures at

identical concentrations. In the presence of a control surfactant (Tween20) however, negative effects on *Methylobacterium* growth returned. We postulate that this effect is likely a result of disrupting the integrity of the OM which renders efflux pumps overwhelmed by the rapid ingress of exogenous glyphosate induced by the presence of Tween20.

The effect of the surfactant is particularly evident in experiments where the concentration of glyphosate was static, and lower biomass was recovered as a function of increasing the concentration of Tween20 (Figure 3.5.). In practice, the precise cause for the negative effects of commercial GBH's on *Methylobacterium* can only be speculated because the proprietary nature of both product formulations tested, prevent direct testing of specific ingredients. Crucially though, in both the Kirby Bauer assay and cell viability tests, *E. coli* did not indicate sensitivity to GBH's, mixes of glyphosate and Tween20, or glyphosate alone. This observation contradicts the hypothesis that surfactants enhance glyphosate toxicity in *Methylobacterium* by enabling bypass of cellular infrastructure designed for detoxification leading to a cessation of AAA biosynthesis.

While the restoration of AAA biosynthesis through eventual elimination of glyphosate may be remarkably slow in *Methylobacterium*, the loss of culturability of *Methylobacterium*, observed even when transferred to fresh nutrient-rich agar, suggests a potential secondary mechanism through which permanent cell damage may occur. We therefore suggest that intracellular glyphosate may not only reduce AAA biosynthesis through inhibition of EPSP synthase, but it also might be metabolized to more toxic products including formaldehyde, through alternate pathways. Through review of the

metabolic capabilities of several *Methylobacterium* strains, we postulate that intracellular glyphosate may contribute to increases in cytosolic formaldehyde by two candidate pathways: (a) C-N cleavage by oxidoreductase, and/or (b) C-P cleavage by phosphatase (Figure 3.7.).

Proposed mechanism of toxicity

Two enzymes previously isolated from *Methylobacterium* spp., carbon-phosphorus lyase (C-P lyase) (Kwak et al. 2014) and sarcosine oxidase (SO) (Gruffaz et al. 2014; Nayak and Marx, 2014), may cause the detected rises in intracellular formaldehyde through a two-step process. Briefly, once inside the cell, glyphosate may initially be cleaved by CP lyase into phosphate and sarcosine, which in turn may be further cleaved into glycine and formaldehyde in a second step by SO (Figure 3.7.). Criticism for this postulation may stem from the fact that the mere presence of CP lyases do not necessarily imply activity against glyphosate, as a higher degree of substrate specificity for glyphosate degradation has been suggested (White and Metcalf, 2004). Additionally, our work indicates no detectable levels of sarcosine were observed in any of the tested *Methylobacterium* strains, irrespective of glyphosate dose. However, the mechanics and kinetics which underly glyphosate degradation by CP lyases remains poorly understood. Available reports suggest that the ability for some CP lyases to degrade glyphosate may either be a result of isoforms with inherently lower substrate specificity in some organisms, or the presence of glyphosate-like molecular analogues naturally present in the environment which necessitate isoforms of narrow substrate specificity that also happen to be capable of degrading glyphosate (Sviridov et al. 2015).

More likely however, the concomitant presence of an oxidoreductase (OR), transaminase (TA), and hydrolase, may metabolize glyphosate in a three-step pathway, whereby glyphosate is first degraded into AMPA by OR, then converted into phosphonoformaldehyde through transamination by TA, and finally cleaved to produce phosphate and formaldehyde by a hydrolase (phosphonatase). Such degradation pathways were suggested for a range of glyphosate-metabolizing organisms (White and Metcalf, 2004; Zhan et al. 2018), and the results of our mass spectrometry work in combination with intracellular formaldehyde measurements, indicate that degradation by this pathway is predominant in our tested *Methylobacterium*. However, a search of the annotated genomes of *Methylobacterium* currently available through the National Center for Biotechnology Information (NCBI) did not indicate the presence of the glyphosate oxidoreductase (goxA) in the *Methylobacterium* strains used in sensitivity experiments. Evidence of mutation, whether random or intentional, enhancing or otherwise altering the sensitivity of *Methylobacteriuum* to glyphosate or GBH's have not yet been reported.

Formaldehyde is a well-established metabolic by-product of normal enzymatic activity, with known toxicity to several critical components of the cell including proteins and nucleic acids (Metz et al. 2004; Toews et al. 2008; Kawanishi et al. 2014; Ortega-Atienza et al. 2016). In a range of bacteria, detoxification of formaldehyde is often achieved through a thiol-dependant pathway involving glutathione to produce the less cytotoxic, formate (Chen et al. 2016). Methylotrophs, especially *Methylobacterium*, are unique in their ability to withstand transient intracellular loads of formaldehyde up to 1 mM which are produced as a result of single-carbon catabolism (methanol, methane, trimethylamine) (Vorholt et al. 2000). In the cytoplasm, formaldehyde undergoes

condensation with dephospho-tetrahydromethanopterin (dH4MPT) catalyzed by formaldehyde-activating enzyme (Fae) (Vorholt et al. 2000; Marx et al. 2003). As with other microorganisms, formaldehyde is eventually oxidized to produce formate. Crucially however, the dH4MPT complex appears to be responsible for handling both formaldehyde produced during growth, and formaldehyde contributions from degradation of xenobiotics (Marx et al. 2003; Chen and Jean, 1982; Christosedova et al. 1998; Laukel et al. 2004; Bosch et al. 2008). In a study conducted by Bazurto et al. a previously unidentified member of the DUF336 domain family called efgA (enhanced formaldehyde growth), present in *Methylorubrum extorquens* (previously, *Methylobacterium extorquens*) and exclusive to methylotrophic taxa, was determined to encode EfgA, a formaldehyde sensor protein (Bazurto et al. 2021). From the available evidence, accumulation and subsequent binding of formaldehyde to EfgA directly, results in reduced growth rate by triggering a reduction in global protein translation through the up-regulation of chaperone-encoding genes (Bazurto et al. 2021; Bazurto et al. 2021b). This suggests that activation of EfgA by endogenous sources of formaldehyde works as part of a negative feedback loop to throttle enzymatic activity preventing overaccumulation of formaldehyde.

However, when glyphosate can gain entry to the cytosol through the lipid bilayer – mediated by the presence of a surfactant – the unchecked degradation of the herbicide in pathways governed by GOR-like enzymes, may lead to increases in formaldehyde levels as seen in *M. organophilum* (64.5 nM/mg) and *M. gnaphali* (72.7 nM/mg). As a result, the EfgA safety switch would respond to the increase in formaldehyde concentration and call for a reduction in translation. However, the enzymes involved in glyphosate degradation may not fall under the control of the EfgA feedback mechanism

and proceed with glyphosate metabolism, unrestrained. In essence, the metabolism of glyphosate may contribute formaldehyde by several potential candidate pathways, including the oxidoreductase pathway indicated by our detection of the primary metabolite, AMPA. If these detoxification pathways do not sense the EfgA arrest signal, this molecular safety switch may become inadvertently and permanently flipped. Macromolecule damage may similarly occur if rapid detoxification of formaldehyde to formate is attempted, resulting in a sharp change in intracellular pH.

With the EfgA alarm activated, prolonged inhibition of growth and translation of critical proteins required for survival eventually reaches a point to which recovery from steadily accumulating formaldehyde and formate is irreversible and cell death becomes inevitable. Although the presence of EfgA in all the strains used in experiments has not been confirmed through polymerase chain reaction (PCR), this unique signalling relay could explain the inconsistency surrounding cytotoxicity of glyphosate and GBH's across many taxa of plant-associated microorganisms. The recurring debate regarding cytotoxicity of glyphosate formulations to microorganisms surrounds whether toxicity is due to the herbicide itself or to their co-formulants, notably the surfactants.

In our work, neither Tween-20 in concentrations up to 4% or glyphosate in concentrations up to 0.1% showed cytotoxicity when administered alone. Yet, even at low concentrations of glyphosate (0.05%), the coadministration of an otherwise non-toxic surfactant, resulted in markedly reduced biomass. Moreover, if present, the prolonged activation of the EfgA molecular switch by formaldehyde contributed from glyphosate degradation, may also explain the observed negative impact on culturability when transferred to fresh medium after GBH exposure. This may be because, despite

the fact that several of the *Methylobacterium* do likely contain a GS-EPSP synthase isoform, allosteric inhibition by glyphosate has been established as transitory and AAA biosynthesis should readily be restored after transfer to glyphosate-free growth media [109–111]. Additionally, several other microorganisms have demonstrated the ability to up-regulate the synthesis of EPSPS up to 30-fold, in an effort to compensate for blockade of the shikimate pathway caused by exogenous applications of glyphosate [112]. Taken together, the temporary and reversable disruption to AAA biosynthesis should be overcome with relative ease, however, the weak growth of *Methylobacterium* cells previously exposed to commercial GBH formulations and glyphosate-Tween20 mixes may be a result of more severe damage incurred to macromolecules by formaldehyde and a coinciding sustained arrest signal generated by EfgA. Importantly, in the absence of a time course study with greater granularity, the differences in detectable levels of intracellular formaldehyde and resultant culturability between strains may indicate variation in formaldehyde decomposition just as much as formaldehyde formation through degradation of intracellular glyphosate. While the presence of glyphosate-degradation pathways in other soil-borne or plant-associated microorganisms may result in transient toxicity, the reported near complete exclusivity of *efgA* and *efgB* loci to methylotrophs, may explain the apparent high sensitivity of *Methylobacterium* to GBH's and glyphosate-Tween20 mixes.

Implications

Host-plant pathogen protection following *Methylobacterium* inoculation was reported previously in studies involving potatoes and tomatoes, where the presence of *Methylobacterium* was also found to induce changes in plant microbiome composition (Ryan et al. 2008; Holland, 1997). Next to resource competition caused by invasive

weeds, infection and disease present a significant threat to crop development, for which future studies may find *Methylobacterium* to be particularly helpful. Primarily, five species of pathogenic fungi are particularly problematic to field crop cultivation (*Phytophthora sojae*, *Phomopsis longicolla, Rhizoctonia solani, Pythium* spp., and *Fusarium solani*) causing a range of diseases including seedling rot, seedling blight, and root rot. Interestingly, correlations between glyphosate application and the susceptibility of field crops including soybean to phytopathogens have previously been reported. For example, application of GBH's has been linked to increased incidence of infection by *Phytophthora* spp. (Keen et al. 1982; Holliday and Keen, 1982), *Glomus* spp. (Morandi, 1989), and *Fusarium* spp. (Sanogo et al. 2000). Similarly, infection by *Gaeumannomyces tritici*, a known opportunistic pathogen affecting wheat, was found to increase following field pre-treatments with glyphosate (Mekwatanakarn and Sivasithamparam, 1987,1987b). Use of glyphosate to control weed cover in barley was found to result in significant colonization of germinating seed by *Pythium* spp. (Blowes, 1987), and *Fusarium culmorum* (Lynch and Penn, 1980; Lévesque et al. 2011), causing poor crop performance. Vigor of winter rape seeded in soil pre-treated with glyphosate for the control of invasive weeds including quack grass, was clearly diminished with yields reduced by three-fold relative to untreated conditions (Nilsson and Hallgren, 1990). Infection by *Fusarium* was also documented to increase in common waterhemp (*Amaranthus rudis*) following glyphosate treatment where survivability recorded for both glyphosate-sensitive (GS) and glyphosate-resistant (GR) varieties were reduced when grown in non-sterile (NS) soils relative to sterile conditions (GS: 29% reduced to 10% and GR: 83% reduced to 61%) (Rosenbaum et al. 2014). Nearly identical infection dynamics were also documented in TopCrop beans and McIntosh Apples (*Malus domestica* Borkh.) where seedlings were treated with glyphosate and subsequently

grown in both sterile and non-sterile conditions. The LD50 for glyphosate was markedly decreased in the presence of infections caused by *Pythium* spp. and *Fusarium* spp. in McIntosh seedlings (unchallenged: 40 µg, pathogen challenge: 10–15 µg) (Lévesque et al. 1992). Furthermore, increases in disease incidence was associated with glyphosate application in more unique and geographically diverse crop systems including: banana (Anderson and Aitken, 2021), canola (Rashid et al. 2013), cotton (Savin et al. 2009), maize (Carranza et al. 2019), sugar beet (Larson et al. 2006), and tomato (Brammall and Higgins, 1988). Studies examining the soybean rhizosphere in response to application of GBH's also indicate significant disruption to population and diversity (Kremer et al. 2005), including certain members responsible for reducing manganese (Mn) and secreting indole acetic acid (IAA) (Zobiole et al. 2010). Importantly, both studies report higher incidence of disease caused by *Fusarium* spp. in soybean following glyphosate application, relative to controls.

Several hypotheses surrounding the increased incidence of fungal disease in plants following glyphosate application have been proposed and include: (a) pathogen exposure to glyphosate predisposes the infectious agent to exogenous synthetic chemicals heightening virulence and fungicide resistance, (b) glyphosate may provide an alternate carbon source for pathogens, and (c) glyphosate-induced death of problematic weeds that act as reservoirs for certain phytopathogens cause sudden increases to field colony density during decay (Johal and Huber, 2009). In addition to these, we propose that glyphosate may harm components of the phyllosphere, including the *Methylobacterium*, which prevent infection irrespective of the GR status of the host. Combined with the results presented herein, the relevant literature supports our theory that GBH's may increase crop susceptibility to infections by selectively attenuating the

protective effects of *Methylobacterium* colonization. Our work also demonstrates that even the presence of the relatively non-toxic surfactant, Tween20, can disrupt the growth of *Methylobacterium*.

This is supported by the fact that, in addition to soybean, the *Methylobacterium* genus comprises a major part of the phyllosphere in several of the same food crop systems that exhibit increased susceptibility to infectious disease when exposed to GBH's including: apple (Doronina et al. 2004; Arrigoni et al. 2018;Zervas et al. 2019), wheat (Meena et al. 2012; Kalyaeva et al. 2002; Senthilkumar and Krishnamoorthy, 2017), tomato (Longoria-Espinoza et al. 2020; Ryu et al. 2006; Ribeiro et al. 2015), hemp (Nalayini et al. 2014), cotton (Madhaiyan et al. 2011; Periyasamy et al. 2016), banana (Suhaimi et al. 2017; Senthilkumar et al. 2021; Pushpakanth et al. 2021), and soybean (Kremer et al. 2005; Zobiole et al. 2010). Importantly, the application of glyphosate has also been previously found to decrease host phytoalexin levels, even when attempting to elicit an immune response through deliberate pathogen challenge (Johal and Rahe, 1990; Indiragandhi et al. 2008). As it so happens, *Methylobacterium* spp. have been documented to contribute to ISR and directly stimulate the synthesis of phytoalexins in plants (Madhaiyan et al. 2006; Indiragandhi et al. 2008; Lu et al. 2019).

3.7. CONCLUSION

Our work on GBHs and surfactants indicate that glyphosate is toxic to *Methylobacterium* and exhibits bactericidal activity. While not harmful in its pure form alone, the toxic effects of glyphosate are observed when bacterial cell wall permeability is enhanced by the presence of a surfactant. The significance of our work is that while *Methylobacterium* species can be beneficial for plant growth, development, and disease protection, there are coinciding reports of greater disease activity when glyphosate is used for weed control. Our findings of heightened levels of intracellular formaldehyde in response to exogenously-applied GBH's, with possible EfgA participation also lays the foundation for further investigation of the precise mechanics by which selective inhibition of discrete components of the phyllosphere is mediated. Our detection of AMPA, indicates conclusively that the tested *Methylobacterium* strains are metabolically active against glyphosate through an oxidoreductase-like pathway. Continued investigation of PGPB such as *Methylobacterium* spp. presents a realistic path forward in the development of broad-spectrum biological fertilizers that not only make use of the natural biochemistry of the plant but do so in an ecologically and toxicologically appropriate way. Future studies should involve targeted sequencing of glyphosate oxidoreductase (*goxA*) and enhanced formaldehyde growth (*efgA*) genes in *Methylobacterium* – and later, their knockouts – to compliment the metabolite results presented herein and further understand the mechanics of glyphosate toxicity in bacteria.

3.8. TABLES AND FIGURES

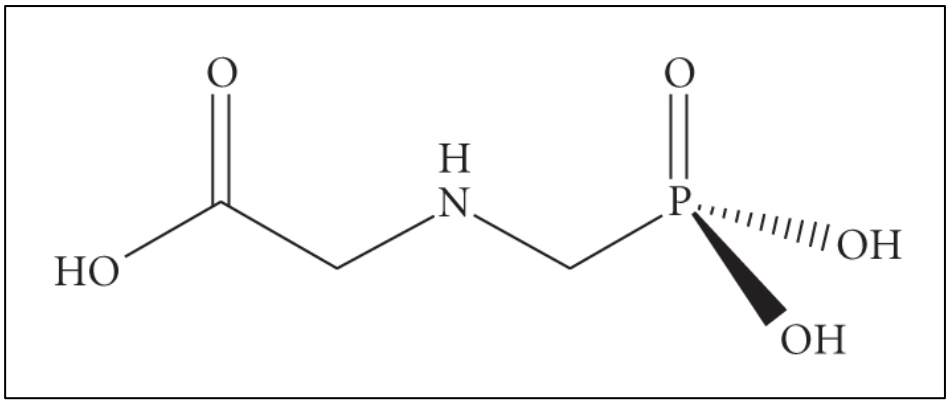

Figure 3.1: chemical structure of glyphosate [N-(phosphonomethyl)glycine].

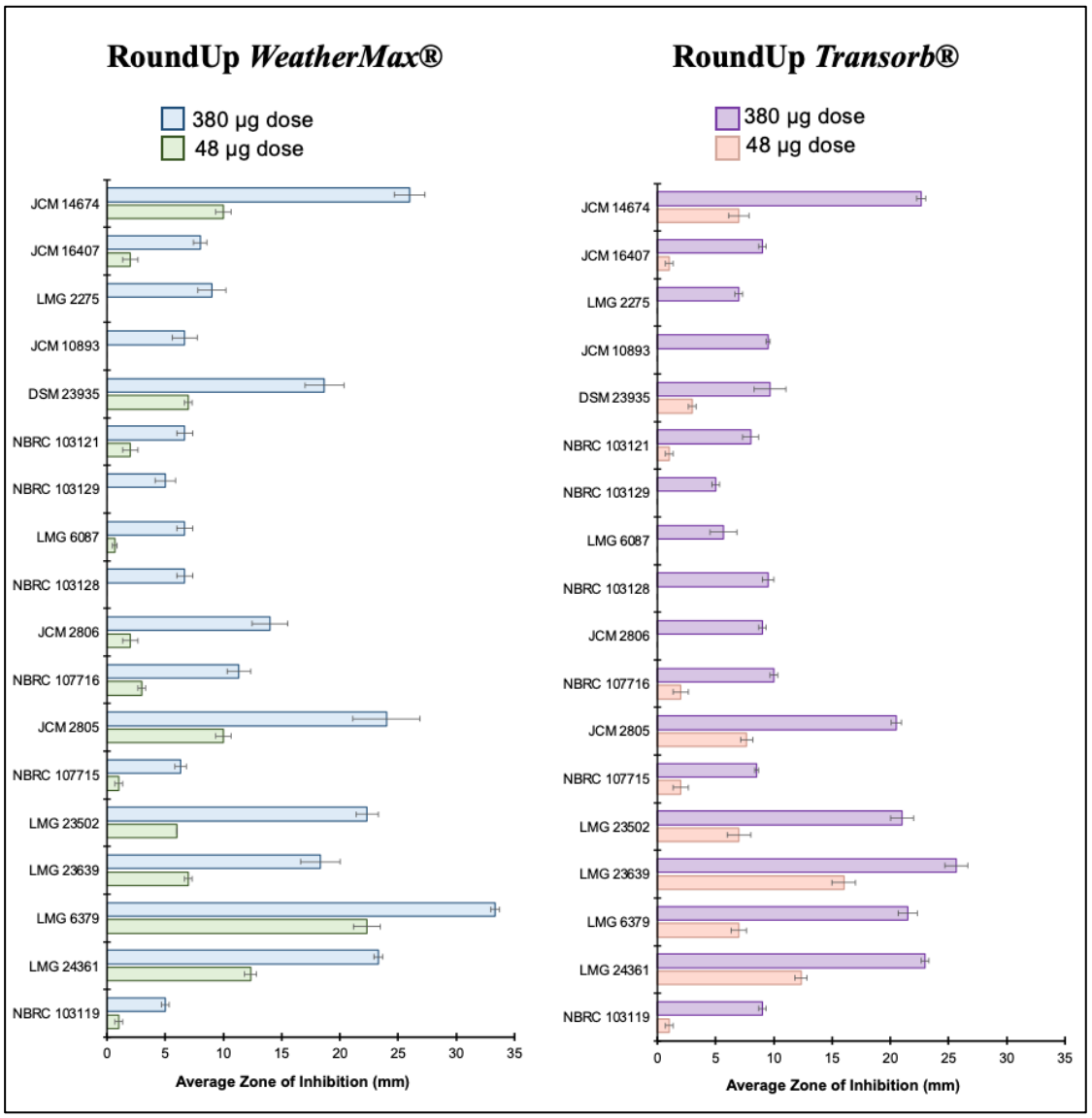

Figure 3.2: Average (n=3) zone of inhibition of each tested strain of *Methylobacterium* spp. (Table 1) against maximum and minimum concentrations glyphosate (380 µg and 95 µg, respectively) in the *WeatherMax*® [left] and the *Transorb*® [right] products tested.

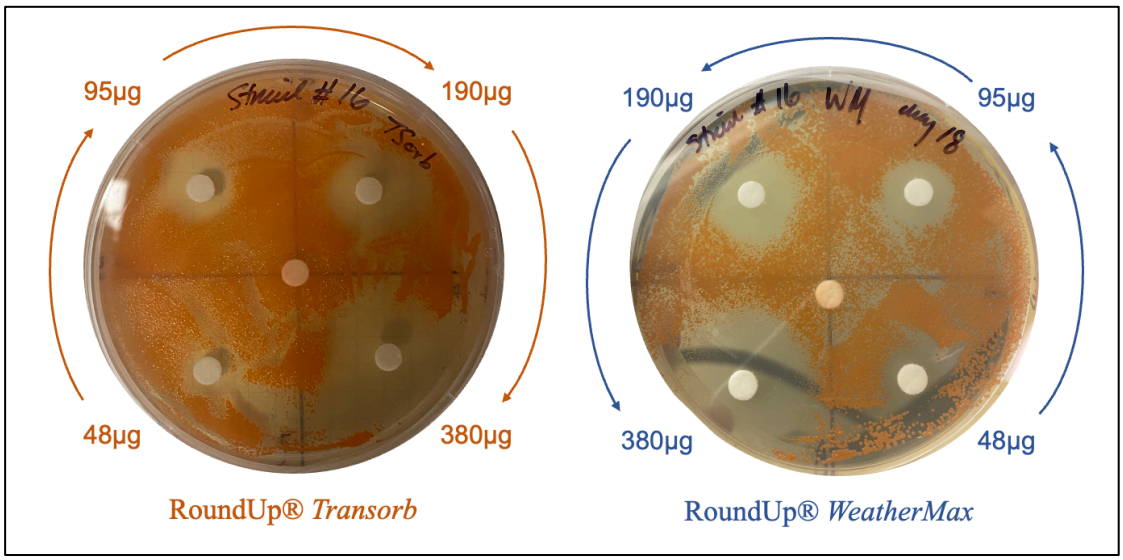

Figure 3.3: Representative photograph illustrating zone of inhibition of *Methylobacterium gnaphali* (NBRC 107716) to four concentrations of *Transorb*® [left] and *WeatherMax*® [right].

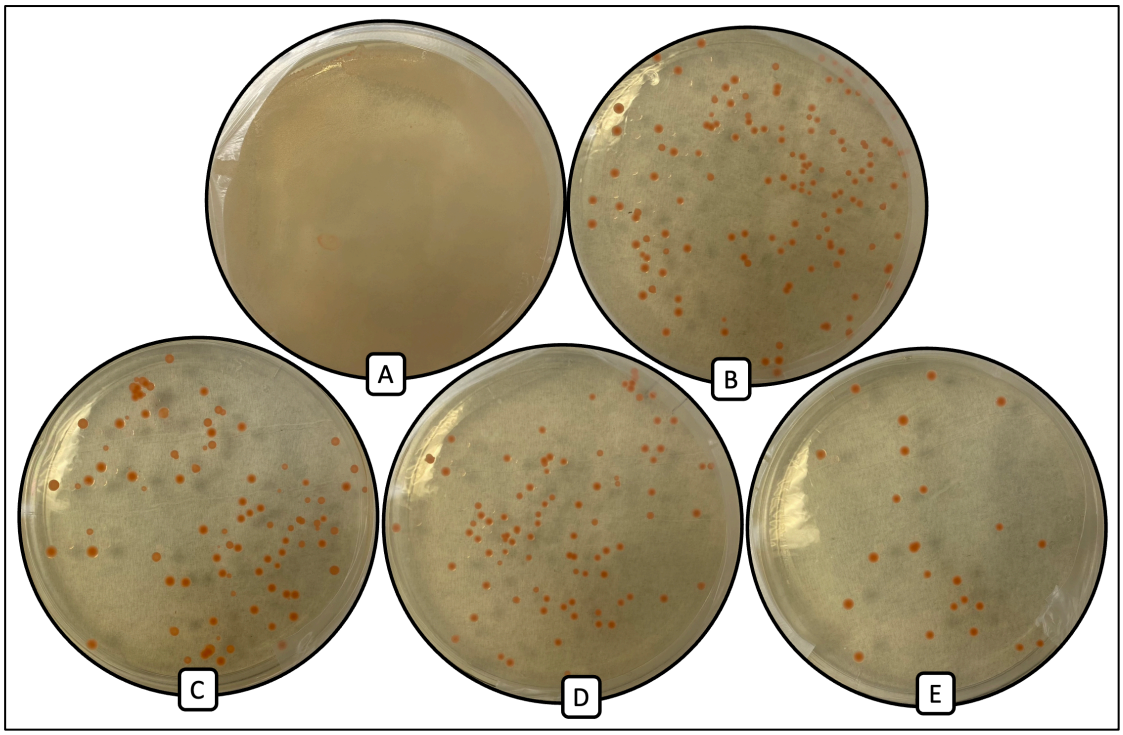

Figure 3.4: Representative photograph illustrating results of cell viability test from cultures containing 0.1% pure glyphosate (1 mg/mL) with *Methylobacterium gnaphali* (NBRC 107716) with varying concentrations of Tween20 (polysorbate-20); (A) control, (B) 0.5%, (C) 1.0%, (D) 2.0%, (E) 4.0% (v/v). Frame (A) depicts confluent growth of NBRC 107716.

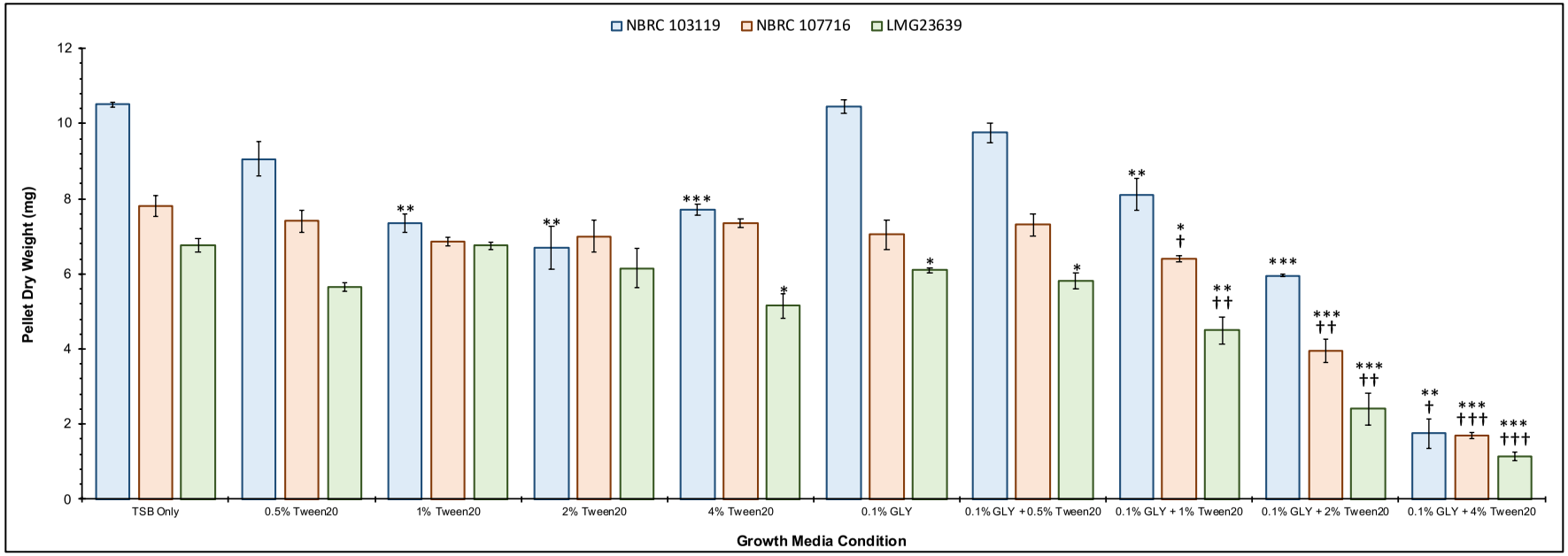

Figure 3.5: Graphical representation of dry pellet weight of three distinct *Methylobacterium* strains when cultured in tryptic soy broth (TSB) containing a fixed quantity of pure glyphosate (0.1% w/v) in relation to changes in the presence of Tween20 (polysorbate-20), relative to controls containing Tween20 alone (*n=4*). The Student's t-test was used to assess statistical difference between groups. A star (*) indicates statistical difference in pellet weight between control conditions (TSB only) and following the application of a treatment ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***). A dagger (†) indicates statistical difference comparing pellet weight between the application of Tween20 and the corresponding application of Tween20 with the addition of glyphosate ($p < 0.05$ = †, $p < 0.01$ = ††, $p < 0.001$ = †††).

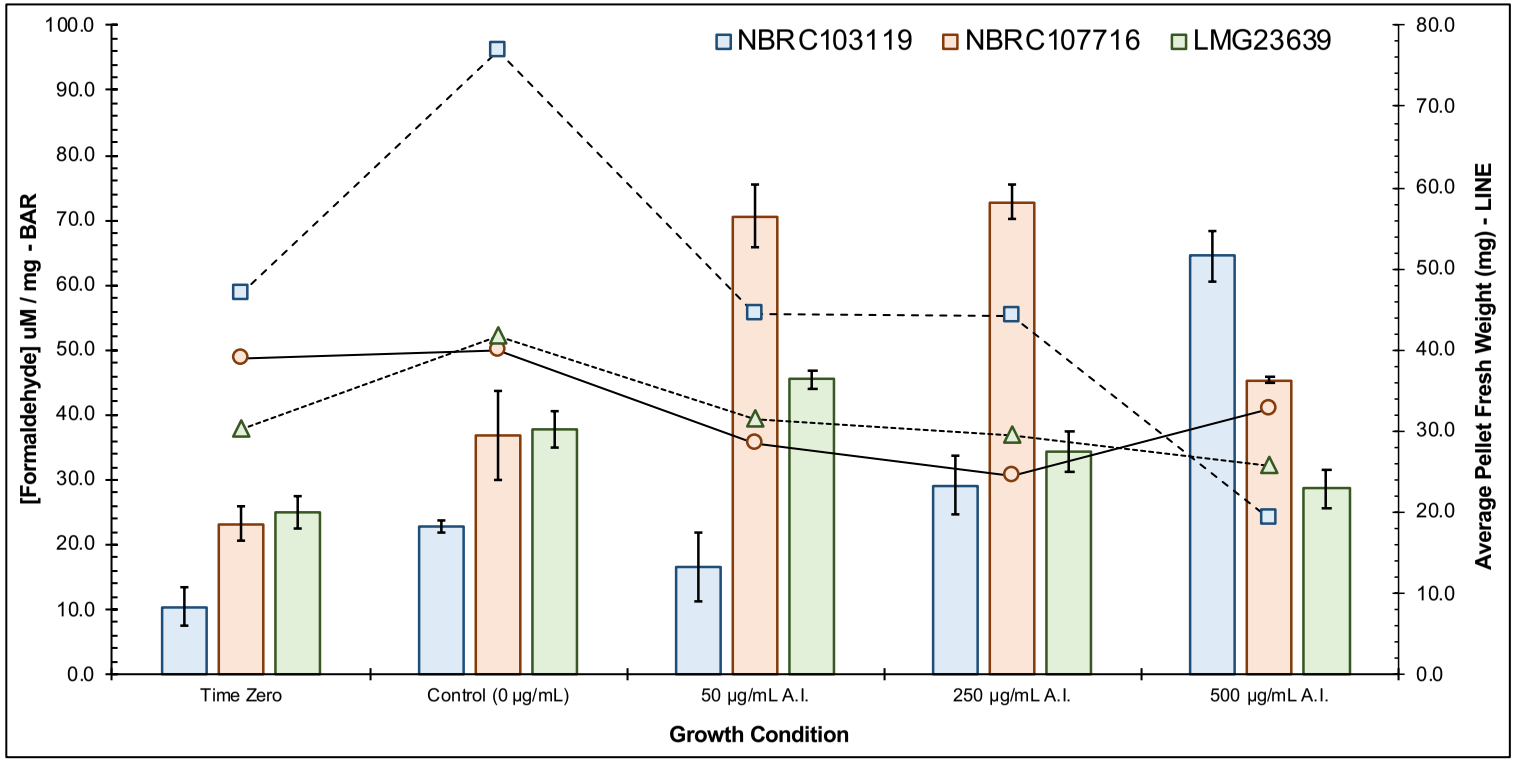

Figure 3.6: Graphical representation of average pellet fresh weight (line, right axis) of three distinct *Methylobacterium* strains when cultured in tryptic soy broth (TSB) containing fixed quantities of the active ingredient (AI), glyphosate, obtained from the *Transorb*® commercial product, and corresponding intracellular formaldehyde concentrations (bar, left axis) after 4 days of growth at 27°C (*n = 4*).

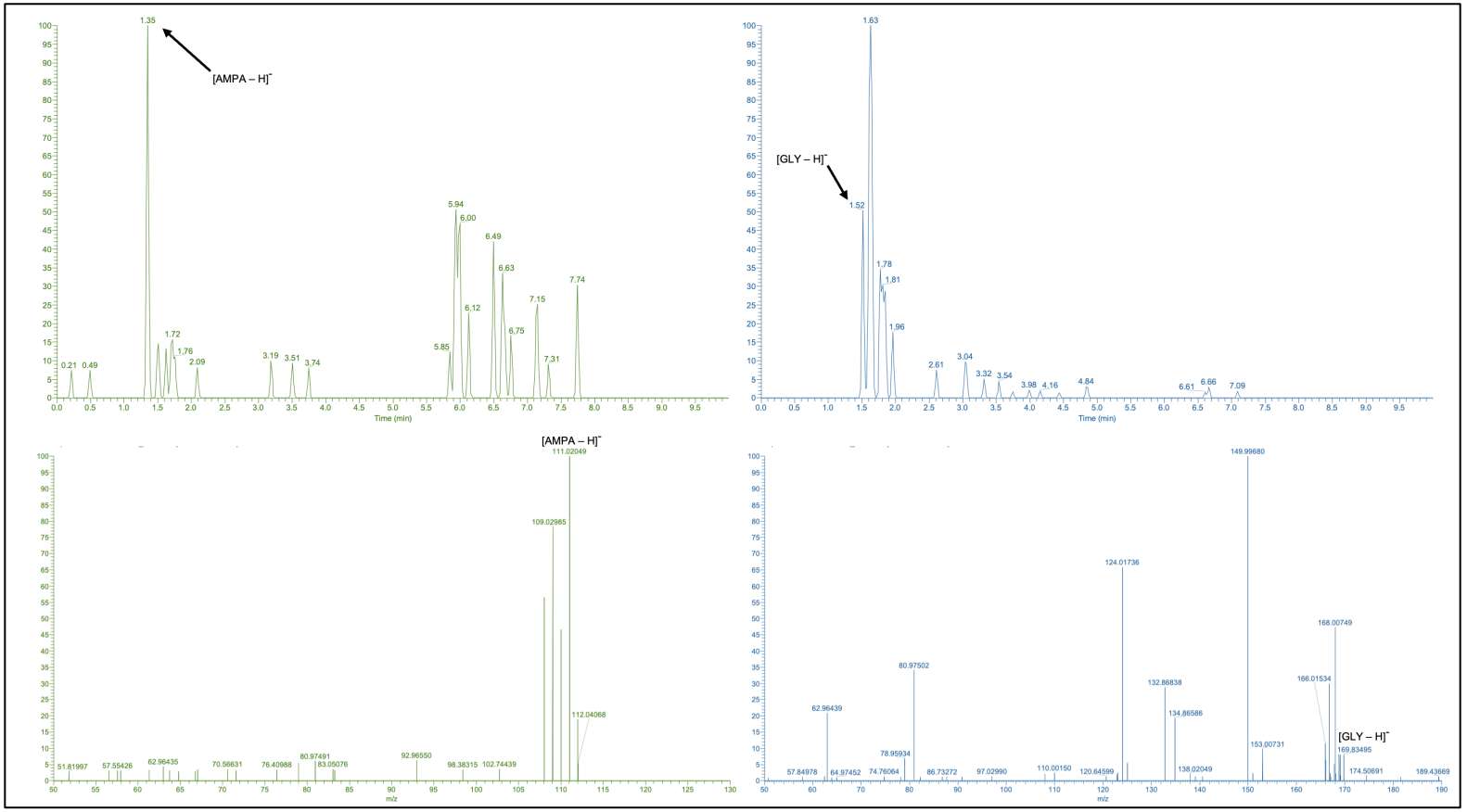

Figure 3.7: Mass spectrum of target metabolite AMPA and associated chromatogram (left), and glyphosate (right) detected intracellularly from a pellet of *M. gnaphali* (NBRC 107716) after 4 days incubation in TSB spiked with *Transorb*® formulation (500 ug/mL A.I.).

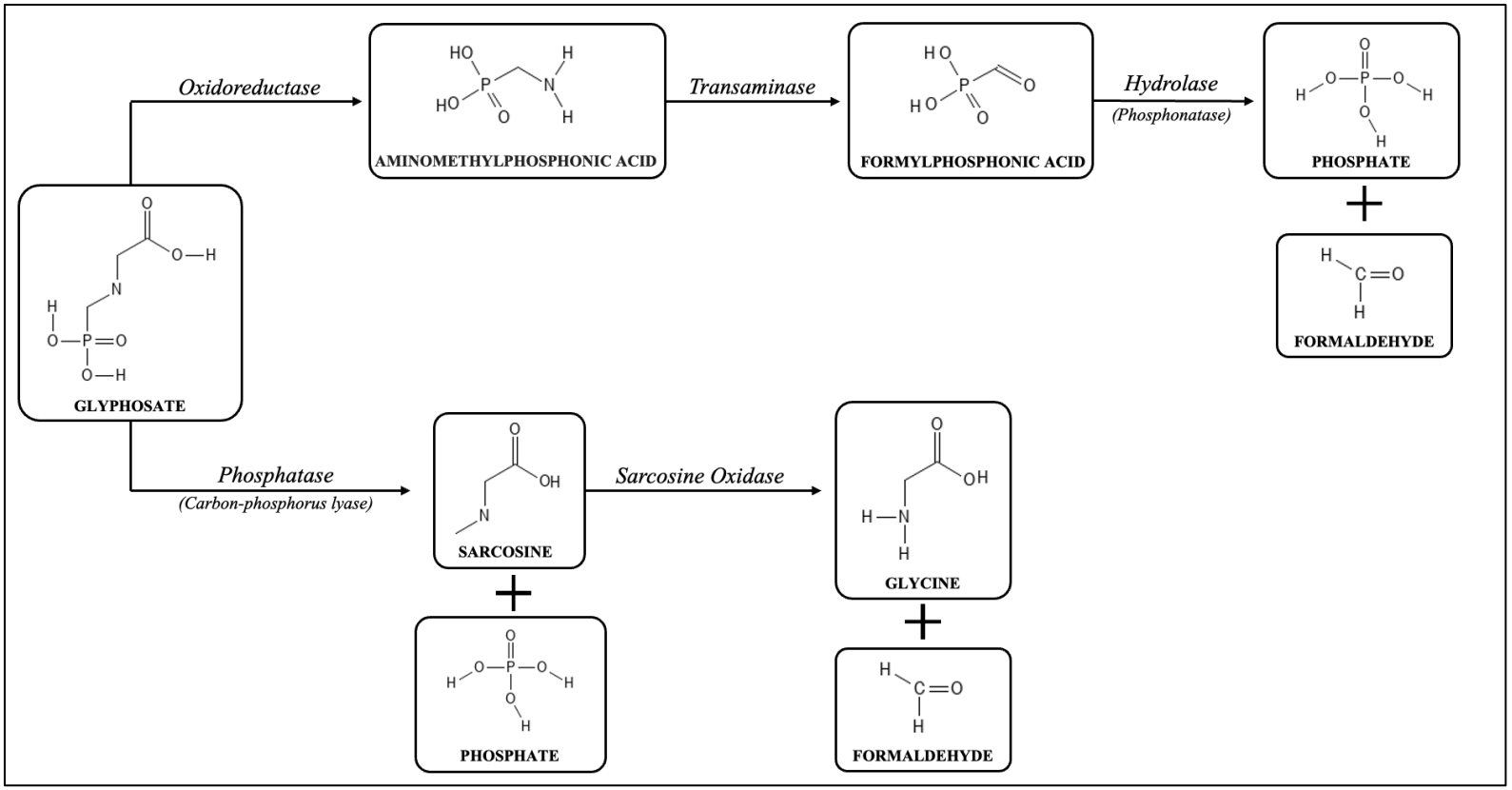

Figure 3.8: Schematic illustrating basic metabolic pathways that may lead to increased intracellular formaldehyde load in *Methylobacterium* spp.

Table 3.1: Inventory of *Methylobacterium* strains examined for glyphosate sensitivity.

Species	Strain	Source of Isolation
M. *organophilum*	NBRC103119(T)	*Pelargonium zonale*; phyllosphere
	NBRC 103121	*Begonia sp*.; phyllosphere
M. *phylospharae*	LMG 24361(T)	*Oryza sativa*; phyllosphere
M. *radiotolerans*	LMG 6379(T)	Forest soil
M. *jeotgali*	LMG 23639(T)	Traditional fermented seafood (*jeotgal*)
M. *oryzae*	LMG 23582	*Oryzae sativa*; phyllosphere
M. *oxalidis*	NBRC 107715	*Oxalis corniculata*; phyllosphere
M. *extorquens*	JCM 2805	Air
	JCM 2806	Garden soil, slough
	NBRC 103129	*Eucalyptus sp*.; phyllosphere
M. *gnaphali*	NBRC 107716	*Gnaphalium spicatum*; phyllosphere
M. *thiocyanatum*	NBRC 103128	*Mesenbryanthemum sp*.; phyllosphere
M. *zatmanii*	LMG 6087	-
M. *thiocyanatum*	JCM 10893	*Allium aflatuense*; phyllosphere
M. *rhodinum*	LMG 2275	Alder tree (*Alnus*); phyllosphere
M. *iners*	JCM 16407	Air
Methylobacterium spp.	JCM 14674	*Oryza rufipogon:* phyllosphere
	DSM 23935	*Cardamine hirsuta;* phyllosphere
E. *coli*	NM522	-

Table 3.2: Cell viability test (*n = 1*) of each strain of *Methylobacterium* spp. against two concentrations of the *Transorb*® (Tsorb) and *WeatherMax*® (WMax) product formulations. Negative control involved nutrient-rich tryptic soy broth (TSB) with no addition of commercial products.

Species	Strain	Cell Viability				
		Neg. Control	Tsorb 0.05%	Tsorb 0.1%	WMax 0.05%	WMax 0.1%
M. *extoquens*	JCM 2805	+ + +	–	–	–	–
	JCM 2806	+ + +	–	–	–	–
	NBRC 103129	+ + +	–	–	–	–
M. *gnaphali*	NBRC 107716	+ + +	–	–	–	–
M. *iners*	JCM 16407	+ + +	–	–	–	–
M. *jeotgali*	LMG 23639	+ + +	–	–	–	–
M. *organophilum*	NBRC 103119	+ + +	–	–	–	–
	NBRC 103121	+ + +	–	–	–	–
M. *oryzae*	LMG 23502	+ + +	–	–	–	–
M. *oxalidis*	NBRC 107715	+ + +	–	–	–	–
M. *phylospharae*	LMG 24361	+ + +	–	–	–	–
M. *radiotolerans*	LMG 6379	+ + +	–	–	–	–
M. *rhodinum*	LMG 2275	+ + +	–	–	–	–
M. *thiocyanatum*	NBRC 103128	+ + +	–	–	–	–
	JCM 10893	+ + +	–	–	–	–
M. *zatmanii*	LMG 6087	+ + +	–	–	–	–
Methylobacterium spp.	DSM 23935	+ + +	–	–	–	–
	JCM 14674	+ + +	–	–	–	–
E. *coli*	NM522	+ + +	+ + +	+ + +	+ + +	+ + +

(–) no CFU, (+) if < 30 CFU, (+ +) if > 30 CFU or partial lawn, and (+ + +) if complete lawn present and CFU count impossible.

Table 3.3: Cell viability test (n=2) of each strain of *Methylobacterium* spp. against two concentrations of pure glyphosate (GLY), with and without Tween20 (polysorbate-20). Negative control involved nutrient-rich tryptic soy broth (TSB) with no addition of glyphosate or Tween20.

Species	Strain	Cell Viability					
		Neg. Control	2% Tween20	GLY 0.05%	GLY 0.1%	GLY 0.05% + 2% Twen20	GLY 0.1% + 2% Twen20
M. *extoquens*	JCM 2805	+ + +	+ +	+ + +	+ + +	+ +	+
	JCM 2806	+ + +	+ +	+ + +	+ + +	+ +	-
	NBRC 103129	+ + +	+ +	+ + +	+ + +	+ +	+
M. *gnaphali*	NBRC 107716	+ + +	+ +	+ + +	+ + +	+ +	+
M. *iners*	JCM 16407	+ + +	+ +	+ + +	+ + +	+ +	+
M. *jeotgali*	LMG 23639	+ + +	+ +	+ + +	+ + +	+ +	-
M. *organophilum*	NBRC 103119	+ + +	+ +	+ + +	+ + +	+	+
	NBRC 103121	+ + +	+ +	+ + +	+ + +	+ +	+
M. *oryzae*	LMG 23502	+ + +	+ +	+ + +	+ + +	+ +	-
M. *oxalidis*	NBRC 107715	+ + +	+ +	+ + +	+ + +	+	+
M. *phylospharae*	LMG 24361	+ + +	+ +	+ + +	+ + +	+ +	-
M. *radiotolerans*	LMG 6379	+ + +	+ +	+ + +	+ + +	+ +	-
M. *rhodinum*	LMG 2275	+ + +	+ +	+ + +	+ + +	+ +	+
M. *thiocyanatum*	NBRC 103128	+ + +	+ +	+ + +	+ + +	+ +	+
	JCM 10893	+ + +	+ +	+ + +	+ + +	+ +	+
M. *zatmanii*	LMG 6087	+ + +	+ +	+ + +	+ + +	+ +	-
Methylobacterium spp.	DSM 23935	+ + +	+ +	+ + +	+ + +	+ +	-
	JCM 14674	+ + +	+ +	+ + +	+ + +	+ +	-
E. *coli*	NM522	+ + +	+ +	+ + +	+ + +	+ + +	+ + +

(–) no CFU, (+) if < 30 CFU, (+ +) if > 30 CFU or partial lawn, and (+ + +) if complete lawn present and CFU count impossible.

Table 3.4: Glyphosate, target metabolites, and optimized PRM method parameters. Product ions were used for quantification of the compounds by Xcalibur (v. 3.0.63) data processing module (Quan Browser).

Target Analyte	Chemical Formula (M)	Precursor $[M-H]^-$ (*m/z*)	Normalized Collision Energy, eV (NCE)	Product Ions (*m/z*)
Glyphosate	$C_3H_8NO_5P$	168.00673	30	78.95902 80.97466 110.00119 124.01685 149.99619
Aminomethylphosphonic Acid (AMPA)	CH_6NO_3P	110.00125	30	62.96417 78.95901 80.97469
Sarcosine	$C_3H_7NO_2$	88.04040	30	60.99348 71.01444 87.00938

Table 3.5: Average intracellular concentration [pMol / mgDW] and standard error of glyphosate and target metabolites in cell pellets of *M. organophilum*, M. *gnaphali,* and *M. jeotgali* determined by HPLC-[ESI]-HRMS/MS (*n=5*). Compounds that were unable to be detected are indicated as not-detected (n.d.). A dagger (†) indicates statistical difference comparing concentration of detected compounds between the application of *Transorb*® commercial GBH and identical negative controls ($p < 0.05$ = †).

Target Analyte	***M. organophilum*** (NBRC 103119)		***M. gnaphali*** (NBRC 107716)		***M. jeotgali*** (LMG23639)	
	Transorb® Media Concentration					
	50 µg/mL	*500 µg/mL*	*50 µg/mL*	*500 µg/mL*	*50 µg/mL*	*500 µg/mL*
Glyphosate	0.23 ± 0.03†	2.36 ± 0.01†	0.03 ± 0.00	1.32 ± 0.11†	0.41 ± 0.02†	2.67 ± 0.18†
Aminomethylpho-sphonic Acid (AMPA)	1.18 ± 0.11†	13.54 ± 0.45†	3.63 ± 0.39†	27.09 ± 1.38†	1.62 ± 0.21†	n.d.
Sarcosine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

3.9. REFERENCES

Abanda-Nkpwatt, D., Müsch, M., Tschiersch, J., Boettner, M., and Schwab, W. 2006. Molecular interaction between *Methylobacterium extorquens* and seedlings: growth promotion, methanol consumption, and localization of the methanol emission site. J. Exp. Bot. 57:4025–4032. https://doi.org/10.1093/jxb/erl173.

Akhtar, N., Rehman, M. U., Khan, H. M. S., Rasool, F., Saeed, T., and Murtaz, G. 2011. Penetration enhancing effect of polysorbate 20 and 80 on the *in vitro* percutaneous absorption of Ascorbic acid. Trop. J. Pharm. Res. 10. https://doi.org/10.4314/tjpr.v10i3.1.

Amrhein, N., Johänning, D., Schab, J., and Schulz, A. 1983. Biochemical basis for glyphosate-tolerance in a bacterium and a plant tissue culture. FEBS Lett. 157:191–196. https://doi.org/10.1016/0014-5793(83)81143-0.

Anderson, J., and Aitken, E. 2021. Effect of in Planta Treatment of "Cavendish" Banana with Herbicides and Fungicides on the Colonisation and Sporulation by *Fusarium oxysporum* f.sp. *cubense* Subtropical Race 4. J. Fungi (Basel) 7:184. https://doi.org/10.3390/jof7030184.

Anesti, V., Vohra, J., Goonetilleka, S., McDonald, I. R., Sträubler, B., Stackebrandt, E., Kelly, D. P., and Wood, A. P. 2004. Molecular detection and isolation of facultatively methylotrophic bacteria, including *Methylobacterium podarium* sp. nov., from the human foot microflora. Environ. Microbiol. 6:820–830. https://doi.org/10.1111/j.1462-2920.2004.00623.x.

Arrigoni, E., Antonielli, L., Pindo, M., Pertot, I., and Perazzolli, M. 2018. Tissue age and plant genotype affect the microbiota of apple and pear bark. Microbiol. Res. 211:57–68. https://doi.org/10.1016/j.micres.2018.04.002.

Aslam, Z., Lee, C. S., Kim, K.-H., Im, W.-T., Ten, L. N., and Lee, S.-T. 2007. *Methylobacterium jeotgali* sp. nov., a non-pigmented, facultatively methylotrophic bacterium isolated from jeotgal, a traditional Korean fermented seafood. Int. J. Syst. Evol. Microbiol. 57:566–571. https://doi.org/10.1099/ijs.0.64625-0.

Balachandar, D., Raja, P., and Sundaram, S. 2008. Genetic and metabolic diversity of pink-pigmented facultative methylotrophs in phyllosphere of tropical plants. Braz. J. Microbiol. 39:68–73. https://doi.org/10.1590/S1517-838220080001000017.

Bazurto, J. V., Nayak, D. D., Ticak, T., Davlieva, M., Lee, J. A., Hellenbrand, C. N., Lambert, L. B., Benski, O. J., Quates, C. J., Johnson, J. L., Patel, J. S., Ytreberg, F. M., Shamoo, Y., and Marx, C. J. 2021a. EfgA is a conserved formaldehyde sensor that leads to bacterial growth arrest in response to elevated formaldehyde. PLoS Biol. 19:e3001208. https://doi.org/10.1371/journal.pbio.3001208.

Bazurto, J. V., Riazi, S., D'Alton, S., Deatherage, D. E., Bruger, E. L., Barrick, J. E., and Marx, C. J. 2021b. Global transcriptional response of *Methylorubrum*

extorquens to formaldehyde stress expands the role of EfgA and is distinct from antibiotic translational inhibition. Microorganisms 9:347. https://doi.org/10.3390/microorganisms9020347.

Bednářová, A., Kropf, M., and Krishnan, N. 2020. The surfactant polyethoxylated tallowamine (POEA) reduces lifespan and inhibits fecundity in *Drosophila melanogaster* - *in vivo* and *in vitro* study. Ecotoxicol. Environ. Saf. 188:109883. https://doi.org/10.1016/j.ecoenv.2019.109883.

Benedetti, A. L., Vituri, C. de L., Trentin, A. G., Domingues, M. A. C., and Alvarez-Silva, M. 2004. The effects of sub-chronic exposure of Wistar rats to the herbicide Glyphosate. Biocarb. Toxicol. Lett. 153:227–232. https://doi.org/10.1016/j.toxlet.2004.04.008.

Beuret, C. J., Zirulnik, F., and Giménez, M. S. 2005. Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses. Reprod. Toxicol. 19:501–504. https://doi.org/10.1016/j.reprotox.2004.09.009.

Bijlani, S., Singh, N. K., Eedara, V. V. R., Podile, A. R., Mason, C. E., Wang, C. C. C., and Venkateswaran, K. 2021. *Methylobacterium ajmalii* sp. nov., Isolated From the International Space Station. Front. Microbiol. 12:639396. https://doi.org/10.3389/fmicb.2021.639396.

Blowes, W. M. 1987. Effect of ryegrass root residues, knockdown herbicides, and fungicides on the emergence of barley in sandy soils. Aust. J. Exp. Agric. 27:785. https://doi.org/10.1071/ea9870785.

Bosch, G., Skovran, E., Xia, Q., Wang, T., Taub, F., Miller, J. A., Lidstrom, M. E., and Hackett, M. 2008. Comprehensive proteomics of *Methylobacterium extorquens* AM1 metabolism under single carbon and nonmethylotrophic conditions. Proteomics 8:3494–3505. https://doi.org/10.1002/pmic.200800152.

Bradberry, S. M., Proudfoot, A. T., and Vale, J. A. 2004. Glyphosate poisoning. Toxicol. Rev. 23:159–167. https://doi.org/10.2165/00139709-200423030-00003.

Brammall, R. A., and Higgins, V. J. 1988. The effect of glyphosate on resistance of tomato to *Fusarium* crown and root rot disease and on the formation of host structural defensive barriers. Can. J. Bot. 66:1547–1555. https://doi.org/10.1139/b88-213.

Bugg, T. D. H., Lloyd, A. J., and Roper, D. I. 2006. Phospho-MurNAc-pentapeptide translocase (MraY) as a target for antibacterial agents and antibacterial proteins. Infect. Disord. Drug Targets 6:85–106. https://doi.org/10.2174/187152606784112128.

Carranza, C. S., Aluffi, M. E., Benito, N., Magnoli, K., Barberis, C. L., and Magnoli, C. E. 2019. Effect of in vitro glyphosate on *Fusarium* spp. growth and disease severity in maize. J. Sci. Food Agric. 99:5064–5072. https://doi.org/10.1002/jsfa.9749.

Chen, B. J., and Jean, G. C. 1982. Active transport of formaldehyde in methanol-utilizing bacteria. Chem. Eng. J. 25:9–20. https://doi.org/10.1016/0300-9467(82)85017-x.

Chen, N. H., Djoko, K. Y., Veyrier, F. J., and McEwan, A. G. 2016. Formaldehyde stress responses in bacterial pathogens. Front. Microbiol. 7:257. https://doi.org/10.3389/fmicb.2016.00257.

Chistoserdova, L., Vorholt, J. A., Thauer, R. K., and Lidstrom, M. E. 1998. C1 transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic Archaea. Science 281:99–102. https://doi.org/10.1126/science.281.5373.99.

Corpe, W. A., and Rheem, S. 1989. Ecology of the methylotrophic bacteria on living leaf surfaces. FEMS Microbiol. Lett. 62:243–249. https://doi.org/10.1111/j.1574-6968.1989.tb03698.x.

Dallegrave, E., Mantese, F. D., Coelho, R. S., Pereira, J. D., Dalsenter, P. R., and Langeloh, A. 2003. The teratogenic potential of the herbicide glyphosate-Roundup in Wistar rats. Toxicol. Lett. 142:45–52. https://doi.org/10.1016/s0378-4274(02)00483-6.

Dill, G. M., Cajacob, C. A., and Padgette, S. R. 2008. Glyphosate-resistant crops: adoption, use and future considerations. Pest Manag. Sci. 64:326–331. https://doi.org/10.1002/ps.1501.

Dimitrijevic, D., Shaw, A. J., and Florence, A. T. 2000. Effects of some non-ionic surfactants on transepithelial permeability in Caco-2 cells. J. Pharm. Pharmacol. 52:157–162. https://doi.org/10.1211/0022357001773805.

Doronina, N. V., Ivanova, E. G., Suzina, N. E., and Trotsenko, Y. A. 2004. Methanotrophs and *Methylobacteria* are found in woody plant tissues within the winter period. Microbiology 73:702–709. https://doi.org/10.1007/s11021-005-0012-0.

Dourado, M. N., Camargo Neves, A. A., Santos, D. S., and Araújo, W. L. 2015. Biotechnological and agronomic potential of endophytic pink-pigmented methylotrophic *Methylobacterium* spp. Biomed Res. Int. 2015:909016. https://doi.org/10.1155/2015/909016.

Duke, S. O., and Powles, S. B. 2008. Glyphosate: a once-in-a-century herbicide: Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci. 64:319–325. https://doi.org/10.1002/ps.1518.

Esitken, A., Yildiz, H. E., Ercisli, S., Figen Donmez, M., Turan, M., and Gunes, A. 2010. Effects of plant growth promoting bacteria (PGPB) on yield, growth and nutrient contents of organically grown strawberry. Sci. Hortic. (Amsterdam) 124:62–66. https://doi.org/10.1016/j.scienta.2009.12.012.

de María, N., Becerril, J. M., García-Plazaola, J. I., Hernandez, A., De Felipe, M. R., and Fernandez-Pascual, M. 2006. New insights on glyphosate mode of action in

nodular metabolism: Role of shikimate accumulation. J. Agric. Food Chem. 54:2621–2628. https://doi.org/10.1021/jf058166c.

Fall, R., Benson, A. A. 1996. Leaf methanol-the simplest natural product from plants. Trends Plant Sci. 1(9): 296–301. https://doi.org/10.1016/S1360-1385(96)88175-0

Funke, T., Han, H., Healy-Fried, M. L., Fischer, M., and Schönbrunn, E. 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. Proc. Natl. Acad. Sci. U. S. A. 103:13010–13015. https://doi.org/10.1073/pnas.0603638103.

Galin, R. R., Akhtyamova, I. F., and Pastukhova, E. I. 2019. Effect of Herbicide Glyphosate on *Drosophila melanogaster* Fertility and Lifespan. Bull. Exp. Biol. Med. 167:663–666. https://doi.org/10.1007/s10517-019-04594-x.

Gholizadeh, A. 2012. Molecular evidence for the contribution of *Methylobacteria* to the pink-pigmentation process in pink-colored plants. J. Plant Interact. 7:316–321. https://doi.org/10.1080/17429145.2012.693208.

Gianessi, L. P. 2013. The increasing importance of herbicides in worldwide crop production: The increasing importance of herbicides. Pest Manag. Sci. 69:1099–1105. https://doi.org/10.1002/ps.3598.

Gourion, B., Francez-Charlot, A., and Vorholt, J. A. 2008. PhyR is involved in the general stress response of *Methylobacterium extorquens* AM1. J. Bacteriol. 190:1027–1035. https://doi.org/10.1128/JB.01483-07.

Green, P. N. 2006. *Methylobacterium*. In The Prokaryotes, New York, NY: Springer New York, pp. 257–265.

Gresshoff, P. M. 1979. Growth inhibition by glyphosate and reversal of its action by phenylalanine and tyrosine. Funct. Plant Biol. 6:177. https://doi.org/10.1071/pp9790177.

Gruffaz, C., Muller, E. E. L., Louhichi-Jelail, Y., Nelli, Y. R., Guichard, G., and Bringel, F. 2014. Genes of the N-methylglutamate pathway are essential for growth of *Methylobacterium extorquens* DM4 with monomethylamine. Appl. Environ. Microbiol. 80:3541–3550. https://doi.org/10.1128/AEM.04160-13.

Haney, R. L., Senseman, S. A., Hons, F. M., and Zuberer, D. A. 2000. Effect of glyphosate on soil microbial activity and biomass. Weed Sci. 48:89–93. https://doi.org/10.1614/0043-1745(2000)048[0089:eogosm]2.0.co;2.

Harvey, B. M. R., and Crothers, S. H. 1988. Causes of uneven desiccation of flax by glyphosate: poor penetration of the canopy and variable spray deposition. Ann. Appl. Biol. 112:195–200. https://doi.org/10.1111/j.1744-7348.1988.tb02055.x.

Hietanen, E., Linnainmaa, K., and Vainio, H. 1983. Effects of phenoxyherbicides and glyphosate on the hepatic and intestinal biotransformation activities in the rat.

Acta Pharmacol. Toxicol. (Copenh.) 53:103–112. https://doi.org/10.1111/j.1600-0773.1983.tb01876.x.

Hiraishi, A., Furuhata, K., Matsumoto, A., Koike, K. A., Fukuyama, M. and Tabuchi, K. 1995. Phenotypic and genetic diversity of chlorine-resistant *Methylobacterium* strains isolated from various environments. Appl Envrion Microbiol. 61(6): 2099–107. https://doi.org/10.1007/0-387-30745-1_14

Holland, M. 1997. *Methylobacterium* and plants. Recent Res Devel Plant Physiol;1:207–13.

Holliday, M. J. and Keen, N. T. 1982. The Role of Phytoalexins in the Resistance of Soybean Leaves to Bacteria: Effect of Glyphosate on Glyceollin Accumulation. 72(11):1470–4. https://doi.org/10.1094/Phyto-72-1470

Hua, T., Zhang, X., Tang, B., Chang, C., Liu, G., Feng, L., Yu, Y., Zhang, D., and Hou, J. 2018. Tween-20 transiently changes the surface morphology of PK-15 cells and improves PCV2 infection. BMC Vet. Res. 14:138. https://doi.org/10.1186/s12917-018-1457-5.

Indiragandhi, P., Anandham, R., Kim, K., Yim, W., Madhaiyan, M., and Sa, T. 2008. Induction of defense responses in tomato against *Pseudomonas syringae* pv. tomato by regulating the stress ethylene level with *Methylobacterium oryzae* CBMB20 containing 1-aminocyclopropane-1-carboxylate deaminase. World J. Microbiol. Biotechnol. 24:1037–1045. https://doi.org/10.1007/s11274-007-9572-7.

Irvine, I. C., Witter, M. S., Brigham, C. S. and Martiny, J. B. H. (2013). Relationships between *Methylobacteria* and glyphosate with native and invasive plant species: implications for restoration. Restor Ecol. 21(2):105–13. https://doi.org/10.1111/J.1526-100X.2011.00850.X

Ito, H. and Iizuka, H. 1971. Taxonomic studies on a radio-resistant *Pseudomonas* part xii. Studies on the microorganisms of cereal grain. 35(10): 1566–71. https://doi.org/10.1080/00021369.1971.10860119

Ivanova, E. G., Doronina, N. V., Shepelyakovskaya, A. O., Laman, A. G., Brovko, F. A. and Trotsenko, Y. A. 2000. Facultative and obligate aerobic *Methylobacteria* synthesize cytokinins. Mikrobiologiia. 69(6): 764–9.

Ivanova, E. G., Doronina, N. V. and Trotsenko, Y. A. 2001. Aerobic *Methylobacteria* are capable of synthesizing auxins. Microbiol. 70: 392–7. https://doi.org/10.1023/A:1010469708107

Jacobs, J. L., Carroll, T. L., and Sundin, G. W. 2005. The role of pigmentation, ultraviolet radiation tolerance, and leaf colonization strategies in the epiphytic survival of phyllosphere bacteria. Microb. Ecol. 49:104–113. https://doi.org/10.1007/s00248-003-1061-4.

Jiang, R., Wu, X., Xiao, Y., Kong, D., Li, Y., and Wang, H. 2021. Tween 20 regulate the function and structure of transmembrane proteins of *Bacillus cereus*: Promoting transmembrane transport of fluoranthene. J. Hazard. Mater. 403:123707. https://doi.org/10.1016/j.jhazmat.2020.123707.

Johal, G. S., and Huber, D. M. 2009. Glyphosate effects on diseases of plants. Eur. J. Agron. 31:144–152. https://doi.org/10.1016/j.eja.2009.04.004.

Johal, G. S., and Rahe, J. E. 1990. Role of phytoalexins in the suppression of resistance of *Phaseolus vulgaris* to *Colletotrichum lindemuthianum* by glyphosate. Can. J. Plant Pathol. 12:225–235. https://doi.org/10.1080/07060669009500992.

Jorge, G. L., Kisiala, A., Morrison, E., Aoki, M., Nogueira, A. P. O., and Emery, R. J. N. 2019. Endosymbiotic *Methylobacterium oryzae* mitigates the impact of limited water availability in lentil (*Lens culinaris* Medik.) by increasing plant cytokinin levels. Environ. Exp. Bot. 162:525–540. https://doi.org/10.1016/j.envexpbot.2019.03.028.

Kalyaeva, M. A., Ivanova, E. G., Doronina, N. V., Zakharchenko, N. S., Trotsenko, Y. A., and Buryanoc, Y. I. 2003. The Effect of Aerobic Methylotrophic Bacteria on the in vitro Morphogenesis of Soft Wheat (*Triticum aestivum*). Russ. J. Plant Physiol. 50:313–317. https://doi.org/10.1023/a:1023861918193.

Kamo, T., Hiradate, S., Suzuki, K., Fujita, I., Yamaki, S., Yoneda, T., Koitabashi, M., and Yoshida, S. 2018. Methylobamine, a UVA-Absorbing Compound from the Plant-Associated Bacteria *Methylobacterium* sp. Nat. Prod. Commun. 13:1934578X1801300. https://doi.org/10.1177/1934578x1801300208.

Kawanishi, M., Matsuda, T., and Yagi, T. 2014. Genotoxicity of formaldehyde: molecular basis of DNA damage and mutation. Front. Environ. Sci. 2. https://doi.org/10.3389/fenvs.2014.00036.

Keen, N. T. 1982. Effects of glyphosate on glyceollin production and the expression of resistance to *Phytophthora megasperma* f. sp. *glycineain* soybean. Phytopathology 72:1467. https://doi.org/10.1094/phyto-72-1467.

Kepler, R. M., Epp Schmidt, D. J., Yarwood, S. A., Cavigelli, M. A., Reddy, K. N., Duke, S. O., Bradley, C. A., Williams, M. M., II, Buyer, J. S., and Maul, J. E. 2020. Soil microbial communities in diverse agroecosystems exposed to the herbicide glyphosate. Appl. Environ. Microbiol. 86. https://doi.org/10.1128/AEM.01744-19.

Kirkwood, R. C., Hetherington, R., Reynolds, T. L., and Marshall, G. 2000. Absorption, localisation, translocation and activity of glyphosate in barnyardgrass (*Echinochloa crus-galli* (L) Beauv): influence of herbicide and surfactant concentration. Pest Manag. Sci. 56:359–367. https://doi.org/10.1002/(sici)1526-4998(200004)56:4<359::aid-ps145>3.0.co;2-s.

Kremer, R., Means, N., and Kim, S. 2005. Glyphosate affects soybean root exudation and rhizosphere micro-organisms. Int. J. Environ. Anal. Chem. 85:1165–1174. https://doi.org/10.1080/03067310500273146.

Kwak, M.-J., Jeong, H., Madhaiyan, M., Lee, Y., Sa, T.-M., Oh, T. K., and Kim, J. F. 2014. Genome information of *Methylobacterium oryzae*, a plant-probiotic methylotroph in the phyllosphere. PLoS One 9:e106704. https://doi.org/10.1371/journal.pone.0106704.

Lanzarin, G., Venâncio, C., Félix, L. M., and Monteiro, S. 2021. Inflammatory, oxidative stress, and apoptosis effects in zebrafish larvae after rapid exposure to a commercial glyphosate formulation. Biomedicines 9:1784. https://doi.org/10.3390/biomedicines9121784.

Larson, R. L., Hill, A. L., Fenwick, A., Kniss, A. R., Hanson, L. E., and Miller, S. D. 2006. Influence of glyphosate on *Rhizoctonia* and *Fusarium* root rot in sugar beet. Pest Manag. Sci. 62:1182–1192. https://doi.org/10.1002/ps.1297.

Laukel, M., Rossignol, M., Borderies, G., Völker, U., and Vorholt, J. A. 2004. Comparison of the proteome of *Methylobacterium extorquens* AM1 grown under methylotrophic and nonmethylotrophic conditions. Proteomics 4:1247–1264. https://doi.org/10.1002/pmic.200300713.

Lee, H.-L., and Guo, H.-R. 2011. The hemodynamic effects of the formulation of glyphosate-surfactant herbicides. In Herbicides, Theory and Applications, InTech.

Lee, Y., Krishnamoorthy, R., Selvakumar, G., Kim, K., and Sa, T. 2015. Alleviation of salt stress in maize plant by co-inoculation of arbuscular mycorrhizal fungi and *Methylobacterium oryzae* CBMB20. J Korean Soc Appl Biol Chem 58:533–540. https://doi.org/10.1007/s13765-015-0072-4.

Lévesque, C. A., Rahe, J. E., and Eaves, D. M. 1987. Effects of glyphosate on *Fusarium* spp.: its influence on root colonization of weeds, propagule density in the soil, and crop emergence. Can. J. Microbiol. 33:354–360. https://doi.org/10.1139/m87-062.

Lévesque, C. A., Rahe, J. E., and Eaves, D. M. 1992. The effect of soil heat treatment and microflora on the efficacy of glyphosate in seedlings. Weed Res. 32:363–373. https://doi.org/10.1111/j.1365-3180.1992.tb01897.x.

Levy, G., and Anello, J. A. 1969. Effect of polysorbate hydrolysis products on biologic membrane permeability. J. Pharm. Sci. 58:494–495. https://doi.org/10.1002/jps.2600580426.

Li, Z.-M., and Kannan, K. 2022. A method for the analysis of glyphosate, aminomethylphosphonic acid, and glufosinate in human urine using liquid chromatography-tandem mass spectrometry. Int. J. Environ. Res. Public Health 19:4966. https://doi.org/10.3390/ijerph19094966.

Lipka, V., and Panstruga, R. 2005. Dynamic cellular responses in plant-microbe interactions. Curr. Opin. Plant Biol. 8:625–631. https://doi.org/10.1016/j.pbi.2005.09.006.

Liu, Z., Shangguan, Y., Zhu, P., Sultan, Y., Feng, Y., Li, X., and Ma, J. 2022. Developmental toxicity of glyphosate on embryo-larval zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 236:113493. https://doi.org/10.1016/j.ecoenv.2022.113493.

Longoria-Espinoza, R. M., Félix-Gastélum, R., and Cordero-Ramírez, J. D. 2020. Diversity of endophytic bacteria associated with tomato plants (*Solanum lycopersicum*). Rev. Mex. Fitopatol. 38. https://doi.org/10.18781/r.mex.fit.2002-7.

Lu, L., Chai, Q., He, S., Yang, C., and Zhang, D. 2019. Effects and mechanisms of phytoalexins on the removal of polycyclic aromatic hydrocarbons (PAHs) by an endophytic bacterium isolated from ryegrass. Environ. Pollut. 253:872–881. https://doi.org/10.1016/j.envpol.2019.07.097.

Lynch, J. M., and Penn, D. J. 1980. Damage to cereals caused by decaying weed residues. J. Sci. Food Agric. 31:321–324. https://doi.org/10.1002/jsfa.2740310319.

Madhaiyan, M., Poonguzhali, S., Senthilkumar, M., Lee, J.-S., and Lee, K.-C. 2012. Methylobacterium gossipiicola sp. nov., a pink-pigmented, facultatively methylotrophic bacterium isolated from the cotton phyllosphere. Int. J. Syst. Evol. Microbiol. 62:162–167. https://doi.org/10.1099/ijs.0.030148-0.

Madhaiyan, M., Suresh Reddy, B. V., Anandham, R., Senthilkumar, M., Poonguzhali, S., Sundaram, S. P., and Sa, T. 2006. Plant growth-promoting *Methylobacterium* induces defense responses in groundnut (*Arachis hypogaea* L.) compared with rot pathogens. Curr. Microbiol. 53:270–276. https://doi.org/10.1007/s00284-005-0452-9.

Marx, C. J., Bringel, F., Chistoserdova, L., Moulin, L., Farhan Ul Haque, M., Fleischman, D. E., Gruffaz, C., Jourand, P., Knief, C., Lee, M.-C., Muller, E. E. L., Nadalig, T., Peyraud, R., Roselli, S., Russ, L., Goodwin, L. A., Ivanova, N., Kyrpides, N., Lajus, A., Land, M. L., Médigue, C., Mikhailova, N., Nolan, M., Woyke, T., Stolyar, S., Vorholt, J. A., and Vuilleumier, S. 2012. Complete genome sequences of six strains of the genus *Methylobacterium*. J. Bacteriol. 194:4746–4748. https://doi.org/10.1128/JB.01009-12.

Marx, C. J., Chistoserdova, L., and Lidstrom, M. E. 2003. Formaldehyde-detoxifying role of the Tetrahydromethanopterin-linked pathway in *Methylobacterium extorquens* AM1. J. Bacteriol. 185:7160–7168. https://doi.org/10.1128/jb.185.23.7160-7168.2003.

Meena, K. K., Kumar, M., Kalyuzhnaya, M. G., Yandigeri, M. S., Singh, D. P., Saxena, A. K., and Arora, D. K. 2012. Epiphytic pink-pigmented methylotrophic bacteria enhance germination and seedling growth of wheat (*Triticum aestivum*)

by producing phytohormone. Antonie Van Leeuwenhoek 101:777–786. https://doi.org/10.1007/s10482-011-9692-9.

Mekwatanakarn, P., and Sivasithamparam, K. 1987a. Effect of certain herbicides on saprophytic survival and biological suppression of the take-all fungus. New Phytol. 106:153–159. https://doi.org/10.1111/j.1469-8137.1987.tb04799.x.

Mekwatanakarn, P., and Sivasithamparam, K. 1987b. Effect of certain herbicides on soil microbial populations and their influence on saprophytic growth in soil and pathogenicity of take-all fungus. Biol. Fertil. Soils 5. https://doi.org/10.1007/bf00257655.

Metz, B., Kersten, G. F. A., Hoogerhout, P., Brugghe, H. F., Timmermans, H. A. M., de Jong, A., Meiring, H., ten Hove, J., Hennink, W. E., Crommelin, D. J. A., and Jiskoot, W. 2004. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. J. Biol. Chem. 279:6235–6243. https://doi.org/10.1074/jbc.M310752200.

Miller, S. I., and Salama, N. R. 2018. The gram-negative bacterial periplasm: Size matters. PLoS Biol. 16:e2004935. https://doi.org/10.1371/journal.pbio.2004935.

Muller, K., Herrera, K., Talyn, B., and Melchiorre, E. 2021. Toxicological Effects of Roundup® on *Drosophila melanogaster* Reproduction. Toxics 9:161. https://doi.org/10.3390/toxics9070161.

Nakanishi, K., Masada, M., and Nadai, T. 1983. Effect of pharmaceutical adjuvants on the rectal permeability of drugs. III. Effect of repeated administration and recovery of the permeability. Chem. Pharm. Bull. (Tokyo) 31:4161–4166. https://doi.org/10.1248/cpb.31.4161.

Nysanth, N. S., Rajan, S. A., Sivapriya, S. L., and Anith, K. N. 2023. Pink Pigmented Facultative Methylotrophs (PPFMs): Potential Bioinoculants for Sustainable Crop Production. J. Pure Appl. Microbiol. 17:660–681. https://doi.org/10.22207/jpam.17.2.17.

Nalayini, P., Anandham, R., Raj, S. P. and Chidambaram, P. 2014. Pink pigmented facultative methylotrophic bacteria (PPFMB)-a potential bioinoculant for cotton nutrition physico-chemical and biological studies of arecanut YLD disease affected soil. Cotton Res J. 6(1):50–3.

Nassiri, M., Kaykhaii, M., Hashemi, S. H. and Sephai, M. 2018. Spectrophotometric determination of formaldehyde in seawater samples after *in-situ* derivatization and dispersive liquid-liquid microextraction. 37(1): 89–98.

Nayak, D. D., and Marx, C. J. 2014. Methylamine utilization via the N-methylglutamate pathway in *Methylobacterium extorquens* PA1 involves a novel flow of carbon through C1 assimilation and dissimilation pathways. J. Bacteriol. 196:4130–4139. https://doi.org/10.1128/JB.02026-14.

Naznin, H. A., Kimura, M., Miyazawa, M., and Hyakumachi, M. 2013. Analysis of volatile organic compounds emitted by plant growth-promoting fungus Phoma sp. GS8-3 for growth promotion effects on tobacco. Microbes Environ. 28:42–49. https://doi.org/10.1264/jsme2.me12085.

Nemecek-Marshall, M., MacDonald, R. C., Franzen, J. J., Wojciechowski, C. L., and Fall, R. 1995. Methanol emission from leaves (enzymatic detection of gas-phase methanol and relation of methanol fluxes to stomatal conductance and leaf development). Plant Physiol. 108:1359–1368. https://doi.org/10.1104/pp.108.4.1359.

Newman, M. M., Hoilett, N., Lorenz, N., Dick, R. P., Liles, M. R., Ramsier, C., and Kloepper, J. W. 2016. Glyphosate effects on soil rhizosphere-associated bacterial communities. Sci. Total Environ. 543:155–160. https://doi.org/10.1016/j.scitotenv.2015.11.008.

Nikaido, H. 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264:382–388. https://doi.org/10.1126/science.8153625.

Nilsson, H. and Hallgren, E. 1990. Influence on *Elymus repens* of Roundup (glyphosate) in different concentrations (different volume rates): a greenhouse experiment.

Omer, Z. S., Tombolini, R., and Gerhardson, B. 2004. Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). FEMS Microbiol. Ecol. 47:319–326. https://doi.org/10.1016/S0168-6496(04)00003-0.

Ortega-Atienza, S., Rubis, B., McCarthy, C., and Zhitkovich, A. 2016. Formaldehyde is a potent proteotoxic stressor causing rapid heat shock transcription factor 1 activation and Lys48-linked polyubiquitination of proteins. Am. J. Pathol. 186:2857–2868. https://doi.org/10.1016/j.ajpath.2016.06.022.

O'sullivan, P. A., and O'donovan, J. T. 1980. Influence of various herbicides and Tween 20 on the effectiveness of glyphosate. Can. J. Plant Sci. 60:939–945. https://doi.org/10.4141/cjps80-137.

Palberg, D., Kisiała, A., Jorge, G. L., and Emery, R. J. N. 2022. A survey of *Methylobacterium* species and strains reveals widespread production and varying profiles of cytokinin phytohormones. BMC Microbiol. 22:49. https://doi.org/10.1186/s12866-022-02454-9.

Patt, T. E., Cole, G. C., and Hanson, R. S. 1976. *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. Int. J. Syst. Bacteriol. 26:226–229. https://doi.org/10.1099/00207713-26-2-226.

Pedrosa, F. O., Monteiro, R. A., Wassem, R., Cruz, L. M., Ayub, R. A., Colauto, N. B., Fernandez, M. A., Fungaro, M. H. P., Grisard, E. C., Hungria, M., Madeira, H. M. F., Nodari, R. O., Osaku, C. A., Petzl-Erler, M. L., Terenzi, H., Vieira, L. G. E., Steffens, M. B. R., Weiss, V. A., Pereira, L. F. P., Almeida, M. I. M., Alves,

L. R., Marin, A., Araujo, L. M., Balsanelli, E., Baura, V. A., Chubatsu, L. S., Faoro, H., Favetti, A., Friedermann, G., Glienke, C., Karp, S., Kava-Cordeiro, V., Raittz, R. T., Ramos, H. J. O., Ribeiro, E. M. S. F., Rigo, L. U., Rocha, S. N., Schwab, S., Silva, A. G., Souza, E. M., Tadra-Sfeir, M. Z., Torres, R. A., Dabul, A. N. G., Soares, M. A. M., Gasques, L. S., Gimenes, C. C. T., Valle, J. S., Ciferri, R. R., Correa, L. C., Murace, N. K., Pamphile, J. A., Patussi, E. V., Prioli, A. J., Prioli, S. M. A., Rocha, C. L. M. S. C., Arantes, O. M. N., Furlaneto, M. C., Godoy, L. P., Oliveira, C. E. C., Satori, D., Vilas-Boas, L. A., Watanabe, M. A. E., Dambros, B. P., Guerra, M. P., Mathioni, S. M., Santos, K. L., Steindel, M., Vernal, J., Barcellos, F. G., Campo, R. J., Chueire, L. M. O., Nicolás, M. F., Pereira-Ferrari, L., Silva, J. L. da C., Gioppo, N. M. R., Margarido, V. P., Menck-Soares, M. A., Pinto, F. G. S., Simão, R. de C. G., Takahashi, E. K., Yates, M. G., and Souza, E. M. 2011. Genome of *Herbaspirillum seropedicae* strain SmR1, a specialized diazotrophic endophyte of tropical grasses. PLoS Genet. 7:e1002064. https://doi.org/10.1371/journal.pgen.1002064.

Periyasamy, P., Ilamurugu, K., and Senthilkumar, M. 2016. Characterization of endophytic plant growth promoting traits of *Methylobacterium* sp. isolated from banana (*Musa* sp.). International Journal of Forestry and Crop 7:114–120. https://doi.org/10.15740/HAS/IJFCI/7.1/114-120.

Pline, W. A., Price, A. J., Wilcut, J. W., Edmisten, K. L., and Wells, R. 2001. Absorption and translocation of glyphosate in glyphosate-resistant cotton as influenced by application method and growth stage. Weed Sci. 49:460–467. https://doi.org/10.1614/0043-1745(2001)049[0460:aatogi]2.0.co;2.

Pojer, F., Li, S.-M., and Heide, L. 2002. Molecular cloning and sequence analysis of the clorobiocin biosynthetic gene cluster: new insights into the biosynthesis of aminocoumarin antibiotics. Microbiology 148:3901–3911. https://doi.org/10.1099/00221287-148-12-3901.

Pushpakanth, P., Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore-641 003, India, Krishnamoorthy, R., Anandham, R., Senthilkumar, M., Department of Crop Management, Vanavarayar Institute of Agriculture, Pollachi- 642 103, India, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore-641 003, India, and Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore-641 003, India. 2021. Biotization of tissue culture banana plantlets with *Methylobacterium salsuginis* to enhance the survival and growth under greenhouse and open environment condition. J. Environ. Biol. 42:1452–1460. https://doi.org/10.22438/jeb/42/6/mrn-1707.

Rashid, A., Hwang, S. F., Ahmed, H. U., Turnbull, G. D., Strelkov, S. E., and Gossen, B. D. 2013. Effects of soil-borne *Rhizoctonia solani* on canola seedlings after application of glyphosate herbicide. Can. J. Plant Sci. 93:97–107. https://doi.org/10.4141/cjps2012-109.

Reddy, K. N., Rimando, A. M., and Duke, S. O. 2004. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-

resistant soybean. J. Agric. Food Chem. 52:5139–5143. https://doi.org/10.1021/jf049605v.

Renier, A., Jourand, P., Rapior, S., Poinsot, V., Sy, A., Dreyfus, B., and Moulin, L. 2008. Symbiotic properties of *Methylobacterium nodulans* ORS 2060T: A classic process for an atypical symbiont. Soil Biol. Biochem. 40:1404–1412. https://doi.org/10.1016/j.soilbio.2007.12.020.

Ribeiro, A., Pochart, P., Day, A., Mennuni, S., Bono, P., Baret, J.-L., Spadoni, J.-L., and Mangin, I. 2015. Microbial diversity observed during hemp retting. Appl. Microbiol. Biotechnol. 99:4471–4484. https://doi.org/10.1007/s00253-014-6356-5.

Richmond, M. E. 2018. Glyphosate: A review of its global use, environmental impact, and potential health effects on humans and other species. J. Environ. Stud. Sci. 8:416–434. https://doi.org/10.1007/s13412-018-0517-2.

Rosenbaum, K. K., Miller, G. L., Kremer, R. J., and Bradley, K. W. 2014. Interactions between glyphosate, *Fusarium* Infection of common waterhemp (*Amaranthus rudis*), and soil microbial abundance and diversity in soil collections from Missouri. Weed Sci. 62:71–82. https://doi.org/10.1614/ws-d-13-00071.1.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. 2008. Bacterial endophytes: recent developments and applications. FEMS Microbiol. Lett. 278:1–9. https://doi.org/10.1111/j.1574-6968.2007.00918.x.

Ryu, Jeounghyun, Madhaiyan M., Poonguzhali, S., Yim, W., Indiragandhi, P., Kim, K., Anandham, R., Yun, J. and Sa, T. 2006. Plant Growth Substances Produced by *Methylobacterium* spp. and Their Effect on Tomato (*Lycopersicon esculentum* L.) and Red Pepper (*Capsicum annuum* L.) Growth. J. Microbiol. Biotechnol. 16:1622-1628.

Sanogo, S., Yang, X. B., and Scherm, H. 2000. Effects of Herbicides on Fusarium solani f. sp. glycines and Development of Sudden Death Syndrome in Glyphosate-Tolerant Soybean. Phytopathology 90:57–66. https://doi.org/10.1094/PHYTO.2000.90.1.57.

Savin, M. C., Purcell, L. C., Daigh, A., and Manfredini, A. 2009. Response of mycorrhizal infection to glyphosate applications and P fertilization in glyphosate-tolerant soybean, maize, and cotton. J. Plant Nutr. 32:1702–1717. https://doi.org/10.1080/01904160903150941.

Schweizer, M., Brilisauer, K., Triebskorn, R., Forchhammer, K., and Köhler, H.-R. 2019. How glyphosate and its associated acidity affect early development in zebrafish (*Danio rerio*). PeerJ 7:e7094. https://doi.org/10.7717/peerj.7094.

Senthilkumar, M., and Krishnamoorthy, R. 2017. Isolation and characterization of tomato leaf phyllosphere *Methylobacterium* and their effect on plant growth. Int. J. Curr. Microbiol. Appl. Sci. 6:2121–2136. https://doi.org/10.20546/ijcmas.2017.611.250.

Senthilkumar, M., Pushpakanth, P., Arul Jose, P., Krishnamoorthy, R., and Anandham, R. 2021. Diversity and functional characterization of endophytic *Methylobacterium* isolated from banana cultivars of South India and its impact on early growth of tissue culture banana plantlets. J. Appl. Microbiol. 131:2448–2465. https://doi.org/10.1111/jam.15112.

Seo, D.-J., Nguyen, D.-M.-C., Song, Y.-S., and Jung, W.-J. 2012. Induction of defense response against *Rhizoctonia solani* in cucumber plants by endophytic bacterium *Bacillus thuringiensis* GS1. J. Microbiol. Biotechnol. 22:407–415. https://doi.org/10.4014/jmb.1107.07027.

Sharma, S. D., and Singh, M. 2001. Environmental factors affecting absorption and bio-efficacy of glyphosate in Florida beggarweed (*Desmodium tortuosum*). Crop Prot. 20:511–516. https://doi.org/10.1016/s0261-2194(01)00065-5.

Sharon, A., Amsellem, Z., and Gressel, J. 1992. Glyphosate suppression of an elicited defense response : Increased susceptibility of *Cassia obtusifolia* to a mycoherbicide. Plant Physiol. 98:654–659. https://doi.org/10.1104/pp.98.2.654.

Sikorski, J. A., and Gruys, K. J. 1997. Understanding Glyphosate's Molecular Mode of Action with EPSP Synthase: Evidence Favoring an Allosteric Inhibitor Model. Acc. Chem. Res. 30:2–8. https://doi.org/10.1021/ar950122+.

Smejkalová, H., Erb, T. J., and Fuchs, G. 2010. Methanol assimilation in *Methylobacterium extorquens* AM1: demonstration of all enzymes and their regulation. PLoS One 5:e13001. https://doi.org/10.1371/journal.pone.0013001.

Steinrücken, H. C., and Amrhein, N. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94:1207–1212. https://doi.org/10.1016/0006-291x(80)90547-1.

Suhadolnik, R. J., and Chenoweth, R. G. 1958. Biosynthesis of gliotoxin. I.1 incorporation of phenylalanine-1- and -2-C14. J. Am. Chem. Soc. 80:4391–4392. https://doi.org/10.1021/ja01549a071.

Suhaimi, N. S. M., Goh, S.-Y., Ajam, N., Othman, R. Y., Chan, K.-G., and Thong, K. L. 2017. Diversity of microbiota associated with symptomatic and non-symptomatic bacterial wilt-diseased banana plants determined using 16S rRNA metagenome sequencing. World J. Microbiol. Biotechnol. 33:168. https://doi.org/10.1007/s11274-017-2336-0.

Sviridov, A. V., Shushkova, T. V., Ermakova, I. T., Ivanova, E. V., Epiktetov, D. O., and Leontievsky, A. A. 2015. Microbial degradation of glyphosate herbicides (Review). Appl. Biochem. Microbiol. 51:188–195. https://doi.org/10.1134/s0003683815020209.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., and Dreyfus, B. 2001. Methylotrophic

Methylobacterium bacteria nodulate and fix nitrogen in symbiosis with legumes. J. Bacteriol. 183:214–220. https://doi.org/10.1128/JB.183.1.214-220.2001.

Tani, A., Ogura, Y., Hayashi, T., and Kimbara, K. 2015a. Complete Genome Sequence of *Methylobacterium aquaticum* Strain 22A, Isolated from *Racomitrium japonicum* Moss. Genome Announc. 3. https://doi.org/10.1128/genomeA.00266-15.

Tani, A., Sahin, N., Fujitani, Y., Kato, A., Sato, K., and Kimbara, K. 2015b. *Methylobacterium* species promoting rice and barley growth and interaction specificity revealed with whole-cell matrix-assisted laser desorption / ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) analysis. PLoS One 10:e0129509. https://doi.org/10.1371/journal.pone.0129509.

Toews, J., Rogalski, J. C., Clark, T. J., and Kast, J. 2008. Mass spectrometric identification of formaldehyde-induced peptide modifications under *in vivo* protein cross-linking conditions. Anal. Chim. Acta 618:168–183. https://doi.org/10.1016/j.aca.2008.04.049.

Tong, M., Gao, W., Jiao, W., Zhou, J., Li, Y., He, L., and Hou, R. 2017. Uptake, translocation, metabolism, and distribution of glyphosate in nontarget tea plant (*Camellia sinensis* L.). J. Agric. Food Chem. 65:7638–7646. https://doi.org/10.1021/acs.jafc.7b02474.

Van Aken, B., Yoon, J. M., and Schnoor, J. L. 2004. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* x *nigra* DN34). Appl. Environ. Microbiol. 70:508–517. https://doi.org/10.1128/AEM.70.1.508-517.2004.

Ventorino, V., Sannino, F., Piccolo, A., Cafaro, V., Carotenuto, R., and Pepe, O. 2014. *Methylobacterium populi* VP2: plant growth-promoting bacterium isolated from a highly polluted environment for polycyclic aromatic hydrocarbon (PAH) biodegradation. ScientificWorldJournal 2014:931793. https://doi.org/10.1155/2014/931793.

Vorholt, J. A., Marx, C. J., Lidstrom, M. E., and Thauer, R. K. 2000. Novel formaldehyde-activating enzyme in *Methylobacterium extorquens* AM1 required for growth on methanol. J. Bacteriol. 182:6645–6650. https://doi.org/10.1128/JB.182.23.6645-6650.2000.

Watson, M. C., Bartels, P. G., and Hamilton, K. C. 1980. Action of selected herbicides and Tween 20 on oat (*Avena sativa*) membranes. Weed Sci. 28:122–127. https://doi.org/10.1017/s0043174500027910.

White, A. K., and Metcalf, W. W. 2004. Two C-P lyase operons in *Pseudomonas stutzeri* and their roles in the oxidation of phosphonates, phosphite, and hypophosphite. J. Bacteriol. 186:4730–4739. https://doi.org/10.1128/JB.186.14.4730-4739.2004.

Yoshida, S., Hiradate, S., Koitabashi, M., Kamo, T., and Tsushima, S. 2017. Phyllosphere *Methylobacterium* bacteria contain UVA-absorbing compounds. J. Photochem. Photobiol. B 167:168–175. https://doi.org/10.1016/j.jphotobiol.2016.12.019.

Zervas, A., Zeng, Y., Madsen, A. M., and Hansen, L. H. 2019. Genomics of aerobic photoheterotrophs in wheat phyllosphere reveals divergent evolutionary patterns of photosynthetic genes in Methylobacterium spp. Genome Biol. Evol. 11:2895–2908. https://doi.org/10.1093/gbe/evz204.

Zgurskaya, H. I., Löpez, C. A., and Gnanakaran, S. 2015. Permeability barrier of Gram-negative cell envelopes and approaches to bypass it. ACS Infect. Dis. 1:512–522. https://doi.org/10.1021/acsinfecdis.5b00097.

Zgurskaya, H. I., and Rybenkov, V. V. 2020. Permeability barriers of Gram-negative pathogens: Antibiotic permeation. Ann. N. Y. Acad. Sci. 1459:5–18. https://doi.org/10.1111/nyas.14134.

Zhan, H., Feng, Y., Fan, X., and Chen, S. 2018. Recent advances in glyphosate biodegradation. Appl. Microbiol. Biotechnol. 102:5033–5043. https://doi.org/10.1007/s00253-018-9035-0.

Zhang, C., Wang, M.-Y., Khan, N., Tan, L.-L., and Yang, S. 2021a. Potentials, utilization, and bioengineering of plant growth-promoting *Methylobacterium* for sustainable agriculture. Sustainability 13:3941. https://doi.org/10.3390/su13073941.

Zhang, L. L., Chen, J. M., and Fang, F. 2008. Biodegradation of methyl t-butyl ether by aerobic granules under a cosubstrate condition. Appl. Microbiol. Biotechnol. 78:543–550. https://doi.org/10.1007/s00253-007-1321-1.

Zhang, W., Wang, J., Song, J., Feng, Y., Zhang, S., Wang, N., Liu, S., Song, Z., Lian, K., and Kang, W. 2021b. Effects of low-concentration glyphosate and aminomethyl phosphonic acid on zebrafish embryo development. Ecotoxicol. Environ. Saf. 226:112854. https://doi.org/10.1016/j.ecoenv.2021.112854.

Zobiole, L. H. S., Kremer, R. J., Oliveira, R. S., Jr, and Constantin, J. 2011. Glyphosate affects micro-organisms in rhizospheres of glyphosate-resistant soybeans: Glyphosate affects micro-organisms in rhizospheres. J. Appl. Microbiol. 110:118–127. https://doi.org/10.1111/j.1365-2672.2010.04864.x.

CHAPTER 4

4.1. PREFACE

Title:	Compatibility of commercial fungicide formulations with plant-associated *Methylobacterium.*
Authors:	Daniel Palberg and R. J. Neil Emery
Reference:	Published in *Canadian Journal of Plant Science* (2024), 105: 1-10 doi: 10.1139/cjps-2024-0169
Copyright:	This work was originally published under the CC BY license, which permits unrestricted reuse with proper attribution.
Contributions:	Conceptualization, investigation, methodology, formal data analysis, figure and table creation, and writing of the initial draft by D.P. Review of methodology, project supervision, and acquisition of funding by R.J.N.E. All authors read and approved the final manuscript.

CHAPTER 4

Compatibility of commercial fungicide formulations with plant-associated *Methylobacterium*

Published in: *Canadian Journal of Plant Science* (2024), 105: 1-10

4.2. ABSTRACT

Symbiotic *Methylobacterium* comprise a significant part of the plant microbiome and are known to benefit host plant growth, development, tolerance to abiotic stress, and enhanced disease resistance. The wide application of commercial broad-spectrum fungicide formulations in contemporary agriculture practices has necessitated the investigation of compatibility between popular pesticide products and bacterial endophytes, especially as the *Methylobacterium* are increasingly considered for agronomic use, including biocontrol of phytopathogens. This study provides an evaluation of compatibility between an extensive inventory of 40 *Methylobacterium* strains in response to commercial pesticide formulations, each containing different active ingredients: DYNASTY and QUADRIS (azoxystrobin), MAXIM 480 (fludioxonil), and APRON XL LS (metalaxyl-M). Using a diffusion disk assay, no sensitivity of tested *Methylobacterium* strains could be detected against any fungicide product, at doses within and above the recommended therapeutic window (1–100 μg). Potency of formulations across the same range were confirmed using the sensitive phytopathogen *Fusarium graminearum*. As *Methylobacterium* spp. continue to emerge as suitable candidates for various roles in biotechnology, including agriculture, a better understanding on the compatibility between this important genus and commercial fungicide products has become a relevant consideration for integrated pest management practices.

KEYWORDS: azoxystrobin, fludioxonil, metalaxyl-M, plant growth-promoting bacteria, phyllosphere, bioinoculants

4.3. INTRODUCTION

Complex and pervasive fungal phytopathogens, including *Rhizoctonia* spp. and *Fusarium* spp., inflict profound impacts due to their cosmopolitan distribution and wide host range. For example, *Rhizoctonia solani*, is predominantly recognized as the causal agent of sheath blight in rice (*Oryzae sativa*) (Chahal et al. 2003), black scurf and stem rot in potato (*Solanum tuberosum*) (Kiptoo et al. 2021), and hypocotyl rot in soybean (*Glycine max*) (Ajayi-Oyetunde and Bradley 2018), but the soil borne necrotrophy is also pathogenic to over 200 other plant species belonging to the Amaranthaceae (e.g., sugar beet), Brassicaceae (e.g., broccoli, brussel sprouts, cabbage, cauliflower, kohlrabi, radish, and turnip), Fabaceae (e.g., alfalfa, chickpea, lentil, peanut, and soybean), Poaceae (e.g., barley, maize, oat, rice, wheat), Rubiaceae (e.g., coffee), and Solanaceae (e.g., potato, tobacco) (Keijer et al. 1997; Chahal et al. 2003; Zheng et al. 2013). Similarly, members of the *Fusarium graminearum* species complex predominate globally as the causal agents of Fusarium head blight in the socioeconomically important cereals, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oat (*Avena sativa*), and maize (*Zea mays*) (Tóth et al. 2005; Ramirez et al. 2006; Ward et al. 2008; Yang et al. 2008; Yli-Mattilaetal. 2009; Boutigny et al. 2011; VanDerLee et al. 2015).

Synthetic fungicides are therefore often used prophylactically as seed-coatings, foliar sprays, or post-harvest applications on fruit, and newer formulations now exist to treat active outbreaks in the field. Of significant importance are the strobilurin (group 11), acylalanine (group 4), and phenylpyrrole (group 12) fungicide families – of which the most established members include azoxystrobin, metalaxyl-M, and fludioxonil, respectively (Figure 4.1.). The discovery of these compounds represented a break-through in agrochemistry, as the systemic and broad-spectrum activity of metalaxyl-M

and azoxystrobin in particular, provided three tiers of action simultaneously: (a) preventing the ingress, (b) curing existing infections, and (c) eradicating phytopathogen load in the field. Using different mechanisms of action, these three agents are frequently deployed in pairs and sometimes all together (MAXIM QUATTRO, Syngenta, Canada). Studies investigating the impact of fungicide formulations on plant-associated bacteria have focused mainly on population dynamics within the rhizosphere and, to date, generally tend to report two possible outcomes: (a) no perceptible change in the population density or diversity richness of rhizospheric bacteria, or (b) an initial decrease in bacterial population density followed by recovery (Bailey and Coffey 1986; Sukul and Spiteller 2001a, 2001b; Sturz and Peters 2007; Sukul et al. 2008; Wang et al. 2015; Álvarez-Martín et al. 2016; Meena et al. 2020). Interestingly, the degradation of metalaxyl-M by soil microbes has been well established in literature, with some studies suggesting that soil bacteria may become sensitized following repeated exposure (Bailey and Coffey 1986; Adetutu et al. 2008). This presents challenges in the control of phytopathogens, as degradation of fungicides by soil microbes may diminish concentrations to sub-therapeutic levels.

Interestingly, there have even been reported increases to both population density and total endophyte species richness in response to metalaxyl-M compared to controls (Barak et al. 1984). While the mechanism of action of metalaxyl is believed to be partly from interruption of RNA biosynthesis in susceptible fungi, recent studies suggest that the compound may potentially control pathogen bioburden through stimulation of helpful soil endophytes (Sturz and Peters 2007). However, investigation of the impact of fungicides and commercially available formulations on phyllosphere-dwelling bacterial endophytes remains largely unexplored.

The *Methylobacterium* (family Methylobacteriaceae, class Alphaproteobacteria) are generally plant-associated bacteria with cosmopolitan distribution that have been isolated from a variety of natural sources, including soil (Dourado et al. 2015), air (Hiraishi et al. 1995), municipal water (Furuhata et al. 2006), humans (Anestietal.2004), and fermented food (Aslam et al. 2007). Several species have also been collected from a range of seemingly hostile environments, including spacecraft (Bijlani et al. 2021) and contaminated industrial sites (Ventorino et al. 2014; Phukon et al. 2024). *Methylobacterium* are aerobic, gram-negative, and their distinct pink pigmentation is an identifiable attribute of the genus, with some exceptions (*Methylobacterium jeotgali*) (Aslam et al. 2007; Green 2006). Despite consideration for use in biotechnology (Aken et al. 2004; Zhang et al. 2008), the body of work concerning the agronomic potential of *Methylobacterium* continues to expand quickly (Holland 1997a; Madhaiyan et al. 2006; Ryan et al. 2008; Meena et al. 2012; Dourado et al. 2015; Tani et al. 2015; Zhang et al. 2021). Classified as plant growth-promoting bacteria (PGPB), certain strains of *Methylobacterium* produce uniquely high levels of phytohormones including the cytokinins (CK), which boost photosynthetic capability and improve seed setting (Holland 1997b; Ivanova et al. 2000, 2001; Palberg et al. 2022). The longstanding association of *Methylobacterium* to the plant microbiome (Corpe and Rheem 1989), then, is thought most likely to be sustained by methanol emissions from heightened cell wall remodelling stimulated in response to growth-promoting phytohormones of microbial origin (Abanda-Nkpwatt et al. 2006; Šmejkalová et al. 2010). *Methylobacterium* spp. may also provide protection of the host-plant from biotic stress, including phytopathogens, as has been established in their promotion of peroxidase activity in groundnut (Madhaiyan et al. 2006) and inducing microbiome population shifts and phytoalexin biosynthesis in tomato (Ryu et al. 2006;

Indiragandhi et al. 2008; Senthilkumar and Krishnamoorthy 2017). In response to evidence of the role of *Methylobacterium* in crop health, several commercial bioinoculant products have already reached the international market. However, compatibility of biological crop-protection agents with contemporary farming techniques and agrochemicals remains a vitally important consideration. The integrity of the phyllosphere microbiome has been linked to host productivity by improving photosynthetic capacity, flowering, and fruit filling of economically important crops (Stone et al. 2018; Thapa and Prasanna 2018; Abadi et al. 2020).

The integration of bioinoculants with fungicidal treatment therefore presents an optimal path for phytopathogen management: enhancing pathogen control through synergistic effects, extending the duration of control, and reducing fungicidal resistance. It is the objective of this study to examine the sensitivity of a wide range of *Methylobacterium* isolates, unique in source and phenotype, to commercially available products containing three popular broad-spectrum fungicides: azoxystrobin, fludioxonil, and metalaxyl-M. To the knowledge of the authors, this study is the first comprehensive evaluation of compatibility between *Methylobacterium* spp. and commercial fungicide products.

4.4. MATERIALS AND METHODS

Chemicals and materials

Commercial fungicide formulations were used in this study: DYNASTY 100FS (Syngenta Canada, azoxystrobin 10% w/v, PCP Reg. No. 28394), QUADRIS (Syngenta Canada, azoxystrobin 23% w/v, PCP Reg. No. 26153), MAXIM 480FS (Syngenta Canada, fludioxonil 40% w/v, PCP Reg. No. 27001), and APRON XL LS (Syngenta Canada, metalaxyl-M 33% w/v, PCPReg.No.25585). Due to the proprietary nature of the commercial formulations, exact ingredients, including adjuvants including the type and quantity of surfactants used, could not be determined. Pure forms of each fungicidal compound were sourced from Millipore Sigma. Ampicillin sodium salt (BioShop ; Burlington, Canada) was used as a positive control. Ultra-pure water (18.2 MΩ•cm) was obtained from a Milli-Q system. Sterile, blank paper disks (Oxoid, Fisher Scientific) of 6 mm diameter (~1 mm thickness) were obtained to conduct a modified disk diffusion (Kirby Bauer) sensitivity assay.

Selection of bacterial strains and culture conditions

Preparation of bacterial cultures were conducted aseptically in a biological safety cabinet (BSC). Freeze-dried stock cultures of *Methylobacterium* spp. were obtained from four microbe collections: the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), the *Deutsche Sammlung von Mikroorganismenund Zellkulturen* (DSMZ) ("German Collection of Microorganisms and Cell Cultures"), the Japan Collection of Microorganisms (JCM), and the National Institute of Technology and Evaluation's Biological Resource Center (NBRC). Strains were originally collected from different biological (living plants) and natural non-biological sources (soil, water, air). Information on strain taxonomy, origin, and known characteristics is provided in

Table1. The freeze-dried strains were revived in nutrient rich Reasoner's 2A (R2A) broth (VWR, Mississauga, Canada) and cryogenically maintained as 15% (v/v) glycerol stocks at −80 ºC.

Revival of cryogenic stocks

Methylobacterium strains were aseptically streaked on nutrient-rich R2A using a sterile loop. After 5 days of incubation at 27 ºC, single colonies were extracted from each plate and used to inoculate 50 mL of R2A liquid growth media in 250 mL glass Erlenmeyer flasks and maintained in a rotary incubator for 5 days (27 ºC and 120 RPM). When *Methylobacterium* cultures reached the late exponential/early stationary phase after approximately 6 days (OD_{600} = 0.6–1.2, depending on strain, approximately 108 CFU/mL) (Genesys™ 10s Visible Spectrophotometer, Thermo Fisher Scientific) nutrient minimum agar plates were inoculated from the TSB liquid media using a sterile loop and streak method. Selective minimum nutrient medium was prepared in accordance with the DSMZ ("German Collection of Microorganisms and Cell Cultures") recipe for *Methylobacterium* growth media (DSMZ Index 125): KNO3— 1.00 g; $MgSO_4 \times 7H_2O$— 0.2 g; $CaCl_2 \times 2H_2O$— 0.02 g; Na_2HPO_4— 0.23 g; NaH_2PO_4— 0.07 g; $FeSO_4 \times 7H^2O$— 1.00 mg; CuSO4 × 5H2O— 5 µg; H_3BO_3— 10µg;$MnSO_4 \times 5H_2O$— 10µg; $ZnSO_4$ × 7H2O— 70µg;MoO_3— 10 µg; H_2O— 1000 mL; CH_3OH— 5 mL; pH 6.80. Transfer to a minimum nutrient medium was conducted to ensure high selectivity for Methylobacterium and reduce potential for contamination of refrigerated stock plates. After 5 days of incubation at 27 ºC, single colonies of each bacterium were extracted from each plate and used to inoculate culture flasks, as necessary. DSMZ nutrient-minimum plates were kept as stock plates, sealed with parafilm and maintained at 4 ºC for a maximum of 20 days.

Preparation of disk diffusion assay

The Kirby Bauer sensitivity assay is a method for visually assessing sensitivity of microbial strains to specific compounds using impregnated paper disks and agar plates. In this study, sterile 6 mm paper disks (Oxoid, Fisher Scientific) were loaded with each fungicide formulation and deposited on an R2A agar plate immediately post-inoculation with a strain of Methylobacterium. Optimization of the disk loading volume was carried out previously and determined to be 20 µL based on dryness after 120 min. Fungicide formulations were handled aseptically and were not filter-sterilized prior to use. Sterile isotonic solution (0.9% NaCl [w/v]) was used as a diluent to achieve desired concentrations relative to the active ingredient (AI) in each formulation. Final concentrations included in the sensitivity assay were selected with the intent to span the recommended application rate for use on soybean (Glycine max) seed treatment as indicated in the product monograph provided by Syngenta Canada Inc. for each formulation. Based on 100 kg (~6,600 seeds) Syngenta recommends: DYNASTY 1.5 –3.0 µg AI (azoxystrobin), APRON XL 23 µg AI (metalaxyl-M), and MAXIM 6 µg AI (fludioxonil) per seed. The QUADRIS (azoxystrobin) foliar spray monograph recommends an application rate of 500 mL/ha for group 6 crops, including soybean. Assuming a successful stand of 135,000 plants per acre (~335,000 /ha OMAFRA) and the minimum recommended dilution of 500 mL per 45 L freshwater, the maximum expected concentration of azoxystrobin to reach each plant would be ~8–10 µg (0.14 mL/plant of the tank mix).

Concentrations of fungicide formulations, relative to the concentration of the AI in each product, were therefore uniformly tested at final total doses of 1, 10, 50, and 100 µg of each AI using a paper disk sensitivity assay. Positive control of ampicillin

HCl was prepared at the same concentrations as fungicide products. Sterile isotonic solution was used to load a negative control disk. Preparation of paper disks, including serial dilution of fungicide products, and the loading of each disk across all treatment cohorts were carried out aseptically. Loading solutions were prepared by serial dilution and 20 µL of each solution was transferred to paper disks contained in empty sterile glass Petri dishes using a filtered pipette. Each Petri dish was covered and allowed to dry for 2 h.

Fungicide formulation sensitivity assay

As use of a nutrient-minimum DSMZ 125 greatly increased the time required to obtain a confluent lawn of Methylobacterium, nutrient-rich R2A medium was used for the disk diffusion sensitivity assays. Petri dishes (Fisher Brand, 100 × 15mm) containing uniform volumes of 20 mL R2A (1.5% agar [w/v]) were divided into quadrants using permanent marker on the outer surface of the base. 100 µL of a 7-day old (~10^8 CFU/mL) undiluted liquid culture (R2A) was deposited onto the surface of each separate test plate. The inoculum was evenly spread across the surface of the agar using an L-shaped spreader, then covered and allowed to dry for 30 minutes in a BSC in darkness at approximately 22 ºC. Using sterile forceps, dry paper disks containing fungicide formulations (prepared on the previous day) were transferred to the test plates, in the center of each corresponding quadrant. A negative control disk was placed at the center of each Petri dish, at the intersection of quadrant lines. A positive control (ampicillin) was placed in a separate quadrant (Figure 2). Plates were sealed with parafilm and stored inverted in darkness at 27 ºC. The zone of inhibition surrounding each disk was assessed and photographed (Geliance 600 Imaging System, Perkin Elmer) at 3-, 5-, and 7-days post-inoculation.

Basic photograph processing, including adjustment of brightness and contrast for clear visibility of microbial growth, was achieved using GeneSnap (ver. 7.04.06, Synoptics Ltd.) (Figure 4.3.). Zone of inhibition was determined by measuring entire cross section of the region devoid of microbial growth, inclusive of the paper disk diameter. Exposure experiments of each bacterial strain to each fungicide formulation was carried out in triplicate at each of the concentration levels. The experiment was also replicated using Escherichia coli (ß-lactam sensitive, NM522) to validate the performance of ampicillin control disks. Method control plates were developed using identical techniques as assay plates, without inoculation with *Methylobacterium*. Method control plates were used to validate aseptic technique and confirm that commercial products did not contain viable organisms.

Verification of diffusion

To verify the functionality of the modified disk diffusion assay, a second iteration of the experiment was conducted with an isolate of *F. graminearum* obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH)(Gage Research Institute, University of Toronto, Canada) (UAMH 3329). Verification was undertaken with a known susceptible organism to ensure that the material of the paper disks, in combination with the complex formulation of commercial products, did not inhibit the ability of the fungicidal AI to diffuse into the growth area. Briefly, a 2 mm agar plug was collected from the edge of a refrigerated 14-day-old stock plate of *F. graminearum* and transferred to the center of a fresh PDA plate, aseptically. Paper disks containing commercial fungicide formulations, were placed in three respective quadrants, and a negative control (water) in the remaining quadrant. Test plates were sealed with parafilm and incubated at 27 ◦C inverted for 5 days. Confirmation of

effectivity was conducted across the tested dosage range (1–100 μg AI) in triplicate. Diffusion was considered successful if the susceptible organism failed to grow in the quadrant containing fungicide disks after 5 days.

4.5. RESULTS

Insensitiveness of Methylobacterium to commercial fungicides

Across all 40 strains of *Methylobacterium* examined, no measurable zone of inhibition was detected in response to any of the fungicide formulations tested (Figure 4.3.), even at the highest concentration of 100 µg. In several instances, many *Methylobacterium* strains were observed to colonize paper disks treated with formulations containing metalaxyl-M (APRON XL LS) (Figure 4.2.). However, this behaviour was not observed for products containing azoxystrobin or fludioxonil.

Due to the well-documented propensity for *Methylobacterium* isolates to form aggregates in broth, confluent growth of many *Methylobacterium* isolates can form uneven lines and ridges (Figure 4.3.). Efforts to disrupt aggregation using ethylene glycol tetra acetic acid (EGTA) has proven unsuccessful, as this agent, while not lethal and effective at dispersing aggregates, does greatly affect culturability (data not shown). In nearly all cases, however, sensitivity to the ampicillin positive control was observed, although the measurable zone of inhibition was <20 mm across all 40 strains tested in response to a 100 µg dose, suggesting the genus is only moderately sensitive to ß-lactam antibiotics (Figure 3). Notably, despite the variability in ampicillin sensitivity between *Methylobacterium extorquens* (JCM 2805), *Methylobacterium organophilum* (NBRC 103119), and *Methylobacterium radiotolerans* (LMG 2269), all three isolates display total insensitivity to fungicide formulations even at the maximum dose (100µg), which was observed for all other tested isolates (Figure 4.3.). Importantly, the *E. coli* check plate displayed high sensitivity (>20 mm) to ampicillin, as expected, through the tested range (1–100 µg), further verifying the diffusion effectivity of the positive control (Figure 4.3.).

No discernable differences in sensitivity of *Methylobacterium* could be established between formulations designed for seed coating and foliar spray. Control disks loaded with sterile isotonic solution showed no indication of sensitivity in any strain. Importantly, method control plates showed no microbial growth after incubation for 10 days at 27 ºC in darkness, confirming that the commercial formulations did not contain microbial contamination. Replication of the paper disk sensitivity assay using pure forms of each AI could not be conducted due to the inherently low solubility of each compound in deionized water: approximately 6.7, 1.8, and 7.1 mg/L for azoxystrobin, fludioxonil, and metalaxyl-M, respectively. Absent discernible sensitivity to commercial formulations each containing an AI along with a myriad of adjuvants and surfactants, amendment of pure water–fungicide suspensions with additives in an attempt to improve solubility and permeability was unwarranted.

Experiments involving *F. graminearum* confirmed the effectivity of the modified disk diffusion method, using identical methodology as sensitivity assays involving *Methylobacterium* spp. Decreases to fungal proliferation on PDA was immediately evident after 120 h across the tested concentration range (88% decrease in mycelial growth area relative to controls), in addition to obvious preferential growth towards the negative control disk (Figure 4.4.).

4.6. DISCUSSION

Investigating the compatibility of commercial agrochemicals and sustainable biological products is essential for promoting effective farming practices, safeguarding human health, managing pesticide resistance, and ensuring the long-term sustainability of agriculture. Our work has provided the first comprehensive examination of the compatibility of four widely applied fungicide formulations with phyllosphere-associated *Methylobacterium* isolates. Results indicate complete compatibility between *Methylobacterium* and the fungicide formulations tested, through in vitro sensitivity assays.

Proliferation and morphology of all tested strains were undisrupted by the presence of paper disks impregnated with commercial fungicides, and no measurable zone of inhibition in any experimental replicate was discernable, irrespective of the tested AI concentration. Confirmation of potency with the susceptible phytopathogen *F. graminearum* confirms effectiveness of the disk–diffusion method, as mycelial growth was strongly inhibited in quadrants containing disks laced with fungicide formulations. While research concerning the effects of fungicides on the plant microbiome have focused predominantly on the rhizosphere, available studies on phyllospheric bacteria repeatedly report the Proteobacteria (α-, β-, and γ-) as the dominant bacterial colonizers in most plants. Sensitivity of phyllosphere bacteria to fungicides varies greatly based on the AI, the product formulation, and the nature of the host plant. However, the Proteobacteria – to which *Methylobacterium* belongs – consistently show resilience to fungicide treatments both *in situ* and *in vitro*. For example, foliar treatments with formulations containing to enostroburin, kresoxim–methyl, and iprodione resulted in increases to abundance of *Alphaproteobacteria* in wheat, tobacco, and pepper,

respectively (Gu et al. 2010; Katsoula et al. 2020; Xiang et al. 2022). In plate assays exposing epi- and endophytic bacteria isolated from grapevine (*Vitis vinifera*) to seven different commercial fungicide products, 97% of *Firmicutes* (40/41) – formerly "*Bacillota*" – showed sensitivity to four or more formulations, while only 12% of *Proteobacteria* (strictly β- and γ-) isolates (5/42) were similarly affected (Andreolli et al. 2023). In the treatment of the destructive foliar disease, tobacco target spot caused by Rhizoctonia solani, applications of AMISTAR TOP (EPA Reg. 100–1313) – a commercial concentrate containing azoxystrobin (group 11) and difenoconazole (group 3) – induced an increase in the overall diversity of the phyllosphere bacterial community and, importantly, had little effect on the relative abundance of epiphytic *Methylobacterium* over an 18-day treatment period (Sun et al. 2023). This phylum-level resilience, however, is not universal. For example, foliar treatments with dimethachlon (N-(3,5-dichlorophenyl) succinimide) for control of tobacco brown spot (*Alternaria alternata*) resulted in significant decreases to *Alphaproteobacteria* abundance, which the *Methylobacterium* predominate in the natural host (Nicotiana tabacum) (Chen et al. 2021). Research concerning the impact of the phenylamide (metalaxyl-M) and phenylpyrrole (fludioxonil) fungicide classes on the phyllosphere is similarly low, despite their broad application. Polyfluorinated fungicides like fludioxonil in particular, are typified by their slow degradation in natural soil environments with half-lives often exceeding 100 days due to low microbial-driven metabolism (Marinozzi et al. 2013; Alexandrino et al. 2020). In a recent study, a strain of methylotrophic *Methylobacillus* sp. (β-proteobacteria) isolated from soil previously enriched with fludioxonil was unable to meaningfully catabolize the fungicide under controlled conditions but remained otherwise unaffected by its presence (Alexandrino et al. 2021).

With respect to metalaxyl-M, studies examining soil flora generally report reductions in population density, which eventually recovers despite sustained fungicide administration (Sukul and Spiteller 2001a; Sukul et al. 2008; Wang et al. 2015). Prevailing theories suggest that the initial bacterial population decline represents the elimination of metalayl-M intolerant strains, redistributing resource availability for tolerant strains to rebound. Research concerning the effects of metalaxyl-M on the phyllosphere is even more limited. An available study on the response of the pepper (Capsicum annum L. cv Ozho) microbiome to foliar applications of metalaxyl-M (REDOMIL GOLD SL, EPA Reg. 100-1202) revealed a positive response of Enterobacteriaceae (γ-proteobacteria) to treatment (Moulas et al. 2013). Our work, however, reports the first genus-level information for the responses of phyllospheric bacteria to these same ingredients, and provides direct evidence of their compatibility with *Methylobacterium*.

Incorporation of PGPB like the *Methylobacterium* as part of contemporary integrated pest management (IPM) practices present new opportunities to improve nutrient utilization and stress tolerance (Jorge et al. 2019). Interestingly, several products containing one or more strains of *Methylobacterium* are already marketed: M-BOS™ (NutriAg LTD; Ontario, Canada), Utrisha™ and BlueN™ (acquired by Corteva Biologicals; Alberta, Canada originally from Symborg Inc.; California, USA), METILO™ (Tangsons Biotech; Changsha, China), and GreenPlus™ (Farmer's BioFertilizers; Tamil Nadu, India). With wide crop compatibility and application versatility (seed treatment, furrow drench, and foliar spray), products containing PGPB are marketed as a safe and renewable way to fortify crops. Compatibility studies are therefore crucial for determining whether these biological products can be used

effectively alongside synthetic chemicals. Tank-mixing and co-application of products specifically can result in significant cost-savings as overall expenses related to fuel consumption, water utilization, labour, soil compaction, and mechanical damage to crops can be reduced (Gandinietal.2020; Anil et al. 2024). These factors are important considerations for crops with fast vegetative growth and high susceptibility to fungal phytopathogens (strawberry), which require a more aggressive pesticide regimen. Tank-mix restrictions, however, are nearly universal to microbiome-enhancing products as product labels often advise against co-administration with most commercial agrochemicals. Through our work on *Methylobacterium*, we report evidence of compatibility between all isolates and each of the tested commercial products, at both sub-clinical (1 µg) and excessive (100 µg) concentrations of the AI. Our results suggest that under certain situations, the use of fungicides is safe with respect to the viability of PGPB in the phyllosphere. Notably, the fungicides studied here rank among the top 5 AIs by number of registered products in the United States of America (first, second, and fourth, respectively) (Maino et al. 2023). For example, in a search of the plant product database (Crop Data Management Systems Inc.) and the Pesticide Product and Label System of the United States Environmental Protection Agency (US EPA), azoxystrobin appears as the sole systemic fungicidal agent in 34 of 82 products (41%) licensed for use in barley, 42 of 107 (39%) for maize, 27 of 99 (27%) for potato, 48 of 138 for soybean (35%), and 30 of 110 (27%) registered for strawberry. Variability is even more restrictive for specialty crops: out of 19 products licensed for vanilla (*Vanilla* spp.) and saffron (*Crocus sativus*), 18 contain azoxystrobin as the sole AI. Compatibility with biological products promotes practices that cushion the dependence on synthetic fungicides for crop protection. Coined chemically induced endophyte-mediated disease suppression, researchers have found instances where the application of fungicides,

including metalaxyl-M, enhance disease protection by stimulating plant-associated microbes – even against previously metalaxyl-resistant phytopathogens (Sturz and Peters 2007). Reduced input costs, improved soil health, and enhanced disease resistance are the core pillars of a strong IPM architecture – of which, the use of beneficial endophytes has now become part (Ruiu 2020; Maitra et al. 2021; Gomis-Cebolla and Berry 2023; Irsad et al. 2023; Shahwar et al. 2023). The results of the present study support existing literature regarding the general insensitivity and tolerance of the Proteobacteria to fungicides, provide further corroboration of that trend at the genus level in *Methylobacterium*, and report direct evidence for the feasibility of co-application of the tested commercial agents with microbiome-enhancing products containing PGP *Methylobacterium*.

4.7. CONCLUSION

Our work concerning the compatibility of *Methylobacterium* with popular commercial fungicide products indicates that members of this genus tolerate contact with the fungicidal agents in their full formulation, in concentrations that not only simulate, but exceed the recommended AI concentration specified by the manufacturer. The results of our work suggest that proper adherence to product monograph instructions for product dilution would permit tank mixes of *Methylobacterium* as a biocontrol agent alongside contemporary chemical agents, without injury to the bioinoculant. Perseverance in the study of PGPB, including *Methylobacterium* spp., may produce feasible agricultural amendments, which bolster annual yields.

4.8. TABLES AND FIGURES

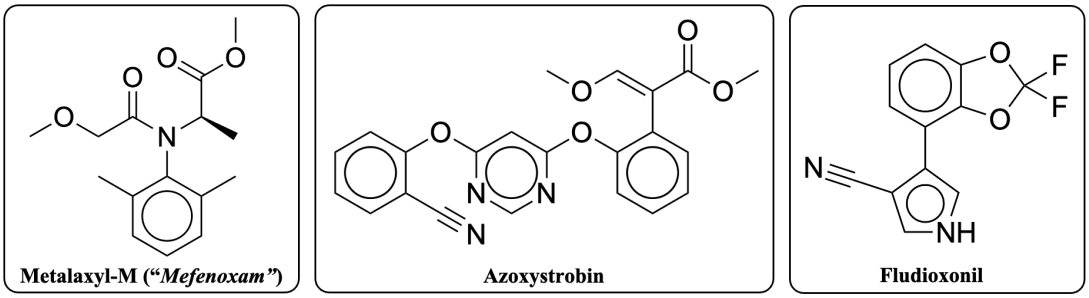

Figure 4.1: chemical structure of three popular fungicidal active agents: metalaxyl-M, azoxystrobin, and fludioxonil.

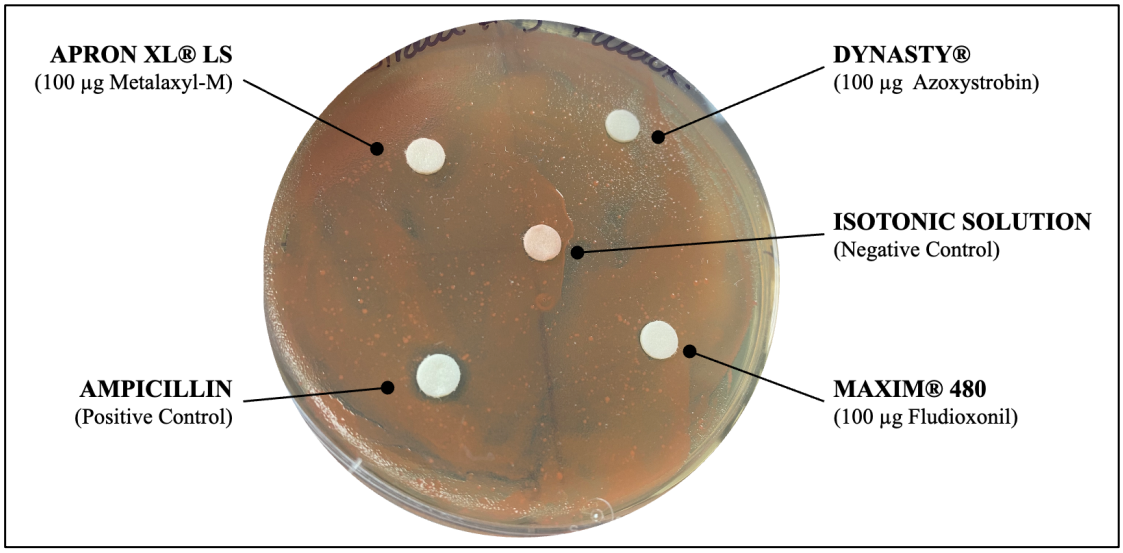

Figure 4.2: Plain light photograph of *Methylobacterium oryzae* (LMG 23582) sensitivity assay against the highest AI concentration after 14-days incubation at 26ºC. Use of ampicillin as positive control (100 µg) produces a small (< 2 mm) zone of inhibition and lack of paper disk colonization. Paper disks with fungicide formulations show no zone of inhibition at 100 µg AI.

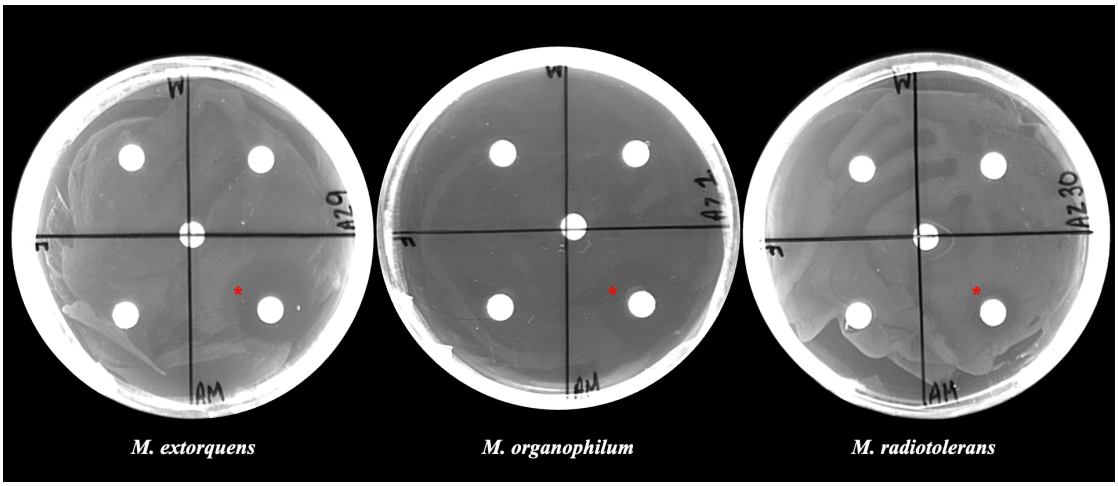

Figure 4.3: Monochromatic photograph of fungicide formulation assay conducted with [from left] *M. extorquens* (JCM 2805), *M. organophilum* (NBRC 103119), and *M. radiotolerans* (LMG 2269) using highest AI concentration (100 µg). Use of ampicillin as positive control produces moderate (> 4 mm) zone of inhibition indicated with a red asterix.

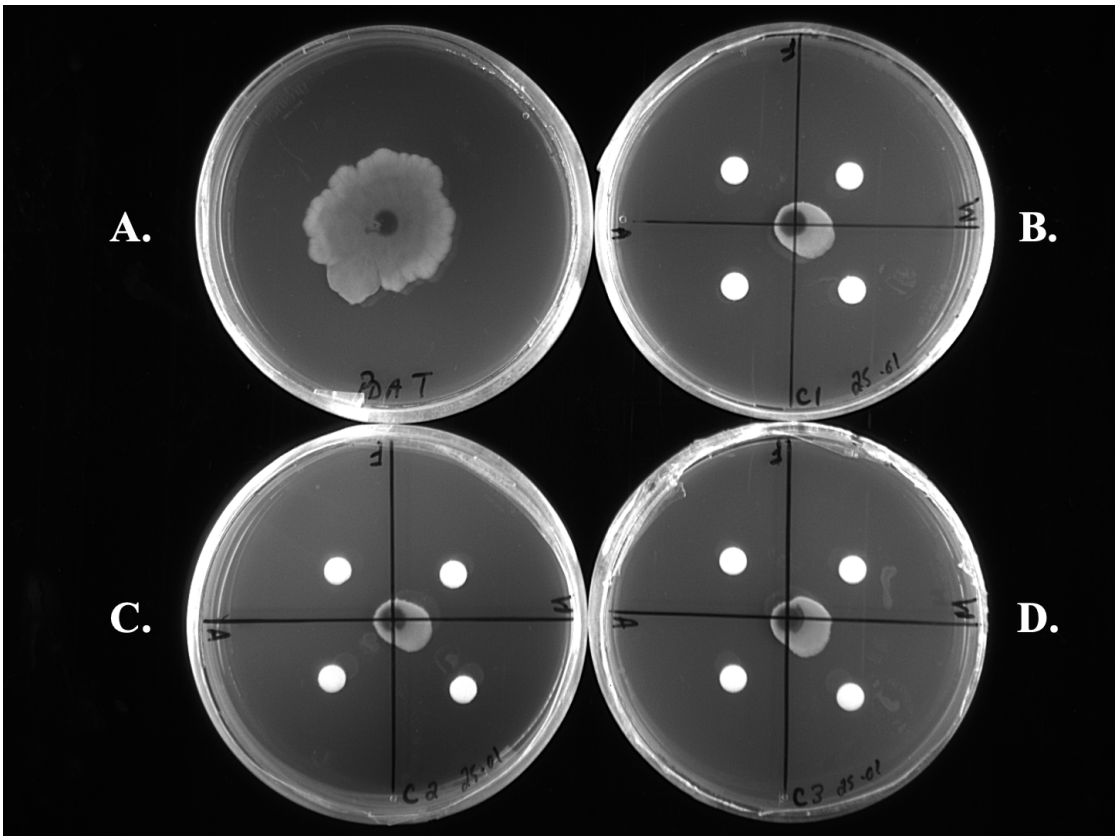

Figure 4.4: Monochromatic photograph of control (A), and test plates (B-D) containing *Fusarium graminearum* (UAMH 3329) agar plugs in response to commercial fungicide formlations using highest AI concentration (100 µg) at 120 hours. From lower left quadrant, clockwise: DYNASTY® (azoxystrobin), MAXIM®480 (fludioxonil), APRON XL® LS (metalaxyl-M), and isotonic (0.9% NaCl) control. Test plates indicating reduced mycelial growth rate and preferential migration towards control disk in each test plate.

Table 4.1: Inventory of *Methylobacterium* strains examined for fungicide formulation sensitivity.

Species	Strain	Isolation Source
M. aerolatum	JCM 16406 *(T)*	Air
M. aminovorans	LMG 21752 *(T)*	Soil
M. aquaticum	B-59286	Phoenix spacecraft surface
M. cerastii	DSM 23679 *(T)*	*Cerasium holosteoides* - phyllosphere
M. extorquens	B-1048	Garden soil enriched with sarcosine
	JCM 2805	Air
	NBRC 15687 *(T)*	Soil
	JCM 2806	Garden soil; slough
	DSM 13060	*Pinus sylvestris* - meristem tissue cultures
	DSM 23939	*Arabidopsis thaliana* - phyllosphere
	JCM 2803	*Psychotria mucronata* - phyllosphere
	JCM 20693	Mine water
	NBRC 103126	Soil-litter close to *Rumex* sp.
	NBRC 103127	Soil-litter close to *Arum* sp.
	NBRC 103129	Soil-litter close to *Eucalyptus* sp.
M. gnaphalii	NBRC 107716 *(T)*	*Gnaphalium spicatum* - phyllosphere
M. gossipiicola	B-51692 *(T)*	*Gossipium hirsutum* - phyllosphere
M. jeotgali	LMG-23639 *(T)*	Traditional fermented seafood (jeotgal)
M. mesophilicum	B-14246 *(T)*	*Lolium perenne* - phyllosphere
	B-2390	Household well water
M. nodulans	LMG-21967 *(T)*	*Crotalaria podocarpa* - phyllosphere
M. organophilum	LMG-6083 *(T)*	Lake water; sediment
M. organophilum	NBRC 103119	*Pelargonium zonale* - phyllosphere
	NBRC 103120	*Ficus elastica* - phyllosphere
	NBRC 103121	*Begonia* sp. - phyllosphere
M. oryzae	LMG-23582 *(T)*	*Oryzae sativa* cv. Nam-Pyeoung - surface-disinfected stem
M. oxalidis	NBRC 107715 *(T)*	*Oxalis corniculata* - phyllosphere
M. phyllosphaerae	LMG-24361 *(T)*	*Oryza sativa* cv. Dong-Jin - leaf tissues
M. platani	JCM 14648 *(T)*	*Platanus orientalis* - phyllosphere
M. radiotolerans	LMG-2269 *(T)*	Japanese unpolished (unhulled) aged commercial rice grain
	LMG-6379	Forest soil
M. rhodinum	LMG-2275 *(T)*	Alder (*Alnus*) - rhizosphere
M. thiocyanatum	JCM 10893 *(T)*	*Allium aflatuense* - rhizosphere soil
	NBRC 103122	*Bryophyllum* sp. - phyllosphere
M. thiocyanatum	NBRC 103128	Soil-litter close to *Mesenbryanthemum* sp.
	NBRC 103130	Soil-litter close to *Rumex* sp.
M. zatmanii	LMG-6087 *(T)*	
Methylobacterium spp.	DSM 23936	*Medicago truncatula* - phyllosphere
	JCM 14673	*Oryza sativa* SC-41 - phyllosphere
	JCM 14674	*Oryza rufipogon* W1964 - phyllosphere

(T) Indicates "type strain" cultures that were descended from a strain designated as the nomenclatural type.

4.9. REFERENCES

Abadi, V.A.J.M., Sepehri, M., Rahmani, H.A., Zarei, M., Ronaghi, A., Taghavi, S.M., and Shamshiripour, M. 2020. Role of dominant phyllosphere bacteria with plant growth–promoting characteristics on growth and nutrition of maize (*Zea mays L.*). J. Soil Sci. Plant Nutr. 20(4): 2348–2363. https://doi.org/10.1007/s42729-020-00302-1.

Abanda-Nkpwatt, D., Müsch, M., Tschiersch, J., Boettner, M. and Schwab, W. 2006. Molecular interaction between *Methylobacterium extorquens* and seedlings: growth promotion, methanol consumption, and localization of the methanol emission site. J. Exp. Bot. 57(15): 4025–4032. https://doi.org/10.1093/jxb/erl173.

Adetutu, E. M., Ball, A. S., and Osborn, A. M. 2008. Azoxystrobin and soil interactions: degradation and impact on soil bacterial and fungal communities. J. Appl. Microbiol. 105(6): 1777–1790. https://doi.org/10.1111/j.1365-2672.2008.03948.x.

Ajayi-Oyetunde, O. O., and Bradley, C.A. 2018. *Rhizoctonia solani*: taxonomy, population biology and management of rhizoctonia seedling disease of soybean. Plant Pathol. 67(1): 3–17. https://doi.org/10.1111/ppa.12733.

Aken, B. V., Aken, B. V., Yoon, J. M., Yoon, J. M., Schnoor, J. L., and Schnoor, J. L. 2004. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *methylobacterium* sp. associated. Appl. Environ. Microbiol. 70(1): 508–517. https://doi.org/10.1128/AEM.70.1.508.

Alexandrino, D. A. M., Mucha, A. P., Almeida, C. M. R., and Carvalho, M. F. 2020. Microbial degradation of two highly persistent fluorinated fungicides - epoxiconazole and fludioxonil. J. Hazard. Mater. 394: 122545. https://doi.org/10.1016/j.jhazmat.2020.122545.

Alexandrino, D. A. M., Mucha, A. P., Tomasino, M. P., Almeida, C. M. R., and Carvalho, M. F. 2021. Combining culture-dependent and independent approaches for the optimization of epoxiconazole and fludioxonil-degrading bacterial consortia. Microorganisms, 9(10): 2109. https://doi.org/10.3390/microorganisms9102109.

Álvarez-Martín, A., Hilton, S. L., Bending, G. D., Rodríguez-Cruz, M. S., and Sánchez-Martín, M. J. 2016. Changes in activity and structure of the soil microbial community after application of azoxystrobin or pirimicarb and an organic amendment to an agricultural soil. Appl. Soil Ecol. 106: 47–57. https://doi.org/10.1016/j.apsoil.2016.05.005.

Andreolli, M., Lampis, S., Tosi, L., Marano, V., and Zapparoli, G. 2023. Fungicide sensitivity of grapevine bacteria with plant growth-promoting traits and antagonistic activity as non-target microorganisms. World J. Microbiol. Biotechnol. 39(5): 121. https://doi.org/10.1007/s11274-023-03569-5.

Anesti, V., Vohra, J., Goonetilleka, S., McDonald, I. R., Sträubler, B., Stackebrandt, E., Kelly, D. P., and Wood, A. P. 2004. Molecular detection and isolation of facultatively methylotrophic bacteria, including *Methylobacterium podarium* sp. nov., from the human foot microflora. Environ. Microbiol. 6:820–830. https://doi.org/10.1111/j.1462-2920.2004.00623.x.

Anil, M., Hugar, S. V., Channakeshava, R. and Huilgol, S. N. 2024. Compatibility studies on selective insecticides, fungicides and water-soluble fertilizer mixtures in soybean. J. Sci. Res. Rep. 30(4): 95–102. https://doi.org/10.9734/jsrr/2024/v30i41894.

Aslam, Z., Lee, C. S., Kim, K. H., Im, W. T., Ten, L. N. and Lee, S. T. 2007. *Methylobacterium jeotgali* sp. nov., a non-pigmented, facultatively methylotrophic bacterium isolated from jeotgal, a traditional Korean fermented seafood. Int. J. Syst. Evol. Microbiol. 57(3): 566–571. https://doi.org/10.1099/ijs.0.64625-0.

Bailey, A.M., and Coffey, M.D. 1986. Characterization of microorganisms involved in accelerated biodegradation of metalaxyl and metolachlorin soils. Can. J. Microbiol. 32(7): 562–569. https://doi.org/10.1139/m86-105.

Barak, E., Edgington, L.V., and Ripley, B.D. 1984. Bioactivity of the fungicide metalaxyl in potato tubers against some species of phytophthora, fusarium, and aiternaria, related to polyphenoloxidase activity. Can. J. Plant Pathol. 6: 304–308. https://doi.org/10.1080/07060668409501533.

Bijlani, S., Singh, N.K., Eedara, V.V.R., Podile, A.R., Mason, C.E., Wang, C.C.C., and Venkateswaran, K. 2021. *Methylobacterium ajmalii* sp. nov., isolated from the International Space Station. Front. Microbiol.12: 1–14. https://doi.org/10.3389/fmicb.2021.639396.

Boutigny, A.-L., Ward, T.J., Van Coller, G.J., Flett, B., Lamprecht, S.C., O'Donnell, K., and Viljoen, A. 2011. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. Fungal Genet. Biol. 48(9): 914–920. https://doi.org/10.1016/j.fgb.2011.05.005.

Chahal, K.S., Sokhi, S.S., and Rattan, G.S. 2003. Investigations on sheath blight of rice In Punjab. Indian Phytopathol. 56(1): 22–26.

Chen, X., Wicaksono, W. A., Berg, G. and Cernava, T. 2021. Bacterial communities in the plant phyllosphere harbour distinct responders to a broad-spectrum pesticide. Sci. Total Environ. 751: 141799. https://doi.org/10.1016/j.scitotenv.2020.141799.

Corpe, W.A. and Rheem, S. 1989. Ecology of the Methylotrophic bacteria on living leaf surfaces. FEMS Microbiol. Lett. 62(4): 243–249. https://doi.org/10.1016/0378-1097(89)90248-6.

Dourado, M.N., Camargo Neves, A.A., Santos, D.S., and Araújo, W.L. 2015. Biotechnological and agronomic potential of endophytic pink-pigmented methylotrophic *Methylobacterium* spp. Biomed. Res. Int. 2015. https://doi.org/10.1155/2015/909016.

Furuhata, K., Kato, Y., Goto, K., Hara, M., Yoshida, S.I., and Fukuyama, M. 2006.Isolation and identification of *Methylobacterium* species from the tap water in hospitals in Japan and their antibiotic susceptibility. Microbiol. Immunol. 50(1): 11–17. https://doi.org/10.1111/j.1348-0421.2006.tb03765.x.

Gandini, E.M.M., Costa, E.S.P., Dos Santos, J.B., Soares, M.A., Barroso, G.M., Corrêa, J.M., et al. 2020. Compatibility of pesticides and/or fertilizers in tank mixtures. J. Cleaner Prod. 268: 122152. https://doi.org/10.1016/j.jclepro.2020.122152.

Gomis-Cebolla, J., and Berry, C. 2023. *Bacillus thuringiensis* as a biofertilizer in crops and their implications in the control of phytopathogens and insect pests. Pest Manage. Sci. 79(9): 2992–3001. https://doi.org/10.1002/ps.7560.

Green, P. N. 2006. *Methylobacterium*. In The Prokaryotes, New York, NY: Springer New York, pp. 257–265.

Gu, L., Bai, Z., Jin, B., Hu, Q., Wang, H., Zhuang, G., and Zhang, H. 2010. Assessing the impact of fungicide enostrobur in application on bacterial community in wheat phyllosphere. J. Environ. Sci. 22(1):134–141. https://doi.org/10.1016/S1001-0742(09)60084-X.

Hiraishi, A., Furuhata, K., Matsumoto, A., Koike, K.A., Fukuyama, M., and Tabuchi, K. 1995. Phenotypic and genetic diversity of chlorine resistant *Methylobacterium* strains isolated from various environments. Appl. Environ. Microbiol. 61(6): 2099–2107. https://doi.org/10.1128/aem.61.6.2099-2107.1995.

Holland, M.A. 1997a. *Methylobacterium* and plants. Recent Res. Dev. Plant Physiol. 207–213.

Holland, M. A. 1997b. Occam's razor applied to hormonology. Plant Physiol. 115: 865–868. https://doi.org/10.1104/pp.115.3.865.

Indiragandhi, P., Anandham, R., Kim, K., Yim, W., Madhaiyan, M., and Sa, T. 2008. Induction of defense responses in tomato against *Pseudomonas syringae* pv. tomato by regulating the stress ethylene level with *Methylobacterium oryzae* CBMB20 containing 1-aminocyclopropane-1-carboxylate deaminase. World J. Microbiol. Biotechnol. 24:1037–1045. https://doi.org/10.1007/s11274-007-9572-7.

Irsad, Shahid, M., Haq, E., Mohamed, A., Rizvi, P.Q., and Kolanthasamy, E. 2023. Entomopathogen-based biopesticides: insights into unraveling their potential in insect pest management. Front. Microbiol. 14:1208237. https://doi.org/10.3389/fmicb.2023.1208237.

Ivanova, E.G., Doronina, N.V., and Trotsenko, Y.A. 2001. Aerobic *Methylobacteria* are capable of synthesizing auxins. Microbiology, 70(4):392–397. https://doi.org/10.1023/A:1010469708107.

Ivanova, E.G., Doronina, N.V., Shepelyakovskaya, A.O., Laman, A.G., Brovko, F.A., and Trotsenko, Y.A. 2000. Facultative and obligate aerobic *Methylobacteria* synthesize cytokinins. Microbiology, 69(6): 646–651. https://doi.org/10.1023/A:1026693805653.

Jorge, G. L., Kisiala, A., Morrison, E., Aoki, M., Nogueira, A. P. O. and Emery, R. J. N. 2019. Endosymbiotic *Methylobacterium oryzae* mitigates the impact of limited water availability in lentil (*Lens culinaris Medik*.) by increasing plant cytokinin levels. Environ. Exp. Bot. 162(May):525–540. https://doi.org/10.1016/j.envexpbot.2019.03.028.

Katsoula, A., Vasileiadis, S., Sapountzi, M. and Karpouzas, D. G. 2020. The response of soil and phyllosphere microbial communities to repeated application of the fungicide iprodione: accelerated biodegradation or toxicity? FEMS Microbiol. Ecol. 96(6): fiaa056. https://doi.org/10.1093/femsec/fiaa056.

Keijer, J., Korsman, M. G., Dullemans, A. M., Houterman, P. M., de Bree, J., and Van Silfhout, C. H. 1997. In vitro analysis of host plant specificity in *Rhizoctonia solani*. Plant Pathol. 46:659–669. https://doi.org/10.1046/j.1365-3059.1997.d01-61.x.

Kiptoo, J., Abbas, A., Bhatti, A. M., Usman, H. M., Shad, M. A., Umer, M., Atiq, M. N., Alam, S. M., Ateeq, M., Khan, M., Peris, N. W., Razaq, Z., Anwar, N., and Iqbal, S. 2021. *Rhizoctonia solani* of potato and its management: A review. Plant Prot. 5:157–169. https://doi.org/10.33804/pp.005.03.3925.

Madhaiyan, M., Suresh Reddy, B. V., Anandham, R., Senthilkumar, M., Poonguzhali, S., Sundaram, S. P., and Sa, T. 2006. Plant growth-promoting *Methylobacterium* induces defense responses in groundnut (*Arachis hypogaea* L.) compared with rot pathogens. Curr. Microbiol. 53:270–276. https://doi.org/10.1007/s00284-005-0452-9.

Maino, J. L., Thia, J., Hoffmann, A. A., and Umina, P. A. 2023. Estimating rates of pesticide usage from trends in herbicide, insecticide, and fungicide product registrations. Crop Prot. 163:106125. https://doi.org/10.1016/j.cropro.2022.106125.

Maitra, S., Brestic, M., Bhadra, P., Shankar, T., Praharaj, S., Palai, J. B., Shah, M. M. R., Barek, V., Ondrisik, P., Skalický, M., and Hossain, A. 2021. Bioinoculants-natural biological resources for sustainable plant production. Microorganisms 10:51. https://doi.org/10.3390/microorganisms10010051.

Marinozzi, M., Coppola, L., Monaci, E., Karpouzas, D. G., Papadopoulou, E., Menkissoglu-Spiroudi, U., and Vischetti, C. 2013. The dissipation of three fungicides in a biobed organic substrate and their impact on the structure and

activity of the microbial community. Environ. Sci. Pollut. Res. Int. 20:2546–2555. https://doi.org/10.1007/s11356-012-1165-9.

Meena, K. K., Kumar, M., Kalyuzhnaya, M. G., Yandigeri, M. S., Singh, D. P., Saxena, A. K., and Arora, D. K. 2012. Epiphytic pink-pigmented methylotrophic bacteria enhance germination and seedling growth of wheat (*Triticum aestivum*) by producing phytohormone. Antonie Van Leeuwenhoek 101:777–786. https://doi.org/10.1007/s10482-011-9692-9.

Meena, R., Kumar, S., Datta, R., Lal, R., Vijayakumar, V., Brtnicky, M., Sharma, M., Yadav, G., Jhariya, M., Jangir, C., Pathan, S., Dokulilova, T., Pecina, V., and Marfo, T. 2020. Impact of agrochemicals on soil Microbiota and management: A review. Land (Basel) 9:34. https://doi.org/10.3390/land9020034.

Moulas, C., Petsoulas, C., Rousidou, K., Perruchon, C., Karas, P., and Karpouzas, D. G. 2013. Effects of systemic pesticides imidacloprid and metalaxyl on the phyllosphere of pepper plants. Biomed Res. Int. 2013:969750. https://doi.org/10.1155/2013/969750.

Palberg, D., Kisiała, A., Jorge, G. L., and Emery, R. J. N. 2022. A survey of *Methylobacterium* species and strains reveals widespread production and varying profiles of cytokinin phytohormones. BMC Microbiol. 22:49. https://doi.org/10.1186/s12866-022-02454-9.

Phukon, H., Harshvardhan, K., Sarma, N., Kumar, P., Lal, M., and Kalita, D. 2025. Isolation and identification of *Methylobacterium komagatae* and its application in textile industries. Nat. Prod. Res. 39:2831–2841. https://doi.org/10.1080/14786419.2024.2318787.

Ramirez, M. L., Reynoso, M. M., Farnochi, M. C., and Chulze, S. 2006. Vegetative Compatibility and Mycotoxin Chemotypes among *Fusarium graminearum* (*Gibberella zeae*) Isolates from Wheat in Argentina. Eur. J. Plant Pathol. 115:139–148. https://doi.org/10.1007/s10658-006-0009-1.

Ruiu, L. 2020. Plant-growth-promoting bacteria (PGPB) against insects and other agricultural pests. Agronomy (Basel) 10:861. https://doi.org/10.3390/agronomy10060861.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. 2008. Bacterial endophytes: recent developments and applications. FEMS Microbiol. Lett. 278:1–9. https://doi.org/10.1111/j.1574-6968.2007.00918.x.

Ryu, J.H., Madhaiyan, M., Poonguzhali, S., Yim, W.J., Indiragandhi, P., Kim, K.A., et al. 2006. Plant growth substances produced by *Methylobacterium* spp. and their effect on tomato (*Lycopersicon esculentum* L.) and red pepper (*Capsicum annuum* L.) growth. J. Microbiol. Biotechnol. 16(10): 1622–1628.

Senthilkumar, M., and Krishnamoorthy, R. 2017. Isolation and characterization of tomato leaf phyllosphere *Methylobacterium* and their effect on plant growth. Int.

J. Curr. Microbiol. Appl. Sci. 6(11): 2121–2136. https://doi.org/10.20546/ijcmas.2017.611.250.

Shahwar, D., Mushtaq, Z., Mushtaq, H., Alqarawi, A.A., Park, Y., Alshahrani, T.S., and Faizan, S. 2023. Role of microbial inoculants as biofertilizers for improving crop productivity: a review. Heliyon. 9(6): e16134. https://doi.org/10.1016/j.heliyon.2023.e16134.

Šmejkalová, H., Erb, T.J., and Fuchs, G. 2010. Methanol assimilation in *Methylobacterium extorquens* AM1: demonstration of all enzymes and their regulation. PLoS ONE, 5(10). https://doi.org/10.1371/journal.pone.0013001.

Stone, B.W.G., Weingarten, E.A., and Jackson, C.R. 2018. The role of the phyllosphere microbiome in plant health and function. In Annual plant reviews online. 1st ed. Edited by J.A. Roberts. Wiley. pp. 533–556. https://doi.org/10.1002/9781119312994.apr0614.

Sturz, A.V., and Peters, R.D. 2007. Endophyte-mediated disease suppression induced by application of metalaxyl-m to potato foliage. Can. J. Plant Pathol. 29(2): 131–140. https://doi.org/10.1080/07060660709507449.

Sukul, P., and Spiteller, M. 2001a. Influence of biotic and abiotic factors on dissipating metalaxyl in soil. Chemosphere. 45(6–7): 941–947. https://doi.org/10.1016/S0045-6535(01)00010-8.

Sukul, P., and Spiteller, M. 2001b. Persistence, fate, and metabolism of [^{14}C] metalaxyl in typical Indian soils. J. Agric. Food Chem. 49(5): 2352–2358. https://doi.org/10.1021/jf001181r.

Sukul, P., Majumder, A., and Spiteller, M. 2008. Microbial population and their activities in soil as influenced by metalaxyl residues. Fresenius Environ. Bull. 17(1): 103–110.

Sun, M., Wang, H., Shi, C., Li, J., Cai, L., Xiang, L., Liu, T., Goodwin, P. H., Chen, X., and Wang, L. 2022. Effect of azoxystrobin on tobacco leaf microbial composition and diversity. Front. Plant Sci. 13:1101039. https://doi.org/10.3389/fpls.2022.1101039.

Tani, A., Sahin, N., Fujitani, Y., Kato, A., Sato, K., and Kimbara, K. 2015. *Methylobacterium* species promoting rice and barley growth and interaction specificity revealed with whole-cell matrix-assisted laser desorption /ionization-time of-flight mass spectrometry (MALDI-TOF/MS) analysis. PLoS ONE, 10(6): 1–15. https://doi.org/10.1371/journal.pone.0129509.

Thapa, S., and Prasanna, R. 2018. Prospecting the characteristics and significance of the phyllosphere microbiome. Ann. Microbiol. 68(5):229–245. https://doi.org/10.1007/s13213-018-1331-5.

Tóth, B., Mesterházy, Á., Horváth, Z., Bartók, T., Varga, M., and Varga, J. 2005. Genetic variability of Central European isolates of the *Fusarium graminearum*

species complex. Eur. J. Plant Pathol. 113(1): 35–45. https://doi.org/10.1007/s10658-005-0296-y.

Van Der Lee, T., Zhang, H., Van Diepeningen, A., and Waalwijk, C. 2015. Biogeography of *Fusarium graminearum* species complex and chemotypes: a review. Food Additives Contam. 32(4): 453–460. https://doi.org/10.1080/19440049.2014.984244.

Ventorino, V., Sannino, F., Piccolo, A., Cafaro, V., Carotenuto, R., and Pepe, O. 2014. *Methylobacterium populi* VP2: plant growth-promoting Bacterium isolated from a highly polluted environment for polycyclic aromatic hydrocarbon (PAH) Sci. World J. 2014: 1–11. https://doi.org/10.1155/2014/931793.

Wang, F., Zhu, L., Wang, X., Wang, J., and Wang, J. 2015. Impact of repeated applications of metalaxyl on its dissipation and microbial community in soil. Water Air Soil Pollut. 226(12): 1–14. https://doi.org/10.1007/s11270-015-2686-x.

Ward, T. J., Clear, R. M., Rooney, A. P., O'Donnell, K., Gaba, D., Patrick, S., Starkey, D. E., Gilbert, J., Geiser, D. M., and Nowicki, T. W. 2008. An adaptive evolutionary shift in *Fusarium* head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. Fungal Genet. Biol. 45:473–484. https://doi.org/10.1016/j.fgb.2007.10.003.

Xiang, L.-G., Wang, H.-C., Cai, L.-T., Guo, T., Luo, F., Hsiang, T., and Yu, Z.-H. 2022. Variations in leaf phyllosphere microbial communities and development of tobacco brown spot before and after fungicide application. Front. Microbiol. 13:1068158. https://doi.org/10.3389/fmicb.2022.1068158.

Yang, L., van der Lee, T., Yang, X., Yu, D., and Waalwijk, C. 2008. *Fusarium* populations on Chinese barley show a dramatic gradient in mycotoxin profiles. Phytopathology 98:719–727. https://doi.org/10.1094/PHYTO-98-6-0719.

Yli-Mattila, T., Gagkaeva, T., Ward, T. J., Aoki, T., Kistler, H. C., and O'Donnell, K. 2009. A novel Asian clade within the *Fusarium graminearum* species complex includes a newly discovered cereal head blight pathogen from the Russian Far East. Mycologia 101:841–852. https://doi.org/10.3852/08-217.

Zhang, C., Wang, M.-Y., Khan, N., Tan, L.-L., and Yang, S. 2021. Potentials, utilization, and bioengineering of plant growth-promoting *Methylobacterium* for sustainable agriculture. Sustainability 13:3941. https://doi.org/10.3390/su13073941.

Zhang, L. L., Chen, J. M., and Fang, F. 2008. Biodegradation of methyl t-butyl ether by aerobic granules under a cosubstrate condition. Appl. Microbiol. Biotechnol. 78:543–550. https://doi.org/10.1007/s00253-007-1321-1.

Zheng, A., Lin, R., Zhang, D., Qin, P., Xu, L., Ai, P., Ding, L., Wang, Y., Chen, Y., Liu, Y., Sun, Z., Feng, H., Liang, X., Fu, R., Tang, C., Li, Q., Zhang, J., Xie, Z., Deng, Q., Li, S., Wang, S., Zhu, J., Wang, L., Liu, H., and Li, P. 2013. The

evolution and pathogenic mechanisms of the rice sheath blight pathogen. Nat. Commun. 4:1424. https://doi.org/10.1038/ncomms2427.

CHAPTER 5

5.1. PREFACE

Title: Biological control of phytopathogenic *Fusarium* by plant growth promoting *Methylobacterium* spp.

Authors: Daniel Palberg and R. J. Neil Emery

Reference: This chapter is currently under preparation for submission. The published version will appear different than presented here.

Contributions: Conceptualization, investigation, methodology, formal analysis, figure and table creation, and writing of the initial draft by D.P. Review of methodology, project supervision, and acquisition of funding by R.J.N.E.

All authors read and approved the final manuscript.

CHAPTER 5

Biological control of phytopathogenic *Fusarium* by plant growth promoting *Methylobacterium* spp.

5.2. ABSTRACT

The biocontrol potential of *Methylobacterium* spp. against key phytopathogenic fungi was evaluated through a series of *in vitro* and *in planta* experiments. A broad spectrum of antagonistic activity was observed across 31 bacterial isolates, with suppression efficacy highly dependent on specific fungal-bacterial pairings. *Fusarium graminearum* (UAMH 3329) was most consistently suppressed, with 28 bacterial isolates inducing >20% mycelial growth inhibition, and 16 bacterial isolates exceeding 40%. In contrast, fewer isolates suppressed *F. oxysporum* and *F. fujikuroi* to a comparable extent. Only two bacterial isolates however – *M. thiocyanatum* (NBRC 103124) and *M. aminovorans* (LMG 21752) – demonstrated statistically significant suppression (>30%, $p < 0.05$) across all three fungal species. Growth inhibition was enhanced on minimal high-carbon nutrient agar amended with sterile spent broth from *Methylobacterium* cultures, suggesting secreted metabolites play a key role in fungal suppression. However, strain-specific activity and substrate effects suggest a complex interplay between bioactive metabolite production and fungal sensitivity. Notably however, *M. organophilum* (LMG 6083) conferred partial protection to soybean seeds challenged with *F. graminearum*, significantly improving germination rates and seedling development in both artificial and soil substrates. Co-inoculated seeds exhibited significantly lower disease severity scores (DSS) and fewer necrotic lesions. While not achieving complete disease suppression, the presence of *M. organophilum* consistently reduced fungal colonization, protected seedling anatomy, and mitigated key, early-stage disease symptoms even at relatively low inoculum density. These

findings highlight the selective yet promising biocontrol potential of certain *Methylobacterium* strains and underscore their value in integrated crop protection strategies and emphasizes the need for further mechanistic investigations.

KEYWORDS: *Methylobacterium*, biological control, *Fusarium*, soybeans, phytobiome, crop protection

5.3. INTRODUCTION

The genus *Fusarium* (Phylum *Ascomycota*, Class *Sordariomycetes*) is one of the most extensively investigated of all the fungi and is a persistent threat to agriculture the world over (Johns et al. 2022). With a cosmopolitan distribution, *Fusarium* species are common causal agents of plant disease and, despite investigations spanning more than a century, they continue to cause widespread crop losses. This is facilitated mainly by an ability to subsist on a wide range of substrates – facultatively switching between necrotrophic and saprotrophic behaviour – and the great number of their asexual spores which enhances their dispersibility and survivability (Leslie and Xu, 2010).

Collectively, the genus has an enormous host range extending from cereals and legumes to vegetables and ornamentals. *Fusarium* pathogens invade plant tissues and cause a wide range of symptoms including stem rot of sweet potato (*Ipomoea batatas*) by *F. oxysporum* f. sp. *batatas*, pod rot of peanut (*Arachis hypogaea*) by *F. solani* and *F. graminearum*, wilt of sesame (*Sesamum indicum*) by *F. oxysporum* f. sp. *sesame*, cotton (*Gosspium* spp.) by *F. oxysporum* f. sp. *vasinfectum*, and stalk rot of Sorghum (*Sorghum bicolor*) by *F. moniliforme*. Outbreaks of Panama disease (*F. oxysporum* f. sp. *cubense*) ravaged banana production through the 20th century, making cultivation of the Gros Michel (*Musa acuminata*) – the favored variety for over a century – so unsustainable that it has been replaced by the Cavendish (*Musa cavendishii*) commercially.

Current strategies for the management of *Fusarium* in agricultural settings involve crop rotation, use of resistant varieties, close monitoring of soil health, and judicious use of fungicides; but to date, biological control (BC) of *Fusarium* remains

limited. As a tool, BC relies on the natural enemies of phytopathogens including predators, parasites, pathogens, and competitors, to suppress their proliferation and inhibit the establishment of infection. BC is an important facet in the greater pursuit of sustainable agriculture as, when deployed properly, it can reduce reliance on synthetic pesticides, alleviate use-driven resistance pressure, and minimize harmful residues. Ideal BC agents are typified by a high target specificity, low invader potential, and easy integration into agroecosystems without jeopardizing soil and water quality (Köhl et al. 2019).

The genus *Methylobacterium* (*Alphaproteobacteria*) has been examined for potential roles in agriculture (Ryan et al. 2008; Meena et al. 2012; Dourado et al. 2015; Tani et al. 2015; Zhang et al. 2021). *Methylobacterium* species are gram-negative, rod-shaped, and often pink-pigmented – though some exceptions do exist (*M. jeotgali*). A resilient group, several members of this genus can tolerate gamma radiation, bleach, desiccation, and freezing (Hiraishi et al. 1995; Yoshida et al. 2017). The *Methylobacterium* have a wide distribution, and are most frequently isolated from soil, water, air, and the plant microbiome where, collectively, they play crucial roles in carbon cycling and plant growth promotion. Both epi- and endophytic species have been studied for their potential as biofertilizers, due to their ability to degrade pollutants (Ventorino et al. 2014), fix nitrogen (Sy et al. 2001), and produce uniquely large quantities of phytohormones, including cytokinins (CKs) (Palberg et al. 2022).

Significant efforts to examine the agronomic potential of *Methylobacterium* have been expended especially as certain strains of *Methylobacterium* – subclassified as plant growth-promoting bacteria (PGPB) – improve host resilience to abiotic stress

and encourage seed setting (Holland 1997a; Madhaiyan et al. 2006; Ryan et al. 2008; Meena et al. 2012; Dourado et al. 2015; Tani et al. 2015; Zhang et al. 2021). Members of the genus *Methylobacterium* are also storehouses of enzyme classes including glycosidases, pectinases, and chitinases, which may aid in the deterrence of pathogenic fungi directly (Dourado et al. 2015; Lipka and Panstruga 2005). The long-established predominance of *Methylobacterium* in the plant microbiome (Corpe and Rheem 1989) then, may not only be underpinned by a nutrient relationship with the host-plant (Abanda-Nkpwatt et al. 2006; Šmejkalová et al. 2010) but also by the stimulation of host defences as has already been demonstrated in groundnut (Madhaiyan et al. 2006) and tomato (Medina-Rios et al. 2019).

The overall aim of this work was to examine, *in vitro*, the interactions between *Methylobacterium* isolates from a wide range of species, diverse in source of origin and phenotypic presentation, and three dominant phytopathogenic species of *Fusarium*. This study also evaluated the effects of selected *Methylobacterium* isolates as a protectant for soybean seedlings germinated in both soil and soilless media. This work presents the first expansive and rigorous investigation of the biocontrol capability of *Methylobacterium* against multiple agriculturally relevant species of *Fusarium*.

5.4. MATERIALS AND METHODS

Chemicals, Materials, and Media Preparation

Growth media for isolations and preservation of stocks included tryptic soy broth (TSB), potato dextrose broth (PDB) (Fisher Scientific), Reasoner's 2A (R2A) (VWR). In all cases, solid medium was prepared using agar (Fisher Bioreagents, BP1423-500) at a rate of 1.6% w/v, and uniform fill volume of 20 mL in single-use petri dishes (FisherBrand™, 100 x 15 mm).

Selection, Preservation, and Propagation of Methylobacterium and Fusarium Isolates

Preparation and manipulation of all bacterial and fungal cultures were conducted aseptically in a biological safety cabinet (BSC). Freeze-dried cultures of *Methylobacterium* spp. were obtained from four microbe collections: the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) ["German Collection of Microorganisms and Cell Cultures"], the Japan Collection of Microorganisms (JCM), and the National Institute of Technology and Evaluation's (NITE) Biological Resource Center (NBRC). Strains were originally collected from different biological (living plants) and non-biological sources (soil, water, air). Information on strain taxonomy, origin, and known characteristics is provided in Table 5.1. Freeze-dried bacterial strains were revived in 50 mL of nutrient rich R2A broth (VWR, Mississauga, Canada) at 27 ^{o}C in a rotary incubator (120 RPM). After 5 days, a sterile loop was used to inoculate plates containing DSMZ 125 selective medium, from which single colonies would be selected to inoculate flasks containing 50 mL of DSMZ 125 broth. This series of passages was necessary to ensure potential contaminants were removed and an aliquot

of the final broth was used to develop cryogenic stocks (~ 10^8 CFU/mL) in 15% (v/v) glycerol, subsequently maintained at -80 °C.

Restart of *Methylobacterium* strains was achieved by streaking cryogenic stocks on nutrient-rich tryptic soy agar (TSA) using a sterile loop. After 5 days of incubation at 26 ºC, single colonies were extracted from each plate and used to inoculate 50 mL of tryptic soy broth (TSB) liquid growth media in 250 mL glass Erlenmeyer flasks and maintained in a rotary incubator for 7 days (27 ºC and 110-120 RPM). When *Methylobacterium* cultures reached the late exponential/early stationary phase after approximately 7 days (OD_{600} = 0.6 – 1.2, depending on strain) selective nutrient minimum agar plates (DSMZ 125) were inoculated with 50 µL of diluted (10^{-5}) aliquots. Selective minimum nutrient media were prepared in accordance with the DSMZ recipe for *Methylobacterium* growth media (DSMZ Index #125): KNO_3 1.00 g; $MgSO_4 \cdot 7H_2O$ - 0.2 g; $CaCl_2 \cdot 2H_2O$ - 0.02 g; Na_2HPO_4 0.23 g; NaH_2PO_4 - 0.07 g; $FeSO_4 \cdot 7H_2O$ - 1.00 mg; $CuSO_4 \cdot 5H_2O$ – 5.00 µg; H_3BO_3 – 10.00 µg; $MnSO_4 \cdot 5H_2O$ – 10.00 µg; $ZnSO_4 \cdot 7H_2O$ – 70.00 µg; MoO_3 – 10.00 µg; H_2O - 1,000 mL; CH_3OH - 5 mL; pH 6.80-6.88). This transfer to a minimum nutrient media ensured high selectivity for *Methylobacterium* and minimal contamination risk during transfers from cryogenic stocks. After 5-7 days of incubation at 27 ºC (inverted, darkness), single colonies were used for subsequent experiments. Selective nutrient minimum stock plates were maintained at 4 ºC for a maximum of 21 days.

Live cultures of *Fusarium* spp. were obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH) (Gage Research Institute, University of Toronto, Canada). Information on fungal isolate origin and known characteristics are

provided in Table 5.2. Fungal isolates were maintained in potato dextrose agar (PDA) after transfer from transport container, at 27 ºC in darkness. After 10 days, a spore suspension developed by flooding the surface of the plate with 2 mL of sterile water and gentle agitation with a loop, was subsequently used to produce stocks preserved at -80 ºC in 20% glycerol (v/v) (~ 106 conidia/mL). Revival of isolates were achieved by transferring 50 µL of cryogenic spore suspensions to plates containing nutrient-rich PDA or TSA, as needed. Fungal stock plates were maintained at 26 ºC for a maximum of 10 days before being discarded.

For experiments evaluating the inhibitory effect of *Methylobacterium* spp. in nutrient-minimum conditions, multiple variations of Hoagland's complete nutrient hydroponic solution were used, with modifications to support fungal and bacterial growth (Table 5.3.). Use of this medium was essential to eliminating bias of growth effectors or background metabolites present in nutrient-rich medium like TSB or Murashige and Skoog (MS). Importantly, Hoagland's solution is composed exclusively of inorganic salts and, in this study, was verified through use of blanks and negative controls to be free of the plant hormones targeted by the analytical method, further confirming its suitability.

In contrast, MS medium for example, while being widely used for plant tissue culture, is conventionally supplemented with plant growth regulators (e.g., IAA, BAP, kinetin, myo-inositol), thus introducing background hormone contamination which may interfere not only with analytical measurements but observed interactions between cultured organisms (Murashige and Skoog, 1962). Selecting Hoagland's complete

nutrient broth as the base, provided a simple and inert baseline for *in-vitro* measurement of hormone fluctuations at the plant–microbe–pathogen interface.

The base Hoagland's solution was prepared by adding macronutrients at a rate of 6 mL of 1M $NH_4H_2PO_4$, 4 mL of 1M KNO_3, 2 mL of 1M Ca $(NO_3)_2$, and 1 mL of 1M $MgSO_4$, per liter of deionized water. Subsequently, 1 mL of micronutrient stock (2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2 \cdot 4H_2O$, 0.22 g/L $ZnSO_4 \cdot 7H_2O$, 0.08 g/L $CuSO_4 \cdot 5H_2O$, 0.02 g/L $NaMoO_4 \cdot H_2O$) and 1 mL of iron supplement (10.4 g/L EDTA, 7.8 g/L $FeSO_4 \cdot 7H_2O$, 56.0 g/L KOH) were added. Modified Hoagland's recipe included D-glucose (BioShop) at a rate of 10 g/L. In all cases, the broth was heated to 45 ^oC, and the pH was adjusted to 6.90 ± 0.1 using sodium hydroxide (1M NaOH). 100 mL of broth was transferred into 250 mL Erlenmeyer flasks and autoclaved at 121 ^oC for 20 minutes. In the preparation of solid medium, agar was supplemented at a rate of 1.6% w/v.

Biocontrol Plug Assay

Biocontrol activity of *Methylobacterium* isolates against *F. graminearum*, *F. oxysporum*, and *F. fujikuori*, was determined by measured inhibition (considered effective at > 30% mycelial growth inhibition, and $p < 0.05$ relative to controls) of mycelial growth *in-vitro*. Bacterial isolates were grown for 7 days in tryptic soy broth (TSB) at 27 °C, and tryptic soy agar (TSA) was used for the cultivation of fungi, from a cryogenic spore suspension. Upon reaching exponential growth, 20 µL of bacterial cultures (~ 10^8 CFU/mL) were streaked down the centerline of fresh TSA agar plates using a sterile loop and allowed to dry for 10 minutes at room temperature. Plates were subsequently sealed with parafilm and incubated at 27 ^oC inverted, in darkness, for 48 hours. Mycelial plugs (6 mm diameter) of each fungus were sampled from the leading

edge of mycelial growth in 10-day old TSA cultures and transferred to fresh TSA assay plates 20 mm from the edge uniformly using a plate grid. Co-culture plates were incubated for an additional 10 days at 27 ºC in darkness. The controls consisted of agar plugs of the tested fungi with sterile TSB streaked through the midline. The antifungal activity assay was repeated four times, for each combination of bacterial isolate and fungal pathogen.

Fungal growth was photographed by plain light and monochromatically (Geliance 600 Imaging System, Perkin Elmer) at 48-hour intervals for 10 days post-inoculation. Basic photograph processing, including adjustment of brightness and contrast for clear visibility of microbial growth, was achieved using GeneSnap (ver. 7.04.06, Synoptics Ltd.). Fungal growth area was determined using the pixel area tool in ImageJ2 open-source software for Macintosh OS (Fiji, Ver: 2.9.0/1.53t, Build: a33148d777), against the total plate pixel area. Total plate area (500 mm^2) was determined by calculating the area of a circle (πr^2) and a cross-product equation (Eq 1.) was then used to determine the fungal growth area in both treatment and control cohorts, where A_{total} and P_{total} represent the total culturable area available (500 mm^2 and 165,500 pixels, respectively), and P_{fungi} is the total pixels of fungi growth measured in ImageJ2.

$$A_{fungi} = \frac{A_{fungal} \bullet P_{fungi}}{P_{total}} \qquad \text{(Eq 1.)}$$

The percentage of fungal growth inhibition (FGI) was determined using Eq 2 and normalized against controls, where C represents the total fungal growth (in mm^2) of the control in each experimental group, and T_x is fungal growth in dishes containing bacterial isolates.

$$FGI(\%) = \left(\frac{C - T_x}{C}\right) \bullet 100 \qquad \text{(Eq 2.)}$$

Growth Medium Amendment

The inhibitory effect of *Methylobacterium* spp. spent broth on *Fusarium* growth was evaluated using both tryptic soy broth (TSB) and modified Hoagland's complete nutrient broth (MHCNB), with modifications to support fungal and bacterial growth. *Methylobacterium* isolates were selected for inclusion in this experiment based on performance in direct co-culture experiments, where the 5 strains producing greatest fungal growth suppression per phytopathogen species, were selected (Table 5.4.).

Cultures of *Methylobacterium* strains grown in both TSB and HCNB were inoculated with single colonies from refrigerated DSMZ-125 nutrient-minimum stock plates. Inoculated broth (50 mL) was incubated at 27 ºC for 8 days in darkness, with constant shaking at 120 RPM. Sterile spent media from *Methylobacterium* cultures was obtained by centrifugation of cultures in 50 mL conical flasks (3,000 RCF, 15 minutes) and subsequent filtration (0.22 μm, bottle-top PES, Fisher Brand). Molten growth medium (agar 1.6% w/v) was amended with two different concentrations of spent medium (20 and 40% v/v) at 55 ºC and used to prepare solid growth medium in sterile petri dishes. Seven replicates of each amendment concentration were prepared for each of the selected Methylobacterium strains. Control growth medium was amended (20 and 40% v/v) with sterile isotonic water to offset nutrient depletion of the treatment plates.

Each plate was then uniformly inoculated with 5 µL of a spore suspension (10^8 conidia/mL) developed from a 10-day old stock plate of *F. graminearum* (UAMH 3329) using a plate map. This process was repeated in its entirety for *F. fujikuori* (UAMH 9877) and *F. oxysporum* (UAMH 9013). Inoculated plates were sealed with parafilm and incubated at 26 °C in darkness for 48 hours, then inverted and incubated for an additional 8 days. Mycelial growth was photographed every 48 hours and assessed identically to the biocontrol plug assay at 10-days post-inoculation, using ImageJ2 and Eq. 1. and Eq. 2. Successful growth inhibition was benchmarked at $\geq$ 30% mycelial growth reduction with a $p < 0.05$ relative to controls cultured on medium amended with water alone.

Biocontrol in Germination of Soybean

Two isolates of *F. graminearum* (UAMH 3329 and UAMH 7215) and the soybean cultivar 'ACC Mandor' were used in all experiments. In total, 360 seeds were surface sterilized in 500 mL of 2.5% (w/v) sodium hypochlorite solution diluted with sterile deionized water and amended with 100 µL of the non-ionic surfactant poluoxyethylene sorbitan monolaurate (Tween 20), for 1 minute with constant agitation (90 RPM; VWR Benchtop Model 980303). Seeds were then rinsed with 500 mL sterile deionized water four times, transferring the bulk sanitized seeds to a new sterile beaker each time.

Fungal inoculum was prepared by plate flood method of a 10-day-old culture on PDA using 2 mL of sterile isotonic saline. Cultures of *Methylobacterium organophilum* (LMG 6083) and *M. gnaphalii* (NBRC 107716) – the most and least effective biocontrol isolates against *F. graminearum* (UAMH 3329) *in-vitro*, respectively – were grown in

50 mL of DSMZ 125 nutrient-minimum broth for 10 days, pelleted, and subsequently resuspended in sterile isotonic saline. To inoculate seeds, 250 mL Erlenmeyer flasks containing 50 mL of sterile isotonic saline were inoculated to achieve a final concentration of either $5.0 \bullet 10^6$ CFU/mL of bacteria isolates or $2.0 \bullet 10^6$ conidia/mL of fungal spores (positive controls), while co-inoculation with both the fungal and bacterial isolates constituted the protection challenge. A negative control flask contained sterile isotonic saline alone. A total of 60 surface sterilized seeds were transferred to each flask and incubated at 22 ^{o}C for 16 hours, while agitated gently at 90 RPM (VWR Benchtop Model 980303).

Separately, six aluminum trays (30 x 24 x 6 cm) were prepared with 800 mL of Hoagland's Complete Nutrient Agar (HCNA, 0.6% w/v agar), while an additional six trays contained 1 L of standard potting mix (MiracleGrow®, 0.21-0.11-0.16, 30% organic matter) rehydrated with 750 mL of deionized water. All trays were overwrapped with tin foil and steam sterilized at 121 ^{o}C and 15 psi for 30 minutes. Trays were allowed to cool to room temperature, then refrigerated until use to prevent desiccation.

Following incubation, trays were warmed to room temperature and 30 seeds were transferred from each broth condition to respective trays aseptically. Trays were maintained in darkness for 72 hours in an environmental chamber (Conviron) with 27/22 ^{o}C day/night temperature, 60% relative humidity, then under a 12/12 photoperiod (PPFD: 900 $\mu mol \bullet m^{-2} \bullet s^{-1}$) for an additional 5 days. Seedlings and ungerminated seeds were recovered from each tray on the 8th day, photographed, and measured. The height of seedlings was measured from the start of lateral root structures (soil contact line) to

the tip of the epicotyl, while root length was measured from the soil line to the tip of the primary taproot (Mwenye et al. 2019).

Disease severity was scored using a 5-point ordinal scale. Specifically, 5 = no germination and total fungal colonization of the seed; 4 = germination verified by radicle protrusion, but extensive fungal colonization and lesions or necrosis affecting > 75% of the root; 3 = successful germination, moderate fungal colonization of seed, and lesions affecting 25-75% the root; 2 = successful germination and stand, lesions affecting 10-24% of the root; 1 = successful germination and seedling development with lesions affecting < 10% of root tissues, and 0 = successful germination and healthy seedling development with no visible signs of colonization. The experiment was completed in duplicate with both isolates of *F. graminearum* (UAMH 3329 and UAMH 7215) for a total of 720 seeds. The disease severity data was represented graphically as stacked bars (Figure 5.7.). Significant differences ($p < 0.05$) were determined by using the Student's t-test.

5.5. RESULTS

Direct co-culture and broth filtrate amendments

Examination of the biocontrol potential of *Methylobacterium* revealed broad variability of fungal growth suppression across all isolates tested, although meaningful and significant suppression was highly match-dependant. For example, 28 of 31 bacterial isolates were able to significantly supress (> 30% mycelial growth reduction, and $p < 0.05$ relative to controls) the mycelial growth of *F. graminearum* (UAMH 3329) by > 20% compared to unchallenged controls, while only 16 isolates induced suppression of ≥ 40% (Figure 5.1.). A similar phenomenon was observed in experiments involving *F. oxysporum* and *F. fujikuroi*, where the number of isolates capable of supressing mycelial growth in co-cultures by > 20% or ≥ 40% decreased greatly: 12 and 2, respectively for *F. oxysporum* (Figure 5.2.), and 14 and 5, respectively for *F. fujikuroi* (Figure 5.3.).

Overall, the phytopathogenic strain most sensitive to the presence of *Methylobacterium* was *F. graminearum* (UAMH 3329), with 29 of 31 isolates inducing some degree of statistically significant ($p < 0.05$) mycelial growth suppression relative to unchallenged controls – most notably *M. organophilum* (LMG 6083 and NBRC 103119), *M. radiotolerans* (LMG 2269), and *M. aminovorans* (LMG 21752) (Table 5.4.). Sensitivity to *Methylobacterium* in co-culture was followed closely by *F. fujikuroi*, with significant mycelial growth inhibition induced by 17 isolates, led by *M. oryzae* (LMG 23582), *M. gnaphalii* (NBRC 107716), and *M. thiocyanatum* (NBRC 103124). *F. oxysporum* proved to be the most resistant to the presence of *Methylobacterium* in co-culture, showing significant reductions in growth in response to only 8 of 31 tested

bacterial isolates, led by *M. thiocyanatum* (NBRC 103128 and JCM 20893), and *M. gnaphalii* (NBRC 107716) (Table 5.4.).

Methylobacterium isolates capable of effectively suppressing fungal growth – characterized as > 30% mycelial growth inhibition with statistical significance of at least $p < 0.05$, relative to unchallenged controls – share no trend other than a third of effective bacterial strains were isolated from plant material: 9/25, 2/6, and 5/7, for *F. graminearum* (UAMH 3329), *F. oxysporum*, and *F. fujikuroi*, respectively. Importantly, *M. oryzae*, a well-characterized endophyte isolated from *Oryza sativa*, demonstrated significant antagonism of *F. fujikuroi* (UAMH 9877). This suggests a potentially ecologically relevant symbiotic relationship, whereby rice-associated *M. oryzae* possesses mechanisms to suppress a major rice pathogen, even when isolated from a different crop. Botanical links could not be strongly attributed to other effective matches, and only two isolates were able to suppress the growth of all three phytopathogenic fungi in direct co-culture (plug assay): *M. thiocyanatum* (NBRC 103124) and *M. aminovorans* (LMG 21752). In some instances, despite mycelial growth being inhibited initially, some species of fusaria, particularly *F. oxysporum*, hyphal growth would continue either around or penetrate the agar surface and continue expanding under the line of inoculum (Figure 5.5II.).

Use of modified Hoagland's complete nutrient agar (MHCNA) media (Table 5.3.) was generally tolerated well as a growth substrate for fungal phytopathogens, except for *F. fujikuroi* which exhibited a slight decrease in average total mycelium area when cultured in MHCNA relative to TSA (420.1 ± 25.6 mm^2 and 490.5 ± 14.9 mm^2, respectively) under unchallenged conditions. In almost all cases, fungal growth was

reduced when inoculated on growth medium amended with sterile spent broth harvested from *Methylobacterium* cultures, relative to controls. Notably, inhibition of fungal growth intensified in MHCNA relative to TSA, with all but one study (Figure 5.6a) exhibiting strong (> 30%) and statistically significant ($p < 0.01$) suppression. The same inhibitory effect was not observed in TSA-amended trials, which often displayed interquartile spreads double that of their MHCNA counterparts, and statistically insignificant suppressive effects that rarely exceed 20% (Figure 5.6.). For example, MHCNA amended with spent-broth from *M. thiocyanatum* (JCM 10893) restricted the average total area colonized by *F. oxysporum* to just 178.6 ± 43.4 mm^2 after 10 days, whereas TSA-amended plates were not significantly different when compared to TSA controls (417.8 ± 59.0 mm^2 and 496.2 ± 4.3 mm^2, respectively). A similar effect was observed with *F. graminearum*, where colonization was limited to 201.5 ± 26.4 mm^2 after 10 days on MHCNA amended with spent broth from *M. organophilum* (LMG 6083), while TSA plates amended with broth produced from the same bacterial isolate failed to cause any retardation of growth relative to controls. Most dramatically, biocontrol impacts of *F. fujikuroi* previously observed in direct co-culture were lost completely in TSA-amended plates while remaining generally preserved in MHCNA (Figure 6c). Unexpectedly, inhibition of *F. oxysporum* by *M. organophilum* (NBRC 103128) spent media were not observed in either of the amended substrates, suggesting that either the physical presence of *M. organophilum* is imperative to inhibition or that an inhibitory component of the spent broth is thermally labile and was degraded during amendment.

Biological control of F. graminearum in Soybean (Glycine max)

Protection of soybean seeds by *M. organophilum* (LMG 6083) against both tested isolates of *F. graminearum* showed potential, though microbiome enrichment with the bacterium did not prevent the establishment of infection or the occurrence of lesions, absolutely. For example, the presence of *M. organophilum* in a co-inoculum with *F. graminearum* (UAMH 3329) increased the total number of seeds to successfully germinate in both HCNA (73.3 %) and soil (81.1 %), relative to controls (HCNA: 62.1 %, soil: 43.3 %), and reduced the proportion of seeds deemed unrecoverable – those with a DSS > 2 and unlikely to survive to maturity or have production value – by 46 % and 53 % in HCNA and soil, respectively (Figure 5.7.). Interestingly, while the proportion of seeds with a DSS > 3 was greater for seeds protected by co-inoculation with *M. organophilum* and challenged with *F. graminearum* (UAMH 3329) compared to those challenged with an alternate *F. graminearum* strain (UAMH 7215) – ultimately resulting in a similar proportion of unrecoverable seeds in both groups (Figure 5.7.). Importantly, however, the presence of *M. organophilum* did not yield completely disease-free soybean seedlings (DSS = 0) across any iteration of the experiment. Additionally, protection conferred by *M. organophilum* against both isolates of *F. graminearum* did not differ statistically between growth substrates, and while the co-inoculation with the poor biocontrol (no suppression of mycelial growth in *in vitro* studies; Chapter 5) agent *M. oxalidis* (NBRC 107715) did modestly improve the overall germination rate of soybean in both substrates and phytopathogens, this isolate failed to improve the ultimate proportion of seeds with a DSS > 3 (Figure 5.7.).

For seedlings grown in HCNA with a DSS < 4, and therefore measurable anatomy, the presence of the candidate isolate *M. organophilum* (LMG 6083)

significantly ($p < 0.05$) increased the average root (149 ± 41 mm) and shoot (144 ± 23 mm) lengths during exposure to *F. graminearum* (UAMH 3329) relative to that of the unprotected (root: 86 ± 21 mm; shoot: 100 ± 20 mm) and sterile (root: 133 ± 35 mm; shoot: 141 ± 25 mm) control cohorts. Similar improvements to average root and shoot length were also observed for protected seeds grown in soil (root: 169 ± 38 mm; shoot: 114 ± 33 mm) relative to the unprotected (root: 108 ± 14 mm; shoot: 126 ± 9 mm) and sterile control groups (root: 147 ± 31 mm; shoot: 127 ± 11 mm). When co-inoculated with *F. graminearum* (UAMH 3329), the bacterial isolate *M. oxalidis* (NBRC 107715) demonstrated a weak, and statistically insignificant ability to recover germination or improve seedling development in both HCNA and soil substrates relative to the candidate strain (LMG 6083) (Figure 5.7.).

Macroscopic examination of seeds collected from growth trays revealed that priming the seeds in an isotonic bath containing the candidate biocontrol strain *M. organophilum* (LMG 6083) appeared to prevent heavy colonization and digestion of the seed coat by both strains of *F. graminearum*, resulting in the repeated observation of an intact seed coat in individuals among the protected cohort (Figure 5.8c-d and Figure 5.9c-d), as opposed to seeds inoculated with the phytopathogen alone (Figure 5.8a-b and Figure 5.9a-b).

Another frequent observation was obvious water-soaked lesions, or sites of localized tissue necrosis at the tip of the cotyledons of the few young seedlings to successfully germinate in the unprotected cohort (Figure 5.9a.). This may be caused by inadequate moisture control of the seed coat (Moore, 1971) induced by heavy colonization by fusarium, which causes a shearing injury as the cotyledons are impinged

and as they attempt to expand. Irrespective of the precise mechanism of injury, the wound site presents an opportunity for the further colonization of fusarium in the apical portion of the seedling anatomy (Figure 5.9a-b). This phenomenon, however, was not observed in cohorts co-incubated in a bath with *M. organophilum* (LMG 6083), suggesting that the preservation of the seed coat and reduction of secondary injury to the cotyledons appears to be a critical component of preventing advanced disease. While not entirely unscathed, surviving seedlings from the protected cohort generally showed lower rates of necrosis and tissue injury, an absence of soft-rot or water-soaked lesions, and markedly reduced extent of surface mycelium coverage (Figure 5.9.).

Extensive colonization and eventual rot of the radicle was also a frequent observance in unprotected seeds exposed to *F. graminearum*, particularly with UAMH 7215, while the structures of the early taproot of individuals from the protected cohort, despite some scarring, remained relatively healthy (Figure 5.10.). Inspection of the root structures of protected seedlings also often revealed the presence of extensive mycelial networks encasing the root surface (Figure 5.10b.), seemingly without causing injury.

The mechanism of injury was consistent for both fungal strains across the control cohort, and involved the rapid colonization of the seed coat, resulting in injury to the embryo at least in part by adherence to the seed coat (‘helmet head’) and typified by the presence of necrotic lesions, scars, and deep fissures in the developing cotyledons. Injury to the embryo resulted in growth deformities, delayed development, and open wounds favourable to ingress by *F. graminearum*. In the majority of the control cohort (~ 80%), damping-off disease occurred, and seeds failed to germinate successfully. In contrast, the germination rate and development of seeds co-inoculated

with *M. organophilum* was significantly improved, and though not entirely eradicated, the ability for *F. graminearum* to penetrate plant tissues or otherwise establish a dense mycelial network, was also diminished relative to unprotected controls. The preservation and proper shedding of the seed coat in the protected cohort contrasted starkly to the heavy colonization and eventual complete digestion of the coat in unprotected controls (Figure 5.8. and Figure 5.9.).

5.6. DISCUSSION

One of the most important groups of phytopathogens, the fungal genus *Fusarium* causes crop disease in every major agro-climatic zone in the world (Burgess and Bryden, 2012; Ekwomadu and Mwanza, 2023). The growing resistance of *Fusarium* species to fungicides has resulted in diminished effectivity of chemical control measures, exacerbated by changes in agricultural practices – reduced tillage and monocropping – and by climate trends which favour pathogen persistence and dispersal, particularly milder winters and fluctuating rainfall patterns through the growing season (Hossain et al. 2024). Infections caused by *Fusarium* cause economic turmoil and threaten food security, especially in China and India – the world's largest producers of cereal grain intended for domestic consumption. Despite extensive efforts to combat losses, the demands for nutrient-dense staple crops (wheat, barley, rice, maize, banana, plantain) for a growing global population necessitate new strategies in thwarting disease caused by *Fusarium*.

Our work has provided the first comprehensive examination of the relationship between *Methylobacterium* and phytopathogenic species of *Fusarium*. Comprised of 93 antagonist-pathogen combinations, this study demonstrated that several *Methylobacterium* isolates suppressed fungal growth, and that the magnitude of antagonism was highly match-specific. For example, while the most dramatic growth suppression of *F. oxysporum* was achieved by the presence of *M. thiocyannatum* (NBRC 1031278), this bacterial strain had no suppressive effect against *F. graminearum* or *F. fujikuroi*. Similarly, the greatest suppression of *F. graminearum* was achieved by *M. organophilum* (LMG 6083), yet the growth of *F. fukikuori* and *F. oxysporum* were unimpeded by the presence of this isolate. Moreover, overall sensitivity to the presence

of *Methylobacterium* antagonists also varied greatly between each of species of pathogenic fungi. Specifically, *F. graminearum* (UAMH 3329) was significantly inhibited (> 30 % growth inhibition, and $p < 0.05$, relative to controls) by 29 of 31 isolates, while the suppression of *F. fujikuroi* was statistically significant for only 17 combinations (Figure 5.1. and Figure 5.3.). Uniquely, *F. oxysporum* was highly sensitive to two strains of *M. thiocyanatum* (NBRC 103128 and JCM 10893), but the magnitude of inhibition was diminished greatly for all other tested strains (< 37 %), resulting in only 8 antagonists with statistical suppression (Figure 5.2.).

Here, we also demonstrated that the presence of a bacterial endophyte, namely *M. organophilum*, even at relatively low inoculum density (10^6 CFU/mL) improves germination and seedling development and suppresses the nectrotrophic behaviour of two strains of *F. graminearum* towards soybean (*Glycine max*) seedlings in both soil and artificial growth medium. Importantly, our work has indicated that the presence of *M. organophilum* appears to preserve the health of the seed coat by slowing its digestion by *F. graminearum*, restoring the normal coat shedding process – evidently, this appears essential to preventing secondary injury to the embryonic tissue and further pathogen ingress (Figure 5.8. and Figure 5.9.). Seedlings co-inoculated with *M. organophilum* and *F. graminearum* had overall lower disease severity scores (DSS) (Figure 5.7.), longer taproots and taller stems (43% and 31%, respectively), thinner reduced surface colonization on microscopic examination, and a complete absence of soft rot in the maturing cotyledons (Figure 5.8. and Figure 5.9.). These results suggest that *Methylobacterium* may play an important role in slowing the progression of serious disease by soilborne pathogens like *Fusarium*.

The phenomenon that bacterial isolates on fungal growth are heavily match-dependent however, is unusual and raises questions about how BC may be mediated. For example, if fungal growth was supressed by either the presence of a broad-spectrum, toxic metabolite or the digestion of characteristic fungal tissues, any aggressively inhibitory strain – such as *M. oryzae* against *F. fujikuroi* – should produce similar, if not identical, magnitudes of inhibition in the other tested fungal species. Instead, the most effective bacterial antagonist for each species of *Fusarium* was unique, except for NBRC 103124, NBRC 107716, and NBRC 103119 (Table 5.3.). This finding is strikingly dissimilar to the results of other BC studies involving *Bacillus* and *Pseudomonas*, where the effects of inhibitory metabolites and proteins were generally similar across fungal species and strains (Khan et al. 2018; Schouten et al. 2004).

We posit that these findings collectively suggest that the inhibitory effects observed are not driven by a single, broadly toxic metabolite or the activity of a conserved enzyme, but are instead the result of a multifactorial, context-dependent antagonism that varies between specific pairings. The pronounced match-dependency implies that the efficacy of BC in this system is not predicted solely on the inherent aggressiveness or metabolic activity of any one bacterial isolate, but rather emerges from a combination of environmental, physiological, and biochemical compatibilities.

Several potentially synergistic mechanisms may be responsible for the nuanced patterns of inhibition. For example, in direct co-culture challenges, rapid bacterial colonization of the agar surface likely resulted in a depletion of essential macronutrients in an area localized around the inoculation zone, thus imposing constraints on hyphal extension. In our work, we frequently observed the redirection of hyphal growth of

Fusarium around and under the line of *Methylobacterium* in direct co-culture experiments, potentially as a nutrient-seeking strategy. Yet, nutrient deprivation alone does not fully account for the observed outcomes of broth-filtrate assays, wherein fungal growth on solid medium amended with spent bacterial broth was still worse than controls cultured on medium diluted with up to 40% (v/v) isotonic solution. For nutrient depletion to have such a degree of match-dependency would imply the unlikely case that each fungal species was responding to the loss of a unique nutrient, sequestered by only an equally unique and highly specific range of bacterial isolates.

Other pressures including competition for non-nutrient resources may also form some facet of the observed BC. Fast-growing bacteria can form dense biofilms which physically prevent fungal hyphae from advancing. This phenomenon is a form of passive antagonism, whereby the spatial architecture and physical occupation of the substrate preclude fungal access to nutrient-rich zones and is particularly effective against pathogens with competitive saprophytic behaviour. *Fusarium* species, noted for their aggressive saprophytic nature, readily colonize decaying substrates to secure a robust nutrient base and increase inoculum density. Prior colonization by another microorganism, however, could reduce the potential for successful invasion by facultative saprotrophs like *Fusarium*. Notably, the initial colonizer may not even need to possess high saprophytic competence; rather, early access to, and rapid colonization of the substrate may itself be sufficient to confer an exclusionary advantage. This mechanism is especially relevant in light of our findings: rapid colonization of the seed coat by *Methylobacterium* was associated with delayed digestion of the coat, improved health of the embryo, and a greater rate of germination (Figure 5.8 and Figure 5.9). The competitive advantage of *Methylobacterium* could also be attributed to the passive use

of host-derived metabolites – such as methanol, a byproduct of cell wall synthesis expressed by the host during growth – which are not accessible to *Fusarium*. As the consumption of metabolic byproducts does not harm host tissues, *Methylobacterium* could proliferate rapidly and occupy tissues which the fungal pathogen may require access to in order to extract nutrients enzymatically.

Although the results from both *in planta* and *in vitro* co-culture challenges align with the substrate occupation hypothesis, the outcomes of bacterial broth-filtrate assays again suggest an additional mode of antagonism is at play. In these assays, spent bacterial broth, filtered to remove live cells, was used to develop solid growth medium onto which *Fusarium* species were inoculated. Fungal growth on amended medium was markedly slower and resulted in morphological changes including notable deterioration of pigmentation (Figure 5.5.). These findings indicate that even in the absence of *Methylobacterium*, inhibitory effects on fungal development are retained – independent of physical substrate competition.

The mechanism which underpins BC exerted by *Methylobacterium* likely involves pressures like substrate occupation and nutrient deprivation but is primarily driven by one or more bioactive effector molecules. Critically, we have demonstrated that the inhibitory effects on *Fusarium* are preserved in both direct co-culture and in spent bacterial broth, indicating that the active agents are soluble, generally stable, and effective in the absence of living bacterial cells. The differential responses among *Fusarium* species to live cultures and broth derived from various *Methylobacterium* isolates, also suggests that the bioactivity is not attributable to a single compound, but rather to a cluster of effectors acting in concert.

Several possibilities merit consideration, including modulation of pH by bacterial metabolism which may impose chemical barriers to fungal colonization. Certain endophytic bacteria are known to acidify or alkalinize their surroundings through amino acid catabolism, and alter the rhizosphere in ways that affect pathogen viability. A striking example of this strategy is illustrated by *Rahnella aquatilis*, an endophytic rhizobacterium which suppresses *F. oxysporum* through secretion of gluconic acid (GlcA), thereby acidifying the rhizosphere and counteracting the virulence-associated alkalinization undertaken by *F. oxysporum* (Palmieri et al. 2020). In doing so, *R. aquatilis* not only impairs fungal infection but also exploits the hyphae of *F. oxysporum* as physical conduits to enhance its own root colonization guided by chemotaxis (Palmieri et al. 2020). Though not evaluated in our work, the sensitivity of fungal enzymes to changes in pH would mean that deviations from an optimal range could contribute to developmental arrest or dysregulated morphology. Further, shifts in pH imparted by bacterial growth may also alter the bioavailability of nutrients, or the activity of diffusible secondary metabolites.

The role of secreted antibiosis factors also remains a key avenue of interest. Potential players may include lipopeptides (e.g., surfactin, iturin, fengycin), siderophores (which sequester essential micronutrients), or proteolytic enzymes such as chitinases and glucanases which target fungal cell walls. Though less characterized, bacteria used as part of a BC strategy could also emit volatile organic compounds (VOCs) and other semiochemicals that influence fungal gene expression – including pathways related to stress tolerance, secondary metabolism, and development.

Importantly, in experiments where *Fusarium* was inoculated onto medium amended with *Methylobacterium* broth filtrate, fungal germination was significantly delayed compared to controls, though amended medium was not universally lethal or inhibitory to the tested Fusaria (Figure 5.5). We propose that alongside other pressures, bacterially derived hormones may form a facet in the unique BC characteristics of *Methylobacterium* towards *Fusarium*. The production of intense hormone signals by *Methylobacterium* may saturate sensing functions in *Fusarium*, quenching hormone-driven communication and coordination processes required for growth and development. Hormones produced by *Methylobacterium* may also influence *Fusarium* by producing signal interference at the host-pathogen interface and disrupt the subtle chemical cues which the phytopathogen may rely on to identify a compatible and vulnerable host.

This hypothesis is supported by established evidence that *Fusarium* species not only synthesize phytohormones such as auxins, cytokinins, and gibberellins, but also respond to them (Niehaus et al. 2016). For instance, *F. fujikuroi* and related taxa are known to produce these hormones during plant colonization, suggesting an adaptive advantage in manipulating host physiology (Cen et al. 2020; Niehaus et al. 2016). While specific receptors and hormone-sensing mechanisms in *Fusarium* remain poorly characterized, the potential for these compounds to also act as intra-fungal signals cannot be excluded.

In pathogenic fungi, mitogen-activated protein kinase (MAPK) cascades are central to the transduction of external chemical signals into growth, developmental, and in some cases virulence responses (Xu, 2000). In *F. oxysporum*, MAPKs such as Fmk1

and Mpk1 regulate critical processes including chemotropism, cell wall remodeling, and invasive growth in response to environmental and host-derived cues (Di Pietro et al. 2001; Rispail and Di Pietro, 2010). While specific links between these MAPK pathways and phytohormones in *Fusarium* remain undetermined, recent findings in other fungal systems provide compelling evidence that phytohormones, particularly cytokinins (CKs), can directly modulate fungal signalling, including MAPK-dependent pathways (Gupta et al. 2021).

A landmark study by Gupta et al. (2021) demonstrated that exogenous application of the synthetic CK 6-benzylaminopurine (6-BAP), the natural CK forms Zeatin and kinetin, and the synthetic bacterial-derived CK, thidiazuron (TDZ), impact the growth and development of multiple fungal pathogens, including *F. oxysporum* f. sp. *lycopersici*. Researchers determined that exogenous treatments with CKs could downregulate key cellular pathways – including those governing the cell cycle, cytoskeleton integrity, endocytosis, and MAPK signalling – in *Botrytis cinerea*, another necrotrophic fungal pathogen. Transcriptomic analysis revealed broad suppression of MAPK pathway components in response to CK treatment, alongside dramatic alterations in hyphal morphology, actin distribution, and vesicle trafficking. Importantly, these inhibitory effects were not cytotoxic but fungistatic, implicating CK perception and downstream signalling disruption rather than cellular damage (Gupta et al. 2021).

Given the conservation of MAPK and cytoskeletal regulatory networks in filamentous fungi, it is plausible that *Fusarium* species, may similarly interpret CKs as regulatory cues. Although *Fusarium* displayed a lower sensitivity to CKs compared to

B. cinerea in the same study, this variability may reflect differences in hormone uptake, receptor presence, or downstream signalling integration. The evolutionary familiarity with hormone biosynthesis further supports the hypothesis that *Fusarium* has the capacity to both respond to and modulate hormone signalling – potentially making it susceptible to signal disruption by hormone-producing microbial competitors like *Methylobacterium* (Niehaus et al. 2016; Studt et al. 2013).

Our findings align with this framework: broad-spectrum, yet isolate- and species-specific inhibition of *Fusarium* species by *Methylobacterium*, suggests the involvement of diffusible factors like hormones and their analogs. We suggest that microbiome enrichment with antagonists like *Methylobacterium* could stave off infection by maintaining unfavourable conditions for the pathogen. Evidence of the direct beneficial effects of *Methylobacterium* on plants are multitudinous and range from improving tolerance to salinity (Bradley et al. 2021; Broders et al. 2007; Lee et al. 2015) and drought (Jorge et al. 2019), to enhancing defense responses (Madhaiyan et al. 2006) and improving growth (Abadi et al. 2020; Abanda-Nkpwatt et al. 2006, 2006; Kuklinsky-Sobral et al. 2004; Maneewan and Khonsarn 2017; Meena et al. 2012; Senthilkumar and Krishnamoorthy 2017). Ultimately, incorporation of bacterial endophytes into existing crop protection strategies may offset the number of synthetic fungicides required for adequate – both in extent and stability – control, especially with newfound compatibility between these organisms and commercial formulations (Palberg and Emery 2025) (Chapter 4).

5.7. CONCLUSION

Our findings reveal that the biocontrol efficacy of *Methylobacterium* against *Fusarium* pathogens is highly match-specific, with distinct bacterial isolates demonstrating varying levels of suppression across different *Fusarium* species. Notably, the spatially dependent inhibition observed in co-culture assays suggests that diffusible signal is potentially interfering with fungal communication ultimately disrupting growth and diminishing infectivity. These insights not only deepen our understanding of interkingdom interactions but also underscore the promise of leveraging *Methylobacterium* as part of an integrated pest management strategy to restore phytobiome balance, enhancing crop resilience and food security.

5.8. TABLES AND FIGURES

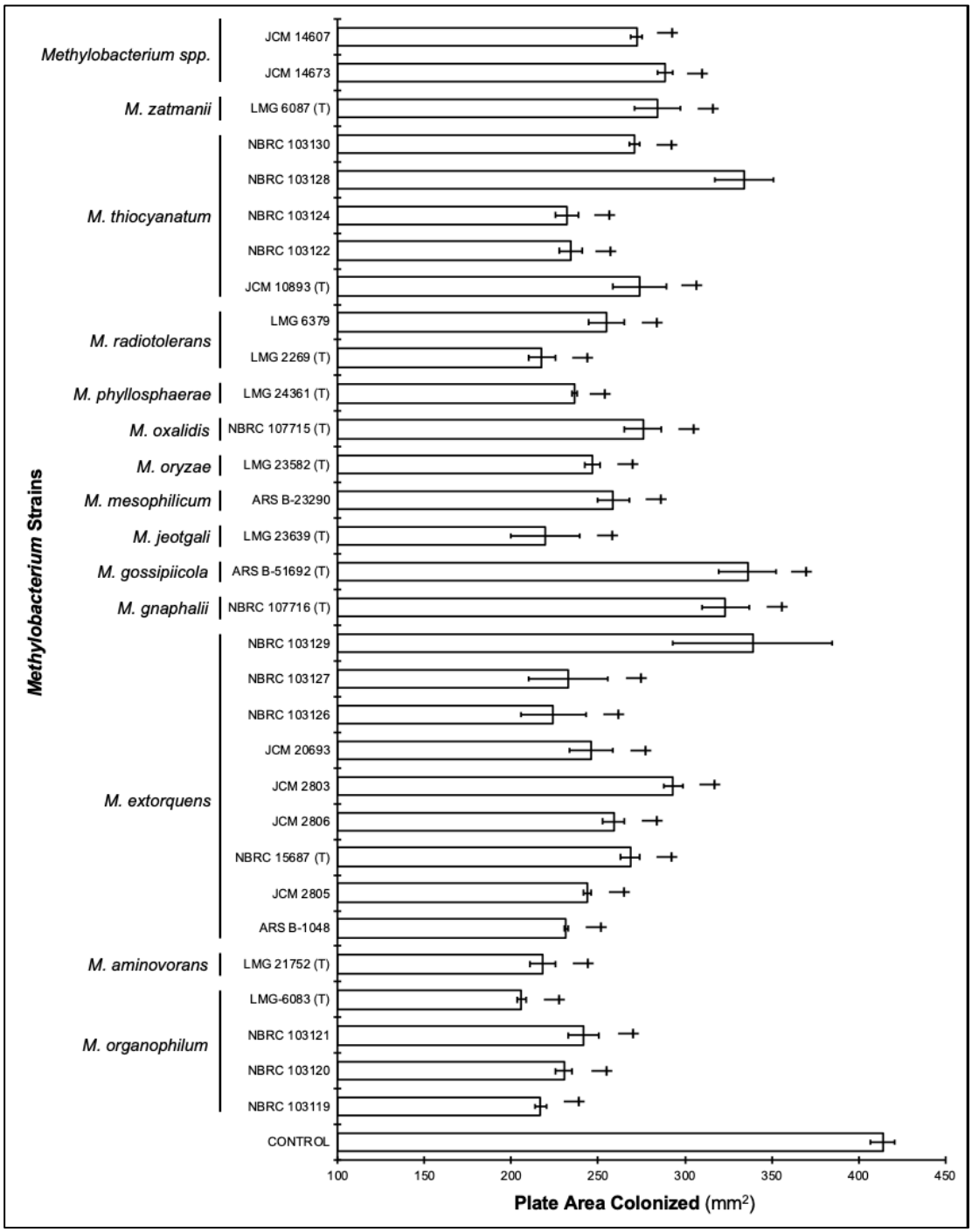

Figure 5.1: Average (n = 3) mycelium colonization area of *F. graminearum* (UAMH 3329) in agar plug co-culture assay with *Methylobacterium* assay after 10 days incubation (27 ºC) in darkness on TSA. Statistical relevance ($p < 0.05$) of each strain compared to the control indicated by (†).

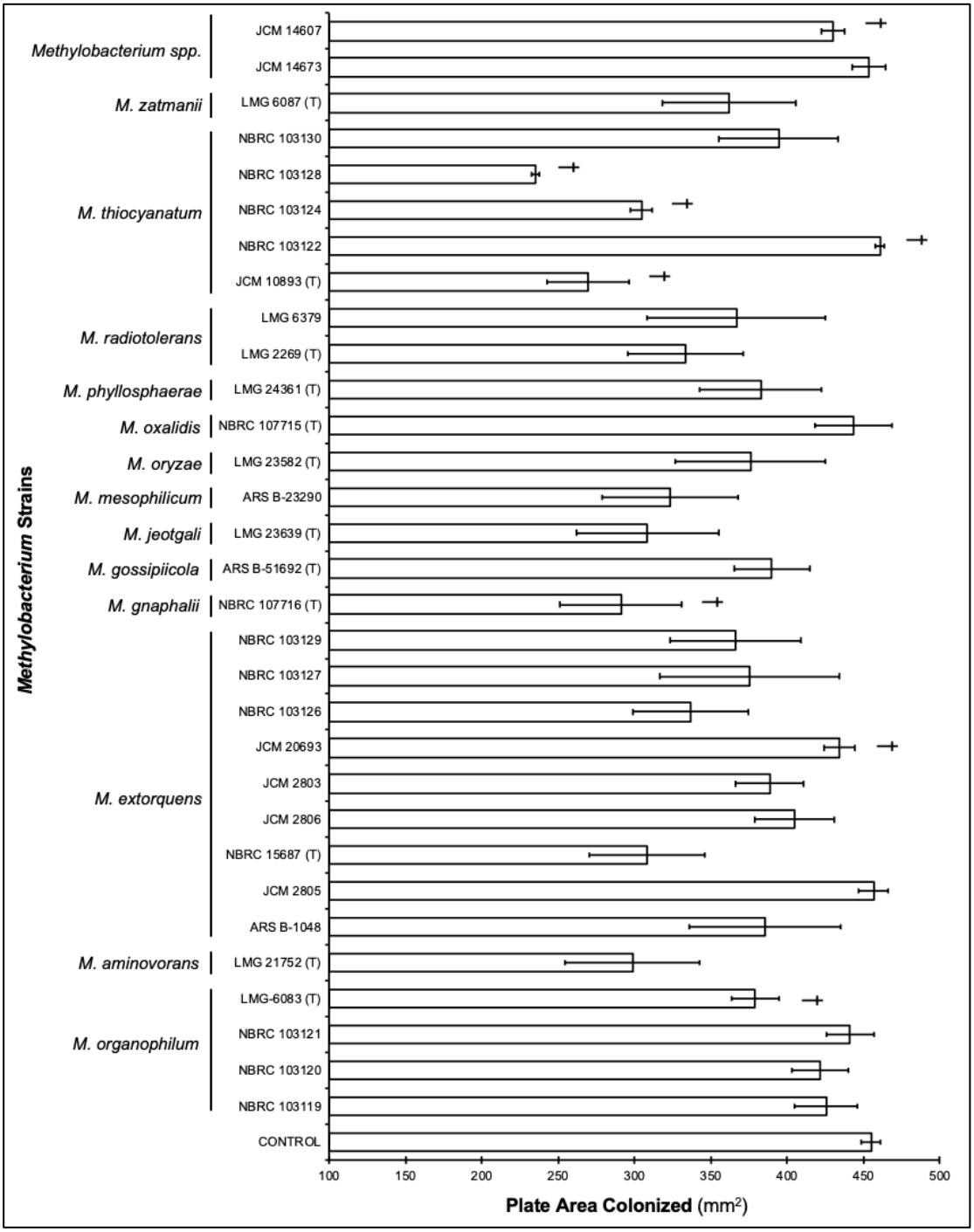

Figure 5.2: Average (n = 3) mycelium colonization area of *F. oxysporum* (UAMH 9013) in agar plug co-culture assay with *Methylobacterium* assay after 10 days incubation on TSA. Statistical relevance ($p < 0.05$) of each strain compared to the control indicated by (†).

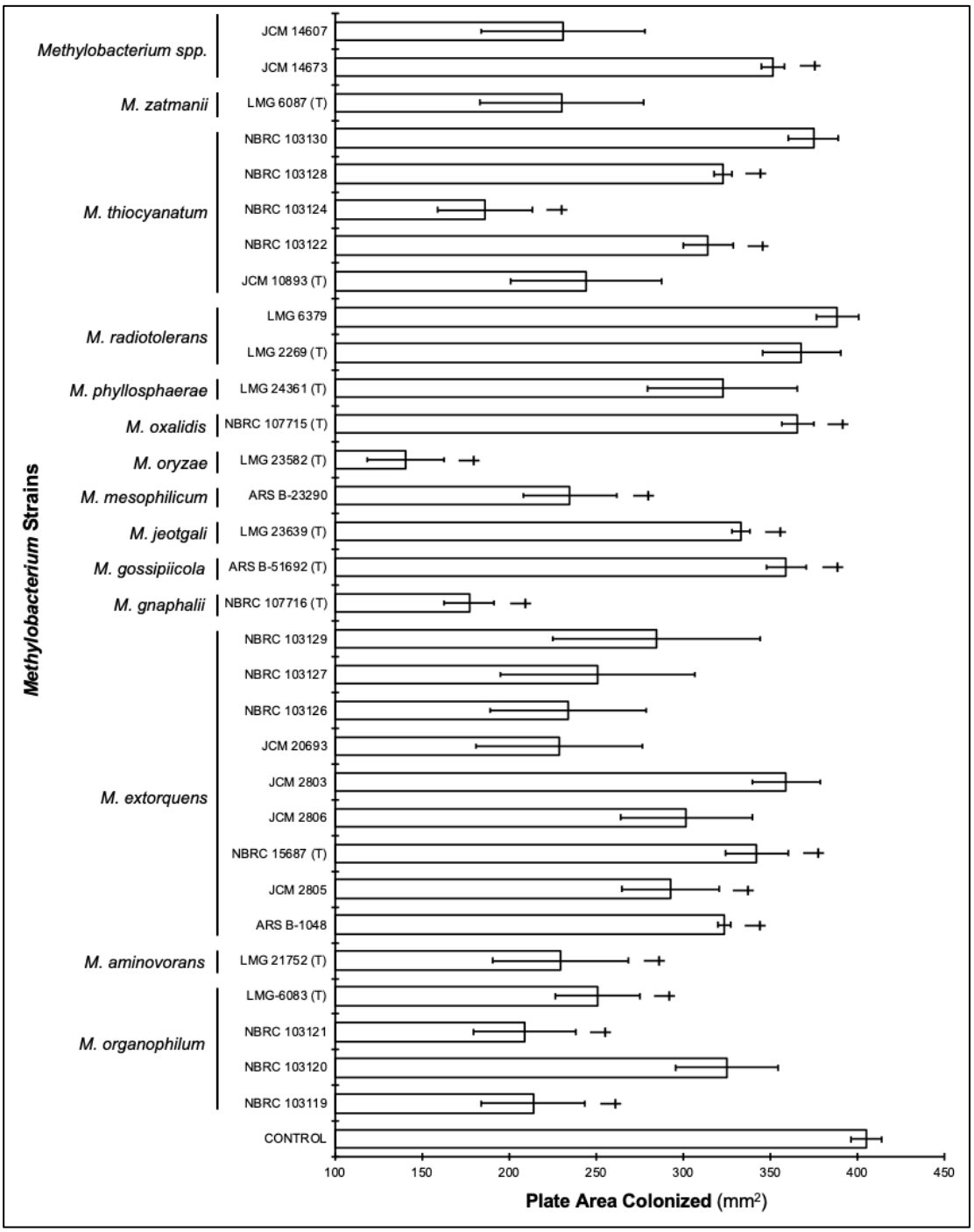

Figure 5.3: Average (n = 3) mycelium colonization area of *F. fujikuroi* (UAMH 9877) in agar plug co-culture assay with *Methylobacterium* assay after 10 days incubation on TSA. Statistical relevance ($p < 0.05$) of each strain compared to the control indicated by (†).

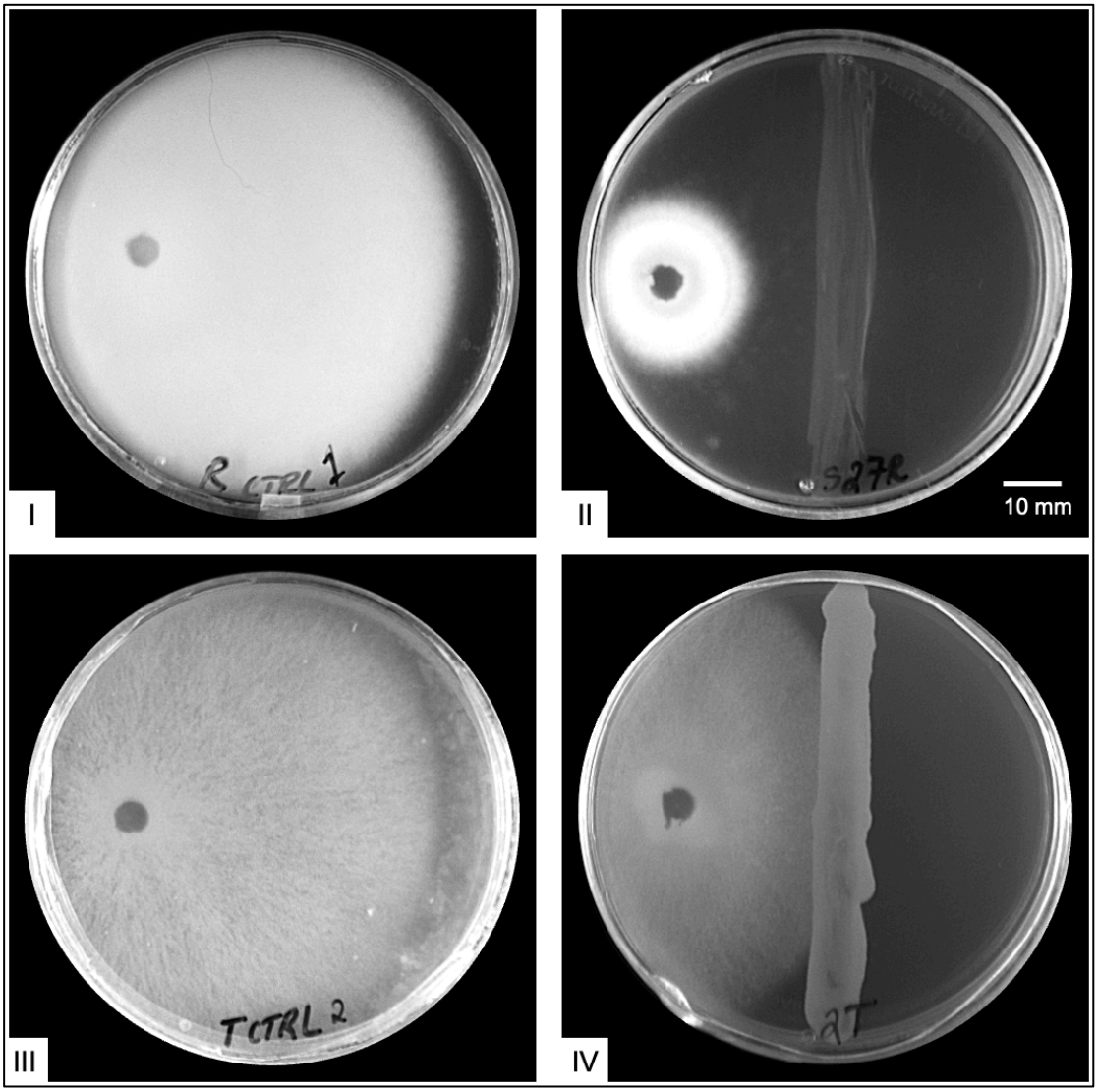

Figure 5.4: Monochromatic photographs of agar plug assay containing (I) *F. fujikuroi* control, (II) *F. fujikuroi* antagonized by *M. oryzae* (LMG 23582), (III) *F. graminearum* (UAMH 3329) control, and (IV) *F. graminearum* antagonized by *M. organophilum* (NBRC 103120), showing varying magnitudes of sensitivity after 14 days of incubation in darkness.

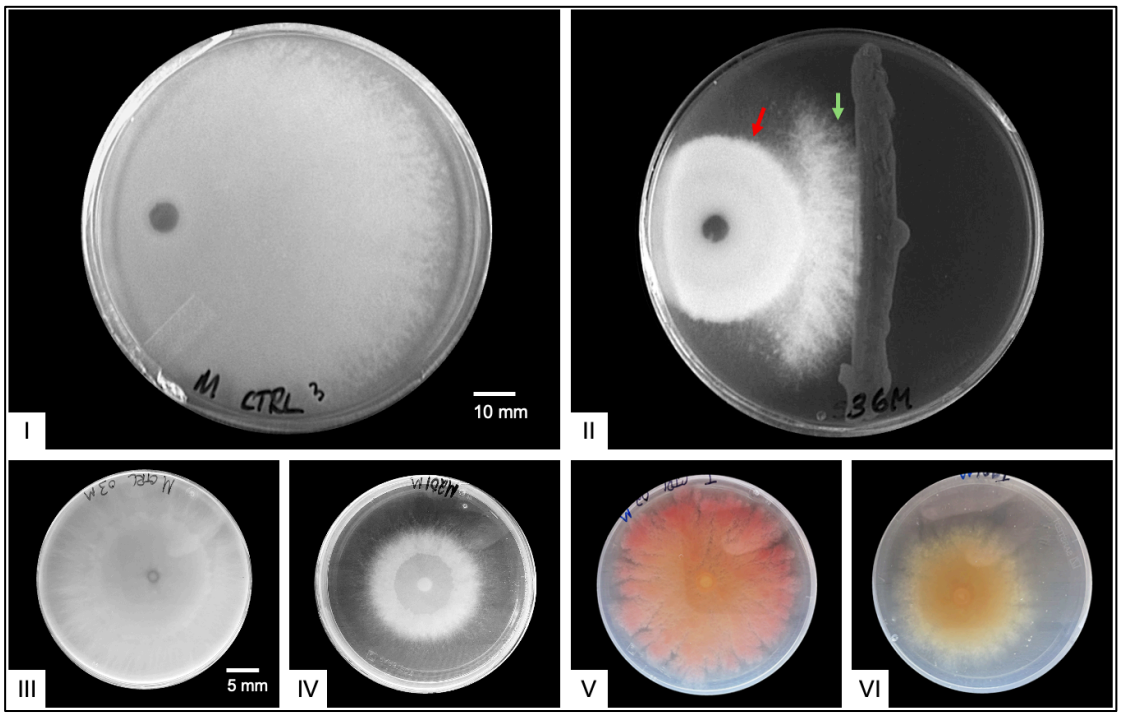

Figure 5.5: Monochromatic image of *F. oxysporum* agar plug growth assay after 10 days on TSA under (I) control and (II) antagonized by *M. thiocyanatum* (NBRC 103128). Additional monochromatic images of *M. oxysporum* cultures after 10 days on MHCNA under (III) control, and (IV) amended with broth from *M. thiocyanatum* (JCM 10893), and full-colour images of *F. graminearum* (UAMH 3329) 10-days post-inoculation on (V) MHCNA, and (VI) MHCNA amended with broth from *M. organophilum* (LMG 6083). Red arrow indicating initial mycelium mat, green arrow indicating area of modified growth as hyphal network of *M. oxysporum* penetrates surface of the growth medium and continues to expand under antagonistic bacteria.

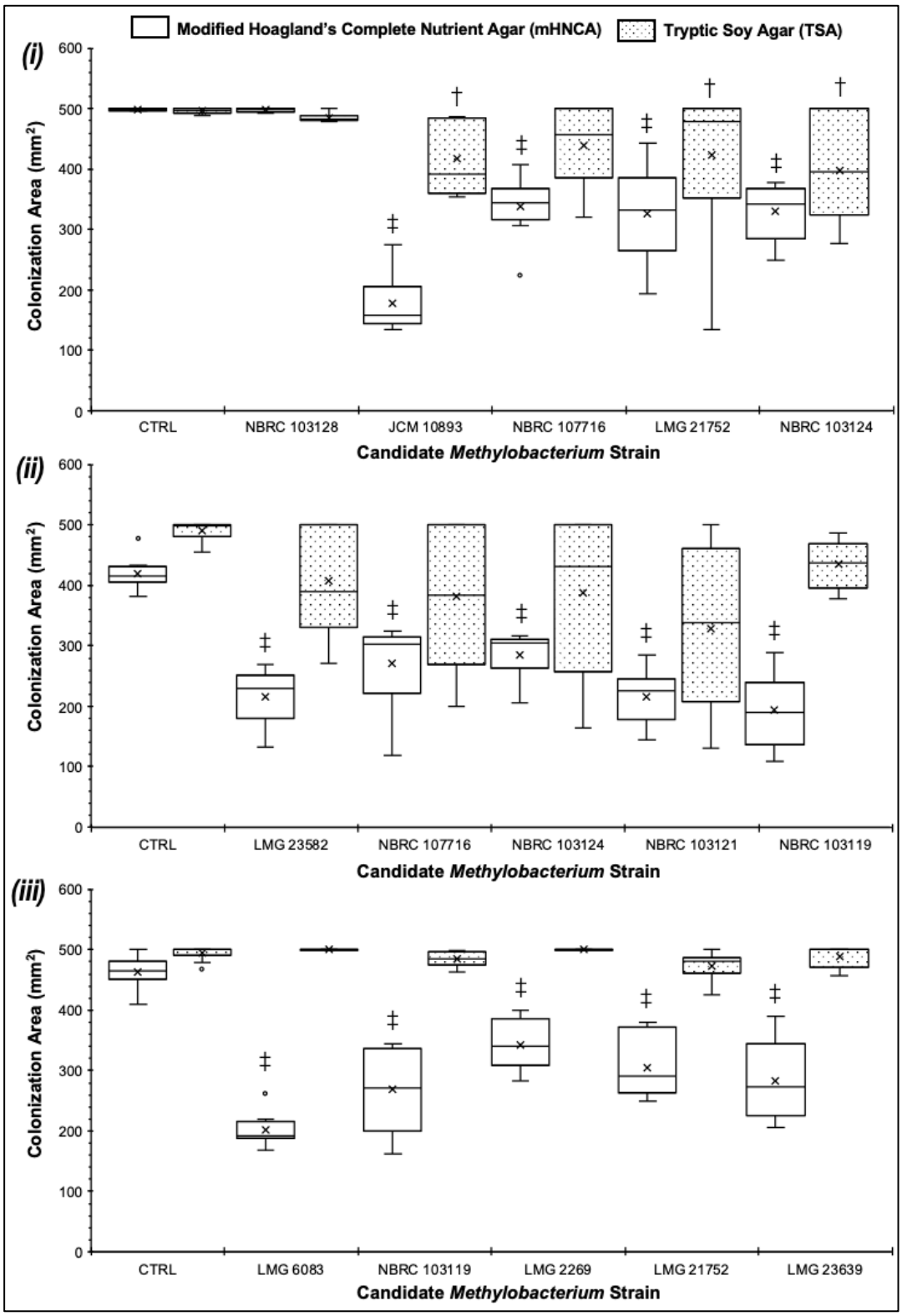

Figure 5.6: Box and whisker plot of mycelium growth of (i) *F. oxysporium* (UAMH 9013), (ii) *F. fujikuroi* (UAMH 9877), and (iii) *F. graminearum* (UAMH 3329) in plates containing MHCNA and TSA amended with spent broth of most inhibitory *Methylobacterium* isolates for each respective fungal phytopathogen, based on initial co-culture assay (Figure 5.1. - 5.3.). Statistical significance ($p < 0.05$ and < 0.01) of tested replicates ($n = 12$) relative to controls indicated by (†) and (‡), respectively. Median represented by horizontal line and exclusive mean represented by (x).

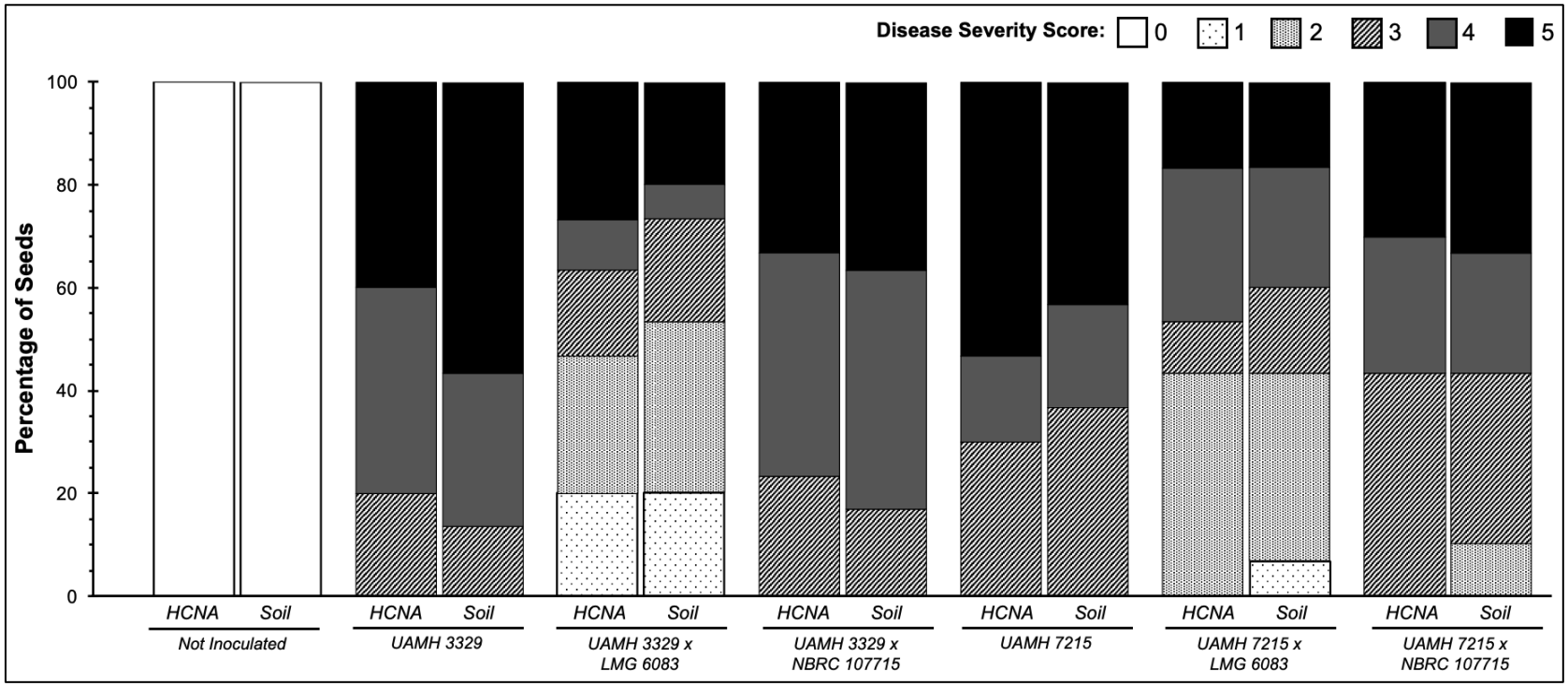

Figure 5.7: Distribution of disease severity score (DSS) across total population per treatment condition (n = 60) in both HCNA and sterile soil. DSS scored on a 5-point ordinal scale: 5 = no germination and total colonization of the seed; 4 = germination verified by radicle protrusion, but extensive colonization and lesions or necrosis affecting > 75% of the root; 3 = successful germination, moderate colonization of seed, and lesions affecting 25-75% the root; 2 = successful germination and stand, lesions affecting 10-24% of the root; 1 = successful germination and seedling development with lesions affecting < 10% of root tissues, and 0 = successful germination and healthy seedling development with no visible signs of colonization.

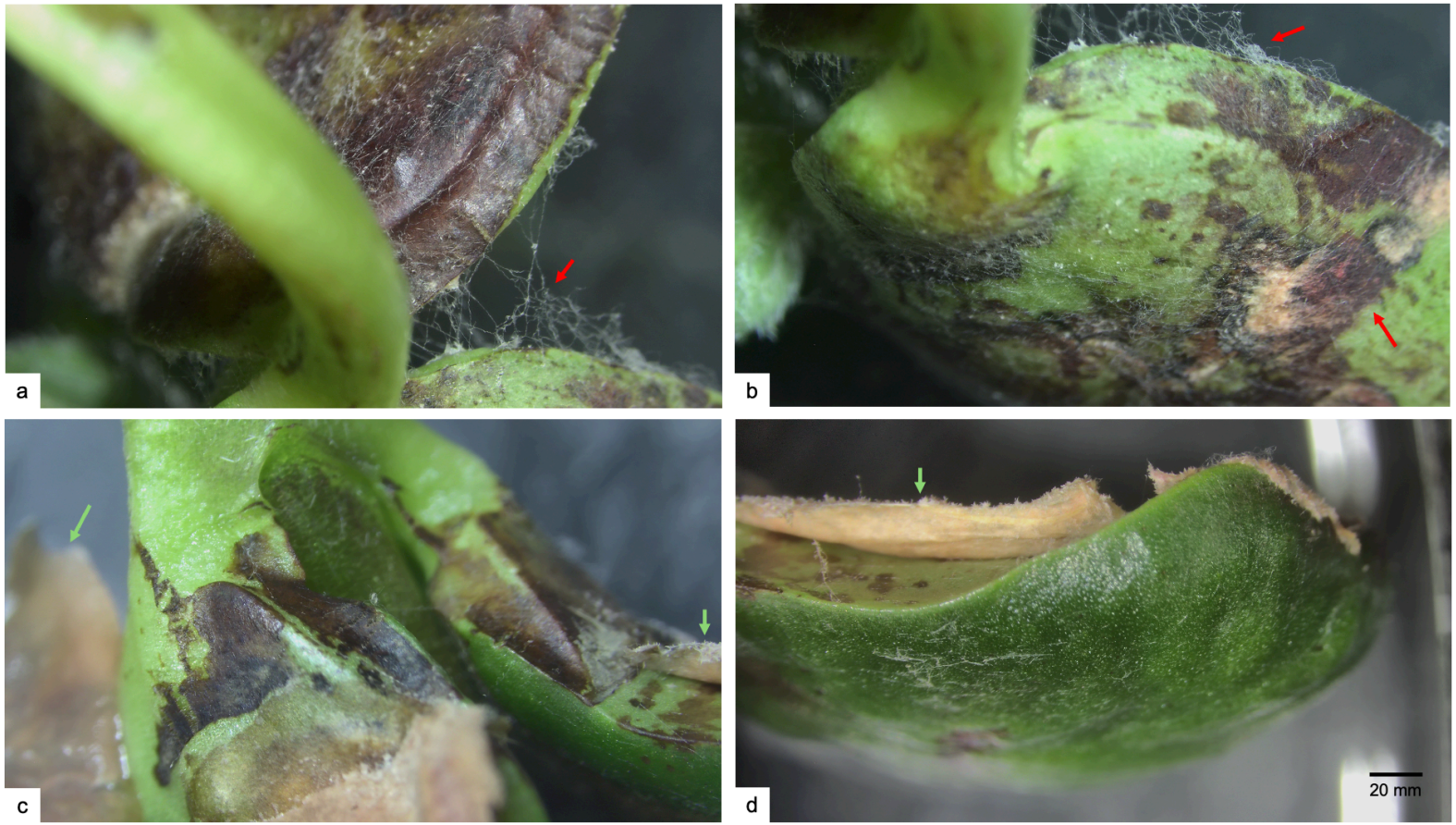

Figure 5.8: Representative photograph of soybean cotyledons after 8 days in soil and inoculated with (a-b) *F. graminearum* (UAMH 3329) alone (DSS. = 4), and (c-d) protective co-culture containing *M. organophilum* (LMG 6083). (DSS = 2). Red arrows indicating fungal structures, while green arrows indicate preserved seed coat.

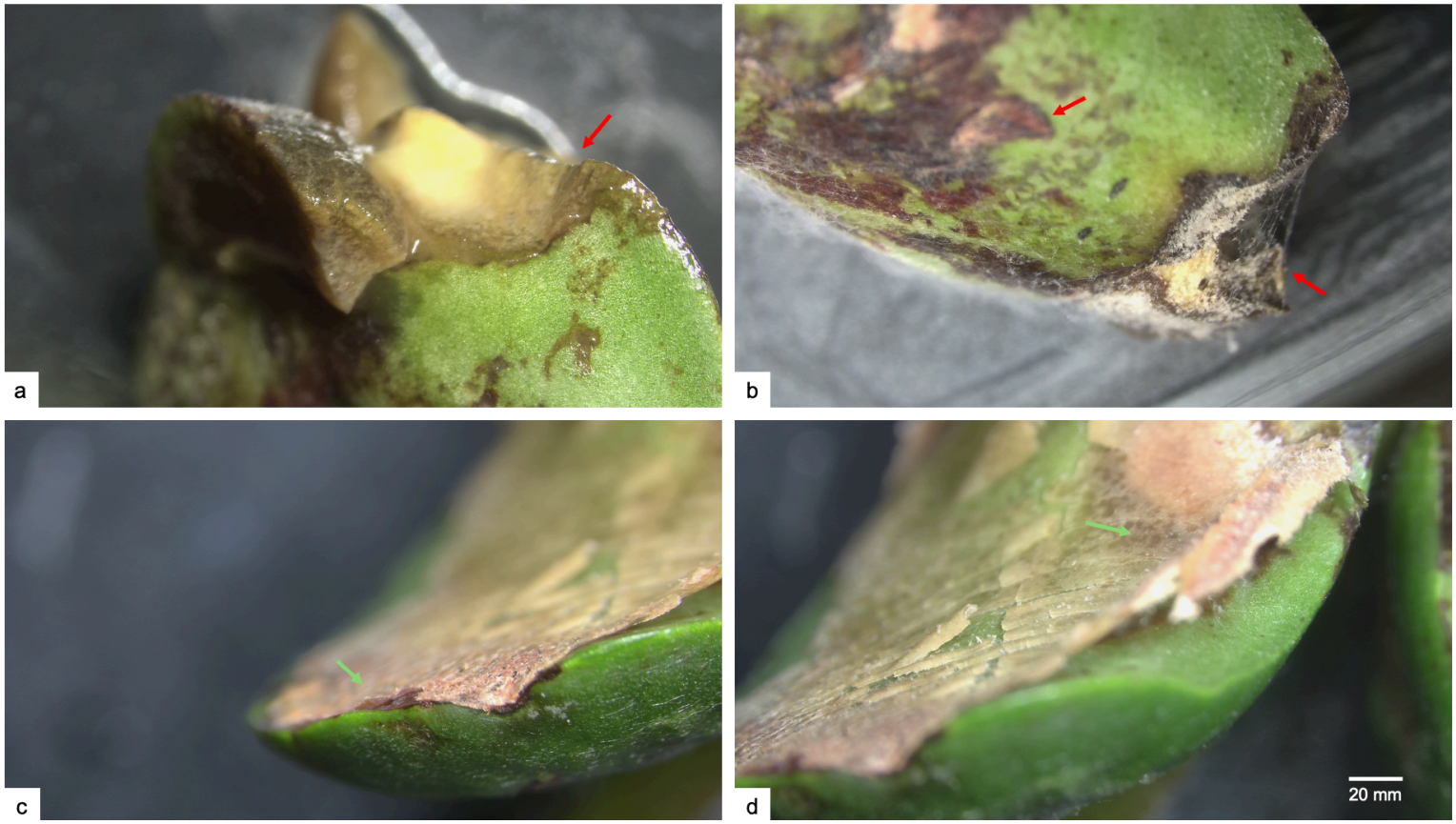

Figure 5.9: Representative photographs of soybean cotyledons after 8 days in HCNA and inoculated with (a-b) *F. graminearum* (UAMH 3329) alone (DSS = 5), and (c-d) protective co-culture containing *M. organophilum* (LMG 6083) (DSS = 2). Red arrows indicating fungal structures, while green arrows indicate preserved seed coat.

Figure 5.10: Stereomicroscopic images captured at 20 x magnification of example soybean radicles grown in HCNA taken after 8-days of growth in HCNA, and post-treatment in isotonic baths containing, (a) *F. graminearum* (UAMH 7215) [DSS = 4], and (b and c) co-culture of *F. graminearum* (UAMH 7215) and *M. organophilum* (LMG 6083) [DSS = 1]. Red arrows indicating fine fungal structures including fruiting bodies and thin mycelial network.

Table 5.1: Inventory of *Methylobaterium* strains examined for biocontrol of *Fusarium*.

Species	Strain	Isolation Source
M. aerolatum	JCM 16406 (*T*)	Air
M. aminovorans	LMG 21752 (*T*)	Soil
M. aquaticum	NRRL B-59286	Phoenix spacecraft surface
M. cerastii	DSM 23679 (*T*)	*Cerasium holosteoides* - phyllosphere
M. extorquens	NRRL B-1048	Garden soil enriched with sarcosine
	JCM 2805	Air
	NBRC 15687 (*T*)	Soil
	JCM 2806	Garden soil, slough
	DSM 13060	Pine (*Pinus sylvestris*) meristem tissue cultures
	DSM 23939	*Arabidopsis thaliana* - phyllosphere
	JCM 2803	*Psychotria mucronata* - phyllosphere
	JCM 20693	Mine water
	NBRC 103126	Soil-litter close to *Rumex* sp.
	NBRC 103127	Soil-litter close to *Arum* sp.
	NBRC 103129	Soil-litter close to *Eucalyptus* sp.
M. gnaphalii	NBRC 107716 (*T*)	*Gnaphalium spicatum* - phyllosphere
M. gossipiicola	NRRL B-51692 (*T*)	Cotton (*Gossipium hirsutum*) - phyllosphere
M. jeotgali	LMG 23639 (*T*)	Traditional fermented seafood (jeotgal)
M. mesophilicum	NRRL B-14246 (*T*)	Perennial rye grass (*Lolium perenne*) - phyllosphere
	NRRL B-2390	Household well water
M. nodulans	LMG 21967 (*T*)	*Crotalaria podocarpa* - phyllosphere
M. organophilum	LMG 6083 (*T*)	Lake water, lake sediment
M. organophilum	NBRC 103119	*Pelargonium zonale* - petiole maceration
	NBRC 103120	*Ficus elastica* - petiole maceration
	NBRC 103121	*Begonia* sp. - petiole maceration
M. oryzae	LMG 23582 (*T*)	*Oryzae sativa* cv Nam-Pyeoung - surface-disinfected
M. oxalidis	NBRC 107715 (*T*)	*Oxalis corniculata* - phyllosphere
M. phyllosphaerae	LMG 24361 (*T*)	*Oryzae sativa* cv. Dong-Jin - leaf tissues
M. platani	JCM 14648 (*T*)	*Platanus orientalis* - leaf
M. radiotolerans	LMG 2269 (*T*)	Japanese unpolished (unhulled) aged commercial rice
	LMG 6379	Forest soil
M. rhodinum	LMG 2275 (*T*)	Alder (*Alnus*) rhizosphere
M. thiocyanatum	JCM 10893 (*T*)	*Allium aflatuense* - rhizosphere soil
	NBRC 103122	*Bryophyllum* sp. - petiole maceration
M. thiocyanatum	NBRC 103128	Soil-litter close to *Mesenbryanthemum* sp.
	NBRC 103130	Soil-litter close to *Rumex* sp.
M. zatmanii	LMG 6087 (*T*)	Fermentor operating with formaldehyde as sole source of carbon
Methylobacterium spp.	DSM 23936	*Medicago truncatula* - phyllosphere
	JCM 14673	*Oryza sativa* SC-41 - phyllosphere
	JCM 14674	*Oryza rufipogon* W1964 - phyllosphere

(T) Indicates "type strain" cultures that were descended from a strain designated as the nomenclatural type.

Table 5.2: Inventory of *Fusarium* spp. isolates.

Species	Strain	Isolation Source
F. graminearum	UAMH 7215	Observably moldy hay (2-years old) used as cattle feed resulting in illness and death of calves. Canada, 1992.
F. graminearum	UAMH 3329	Undocumented.
F. oxysporum f. sp. cubense	UAMH 9013	Rhizome of banana plant (*Musae* sp.) showing symptoms of Panama disease. Jamaica, 1997.
F. fujikuori	UAMH 9877	Infected sugar cane (*Saccharum officinarum*). Taiwan, 2000.

Table 5.3: Inventory of nutrient-minimum growth medium variants based on Hoagland's hydroponic solution.

Hoagland's Medium Variant	Modification
Hoagland's Complete Nutrient Broth (HCNB)	Hoagland's original recipe.
Hoagland's Complete Nutrient Agar (HCNA)	Hoagland's original recipe, solidified with agar 1.6% w/v.
Modified Hoagland's Complete Nutrient Broth (MHCNB)	Hoagland's original recipe, amended with 10 g/L d-glucose.
Modified Hoagland's Complete Nutrient Agar (MHCNA)	Hoagland's original recipe, amended with 10 g/L d-glucose, solidified with agar 1.6% w/v.

Table 5.4: Ranking (greatest to least) of biocontrol of *Fusarium* spp. by *Methylobacterium* by magnitude of average mycelial growth suppression in agar plug co-culture assay (n = 3). Statistical difference from control ($p < 0.05$) indicated by (†).

	Methylobacterium Strain *(% mycelium suppression)*		
Rank	***F. oxysporum*** (UAMH 9013)	***F. fujikuori*** (UAMH 9877)	***F. graminearum*** (UAMH 3329)
1	*M. thiocyanatum* NBRC 103128 (48%)†	*M. oryzae* LMG 23582 (61%)†	*M. organophilum* LMG 6083 (50%)†
2	*M. thiocyanatum* JCM 10893 (41%)†	*M. gnaphalii* NBRC 107716 (51%)†	*M. organophilum* NBRC 103119 (48%)†
3	*M. gnaphalii* NBRC 107716 (36%)†	*M. thiocyanatum* NBRC 103124 (48%)†	*M. radiotolerans* LMG 2269 (47%)†
4	*M. aminovorans* LMG 21752 (34%)	*M. organophilum* NBRC 103121 (42%)†	*M. aminovorans* LMG 21752 (47%)†
5	*M. thiocyanatum* NBRC 103124 (33%)†	*M. organophilum* NBRC 103119 (40%)†	*M. jeotgali* LMG 23639 (47%)†
6	*M. extorquens* NBRC 15687 (32%)†	*M. extorquens* JCM 20693 (36%)	*M. extorquens* NBRC 103126 (46%)†
7	*M. jeotgali* LMG 23639 (32%)	*M. aminovorans* LMG 21752 (36%)†	*M. organophilum* NBRC 103120 (44%)†
8	*M. mesophilicum* ARS B-23290 (29%)	*M. zatmanii* LMG 6087 (36%)	*M. extorquens* ARS B-1048 (44%)†
9	*M. radiotolerans* LMG 2269 (27%)	*Methylobacterium spp.* JCM 14607 (36%)	*M. thiocyanatum* NBRC 103124 (44%)†
10	*M. extorquens* NBRC 103126 (26%)	*M. extorquens* NBRC 103126 (35%)†	*M. extorquens* NBRC 103127 (44%)†

5.9. REFERENCES

Abadi, V. A. J. M., Sepehri, M., Rahmani, H. A., Zarei, M., Ronaghi, A., Taghavi, S. M., and Shamshiripour, M. 2020. Role of Dominant Phyllosphere Bacteria with Plant Growth–Promoting Characteristics on Growth and Nutrition of Maize (*Zea mays* L.). J Soil Sci Plant Nutr 20:2348–2363. https://doi.org/10.1007/s42729-020-00302-1.

Abanda-Nkpwatt, D., Müsch, M., Tschiersch, J., Boettner, M., and Schwab, W. 2006. Molecular interaction between *Methylobacterium extorquens* and seedlings: Growth promotion, methanol consumption, and localization of the methanol emission site. Journal of Experimental Botany 57:4025–4032. https://doi.org/10.1093/jxb/erl173.

Abramowsky, C. R., Quinn, D., Bradford, W. D., and Conant, N. F. 1974. Systemic infection by *Fusarium* in a burned child. The Journal of Pediatrics. 84:561–564. https://doi.org/10.1016/S0022-3476(74)80681-5.

Anaissie, E. J., Kuchar, R. T., Rex, J. H., Francesconi, A., Kasai, M., Müller, F. C., Lozano-Chiu, M., Summerbell, R. C., Dignani, M. C., Chanock, S. J., and Walsh, T. J. 2001. Fusariosis Associated with Pathogenic *Fusarium* Species Colonization of a Hospital Water System: A New Paradigm for the Epidemiology of Opportunistic Mold Infections. Clin. Infec. Dis. 33:1871–1878. https://doi.org/10.1086/324501.

Anaissie, E., Kantarjian, H., Jones, P., Barlogie, B., Luna, M., Lopez, G.-B., and Bodey, G. P. 1986. *Fusarium*. A newly recognized fungal pathogen in immunosuppressed patients. Cancer 57:2141–2145. https://doi.org/10.1002/1097-0142(19860601)57:11<2141::aid-cncr2820571110>3.0.co;2-n

Balendres, M. A. O., Karlovsky, P., and Cumagun, C. J. R. 2019. Mycotoxigenic fungi and mycotoxins in agricultural crop commodities in the Philippines: A review. Foods 8:1–12. https://doi.org/10.3390/foods8070249.

Batista, B. G., Chaves, M. A. D., Reginatto, P., Saraiva, O. J., and Fuentefria, A. M. 2020. Human fusariosis: An emerging infection that is difficult to treat. Rev. Soc. Bras. Med. Trop. 53:e20200013. https://doi.org/10.1590/0037-8682-0013-2020.

Behr, M., Motyka, V., Weihmann, F., Malbeck, J., Deising, H. B., and Wirsel, S. G. R. 2012. Remodeling of Cytokinin Metabolism at Infection Sites of *Colletotrichum graminicola* on Maize Leaves. MPMI 25:1073–1082. https://doi.org/10.1094/MPMI-01-12-0012-R.

Biddeci, G., Donà, D., Geranio, G., Spadini, S., Petris, M. G., Pillon, M., Biffi, A., and Putti, M. C. 2020. Systemic Fusariosis: A Rare Complication in Children with Acute Lymphoblastic Leukemia. JoF 6:212. https://doi.org/10.3390/jof6040212.

Bourguignon, R. L., Walsh, A. F., Flynn, J. C., Baro, C., and Spinos, E. 1976. *Fusarium* species osteomyelitis. Case report. JBJS 58:722–723.

Boutati, E. I., and Anaissie, E. J. 1997. *Fusarium*, a Significant Emerging Pathogen in Patients with Hematologic Malignancy: Ten Years' Experience at a Cancer Center and Implications for Management. Blood 90:999–1008. https://doi.org/10.1182/blood.V90.3.999.

Bradley, C. A., Allen, T. W., Sisson, A. J., Bergstrom, G. C., Bissonnette, K. M., Bond, J., Byamukama, E., Chilvers, M. I., Collins, A. A., Damicone, J. P., Dorrance, A. E., Dufault, N. S., Esker, P. D., Faske, T. R., Fiorellino, N. M., Giesler, L. J., Hartman, G. L., Hollier, C. A., Isakeit, T., Jackson-Ziems, T. A., Jardine, D. J., Kelly, H. M., Kemerait, R. C., Kleczewski, N. M., Koehler, A. M., Kratochvil, R. J., Kurle, J. E., Malvick, D. K., Markell, S. G., Mathew, F. M., Mehl, H. L., Mehl, K. M., Mueller, D. S., Mueller, J. D., Nelson, B. D., Overstreet, C., Padgett, G. B., Price, P. P., Sikora, E. J., Small, I., Smith, D. L., Spurlock, T. N., Tande, C. A., Telenko, D. E. P., Tenuta, A. U., Thiessen, L. D., Warner, F., Wiebold, W. J., and Wise, K. A. 2021. Soybean Yield Loss Estimates Due to Diseases in the United States and Ontario, Canada, from 2015 to 2019. Plant Health Progress 22:483–495. https://doi.org/10.1094/PHP-01-21-0013-RS.

Broders, K. D., Lipps, P. E., Paul, P. A., and Dorrance, A. E. 2007. Evaluation of *Fusarium graminearum* associated with corn and soybean seed and seedling disease in Ohio. Plant Disease 91:1155–1160. https://doi.org/10.1094/PDIS-91-9-1155.

Bryden, W. L. 2007. Mycotoxins in the food chain: Human health implications. Asia Pacific Journal of Clinical Nutrition 16:95–101. https://doi.org/10.6133/apjcn.2007.16.s1.18.

Buhrow, L. M., Cram, D., Tulpan, D., Foroud, N. A., and Loewen, M. C. 2016. Exogenous Abscisic Acid and Gibberellic Acid Elicit Opposing Effects on *Fusarium graminearum* Infection in Wheat. Phytopathology. 106:986–996. https://doi.org/10.1094/PHYTO-01-16-0033-R.

Burgess, L.W., and Bryden, W.L. 2012. *Fusarium*: a ubiquitous fungus of global significance. Microbiol. Aust. 33:22–25. https://doi.org/10.1071/MA12022

Bürger, M., and Chory, J. 2019. Stressed Out About Hormones: How Plants Orchestrate Immunity. Cell Host and Microbe 26:163–172. https://doi.org/10.1016/j.chom.2019.07.006.

Chanclud, E., Kisiala, A., Emery, N. R. J., Chalvon, V., Ducasse, A., Romiti-Michel, C., Gravot, A., Kroj, T., and Morel, J.-B. 2016. Cytokinin Production by the Rice Blast Fungus Is a Pivotal Requirement for Full Virulence ed. Jin-Rong Xu. PLoS Pathog 12:e1005457. https://doi.org/10.1371/journal.ppat.1005457.

Corpe, W. A., and Rheem, S. 1989. Ecology of the methylotrophic bacteria on living leaf surfaces. FEMS Microbiology Letters 62:243–249. https://doi.org/10.1016/0378-1097(89)90248-6.

Di Pietro, A., García-Maceira, F. I., Meglecz, E., and Roncero, M. I. G. 2001. A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. Mol. Microbiol. 39:1140–1152.

Dourado, M. N., Camargo Neves, A. A., Santos, D. S., and Araújo, W. L. 2015. Biotechnological and agronomic potential of endophytic pink-pigmented methylotrophic Methylobacterium spp. Biomed Res. Int. 2015:909016. https://doi.org/10.1155/2015/909016.

Dóczi, I., Gyetvai, T., Kredics, L., and Nagy, E. 2004. Involvement of *Fusarium* spp. in fungal keratitis. Clinical Microbiology and Infection 10:773–776. https://doi.org/10.1111/j.1469-0691.2004.00909.x.

Ekwomadu, T.I., and Mwanza, M. 2023. *Fusarium* fungi pathogens, identification, adverse effects, disease management, and global food security: A review of the latest research. Agriculture 13:1810. https://doi.org/10.3390/agriculture13091810

Fu, J., Liu, H., Li, Y., Yu, H., Li, X., Xiao, J., and Wang, S. 2011. Manipulating Broad-Spectrum Disease Resistance by Suppressing Pathogen-Induced Auxin Accumulation in Rice. Plant Physiology 155:589–602. https://doi.org/10.1104/pp.110.163774.

Garcia, R. R., Min, Z., Narasimhan, S., and Bhanot, N. 2015. *Fusarium* brain abscess: case report and literature review. Mycoses. 58:22–26. https://doi.org/10.1111/myc.12271.

Green, P. N. 2006. Methylobacterium. In The Prokaryotes, New York, NY: Springer New York, pp. 257–265. https://doi.org/10.1007/0-387-30745-1_14.

Guarro, J. 2013. Fusariosis, a complex infection caused by a high diversity of fungal species refractory to treatment. Eur J Clin Microbiol Infect Dis. 32:1491–1500. https://doi.org/10.1007/s10096-013-1924-7.

Gupta, R., Anand, G., Pizarro, L., Laor Bar-Yosef, D., Kovetz, N., Sela, N., Yehuda, T., Gazit, E., and Bar, M. 2021. Cytokinin inhibits fungal development and virulence by targeting the cytoskeleton and cellular trafficking. MBio 12. https://doi.org/10.1128/mbio.03068-20.

Hinsch, J., Vrabka, J., Oeser, B., Novák, O., Galuszka, P., and Tudzynski, P. 2015. *De novo* biosynthesis of cytokinins in the biotrophic fungus *Claviceps purpurea*. Environmental Microbiology. 17: 2935–2951. https://doi.org/10.1111/1462-2920.12838.

Hiraishi, A., Furuhata, K., Matsumoto, A., Koike, K. A., Fukuyama, M., and Tabuchi, K. 1995. Phenotypic and genetic diversity of chlorine-resistant *Methylobacterium* strains isolated from various environments. Applied and Environmental Microbiology 61:2099–2107. https://doi.org/10.1128/aem.61.6.2099-2107.1995.

Holland, M. A. 1997a. Methylobacterium and plants. Recent Research Developments in Plant Physiology 207–213.

Holland, M. A. 1997b. Occam's Razor Applied to Hormonology. Plant Physiology 115:865–868.

Hossain, M. M., Sultana, F., Mostafa, M., Ferdus, H., Rahman, M., Rana, J. A., Islam, S. S., Adhikary, S., Sannal, A., Al Emran Hosen, M., Nayeema, J., Emu, N. J., Kundu, M., Biswas, S. K., Farzana, L., and Al Sabbir, M. A. 2024. Plant disease dynamics in a changing climate: impacts, molecular mechanisms, and climate-informed strategies for sustainable management. Discov. Agric. 2. https://doi.org/10.1007/s44279-024-00144-w.

Jakle, C., Leek, J. C., Olson, D. A., and Robbins, D. L. 1983. Septic arthritis due to *Fusarium solani*. The Journal of Rheumatology. 10:151–153.

Jorge, G. L., Kisiala, A., Morrison, E., Aoki, M., Nogueira, A. P. O., and Emery, R. J. N. 2019. Endosymbiotic *Methylobacterium oryzae* mitigates the impact of limited water availability in lentil (*Lens culinaris* Medik.) by increasing plant cytokinin levels. Environmental and Experimental Botany 162:525–540. .org/10.1016/j.envexpbot.2019.03.028.

Kazan, K., and Manners, J. M. 2009. Linking development to defense: auxin in plant–pathogen interactions. Trends in Plant Science 14:373–382. https://doi.org/10.1016/j.tplants.2009.04.005.

Khan, N., Martínez-Hidalgo, P., Ice, T. A., Maymon, M., Humm, E. A., Nejat, N., Sanders, E. R., Kaplan, D., and Hirsch, A. M. 2018. Antifungal Activity of *Bacillus* Species Against *Fusarium* and Analysis of the Potential Mechanisms Used in Biocontrol. Front. Microbiol. 9:2363. https://doi.org/10.3389/fmicb.2018.02363.

Kimanya, M. E. 2015. The health impacts of mycotoxins in the eastern Africa region. Current Opinion in Food Science 6: 7–11. https://doi.org/10.1016/j.cofs.2015.11.005.

Köhl J, Kolnaar R, Ravensberg WJ. 2019. Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy. Front Plant Sci. 10: 845. https://doi.org/10.3389/fpls.2019.00845.

Kuklinsky-Sobral, J., Araújo, W. L., Mendes, R., Geraldi, I. O., Pizzirani-Kleiner, A. A., and Azevedo, J. L. 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environmental Microbiology 6: 1244–1251. https://doi.org/10.1111/j.1462-2920.2004.00658.x.

Kulkarni, G. B., Sanjeevkumar, S., Kirankumar, B., Santoshkumar, M., and Karegoudar, T. B. 2013. Indole-3-Acetic Acid Biosynthesis in *Fusarium delphinoides* Strain GPK, a Causal Agent of Wilt in Chickpea. Appl Biochem Biotechnol 169: 1292–1305. https://doi.org/10.1007/s12010-012-0037-6

Kutschera, U. 2007. Plant-associated *Methylobacteria* as co-evolved phytosymbionts: A hypothesis. Plant Signalling and Behavior 2: 74–78. https://doi.org/10.4161/psb.2.2.4073.

Lee, Y., Krishnamoorthy, R., Selvakumar, G., Kim, K., and Sa, T. 2015. Alleviation of salt stress in maize plant by co-inoculation of arbuscular mycorrhizal fungi and *Methylobacterium oryzae* CBMB20. Journal of the Korean Society for Applied Biological Chemistry 58:533–540. https://doi.org/10.1007/s13765-015-0072-4.

Leslie, J. F., and Xu, J.-R. 2010. *Fusarium* genetics and pathogenicity. In: Borkovich, K. A., and Ebbole, D. J., eds. Cellular and Molecular Biology of Filamentous Fungi. American Society for Microbiology Press, Washington, DC. pp. 607–609. https://doi.org/10.1128/9781555816636.ch38

Liew, W. P. P., and Mohd-Redzwan, S. 2018. Mycotoxin: Its impact on gut health and microbiota. Frontiers in Cellular and Infection Microbiology 8. https://doi.org/10.3389/fcimb.2018.00060.

Lipka, V., and Panstruga, R. 2005. Dynamic cellular responses in plant-microbe interactions. Current Opinion in Plant Biology 8:625–631. https://doi.org/10.1016/j.pbi.2005.09.006.

Madhaiyan, M., Suresh Reddy, B. V., Anandham, R., Senthilkumar, M., Poonguzhali, S., Sundaram, S. P., and Sa, T. 2006. Plant growth-promoting *Methylobacterium* induces defense responses in groundnut (*Arachis hypogaea* L.) compared with rot pathogens. Current Microbiology 53:270–276. https://doi.org/10.1007/s00284-005-0452-9.

Madhavan, M., Ratnakar, C., Veliath, A. J., Kanungo, R., Smile, S. R., and Bhat, S. 1992. Primary disseminated fusarial infection. Postgraduate Medical Journal 68: 143–144. https://doi.org/10.1136/pgmj.68.796.143.

Maneewan, K., and Khonsarn, N. 2017. Selection of bioinoculants for tomato growth enhancement and pathogen resistance. Asia-Pacific Journal of Science and Technology 22: 1–7. https://doi.org/10.14456/apst.2017.37

Medina-Rios, M., Ceja Torres, L. F., López-Díaz, S., Venegas-González, J., and Sánchez-Hernández, C. V. 2019. Effect of *Methylobacterium extorquens* on tomato development in the presence or absence of *Fusarium oxysporum*. Remexca. 10: 1469–1479. https://doi.org/10.29312/remexca.v10i7.644.

Meena, K. K., Kumar, M., Kalyuzhnaya, M. G., Yandigeri, M. S., Singh, D. P., Saxena, A. K., and Arora, D. K. 2012. Epiphytic pink-pigmented methylotrophic bacteria enhance germination and seedling growth of wheat (*Triticum aestivum*) by producing phytohormone. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology. 101: 777–786. https://doi.org/10.1007/s10482-011-9692-9.

Moore, R. P. 1971. Mechanisms of water damage in mature soybean seed. Proc. Assoc. Off. Seed Anal. 61: 112–118. https://www.jstor.org/stable/23432426

Morrison, E. N., Emery, R. J. N., and Saville, B. J. 2017. Fungal derived cytokinins are necessary for normal *Ustilago maydis* infection of maize. Plant Pathology 66: 726–742. https://doi.org/10.1111/ppa.12629.

Murashige, T. and Skoog, F. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plantarum. 15: 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Mwenye, O. J., Van Rensburg, L., Van Biljon, A., and Van der Merwe, R. (2019). Seedling Shoot and Root Growth Responses among Soybean (*Glycine max*) Genotypes to Drought Stress. Intech Open. https://doi.org/10.5772/intechopen.81101

Nicholson, R. I. D., and Van Staden, J. 1988. Cytokinins and Mango Flower Malformation. I. Tentative Identification of the Complement in Healthy and Malformed Inflorescences. Journal of Plant Physiology 132:720–724. https://doi.org/10.1016/S0176-1617(88)80235-9.

Niehaus, E. M., von Bargen, K. W., Espino, J. J., Pfannmüller, A., Humpf, H. U., and Tudzynski, B. 2016. Comparative "Omics" of the *Fusarium fujikuroi* Species Complex Highlights Differences in Genetic Potential and Metabolite Synthesis Genome Biology and Evolution, 8(11), 3574–3599. https://doi.org/10.1093/gbe/evw259

Nucci, M., and Anaissie, E. 2002. Cutaneous Infection by Fusarium Species in Healthy and Immunocompromised Hosts: Implications for Diagnosis and Management. Clinical Infectious Disease. 35:909–920. https://doi.org/10.1086/342328.

Page, J. C., Friedlander, G., and Dockery, G. L. 1982. Postoperative *Fusarium* osteomyelitis. Journal of Foot Surgery 21:174–6.

Palberg, D., Kisiała, A., Jorge, G. L., and Emery, R. J. N. 2022. A survey of *Methylobacterium* species and strains reveals widespread production and varying profiles of cytokinin phytohormones. BMC Microbiology 22:1–17. https://doi.org/10.1186/s12866-022-02454-9.

Palberg, D., and Emery, N. R. 2025. Compatibility of Commercial Fungicide Formulations with Plant-Associated *Methylobacterium*. Can. J. Plant Sci. 105(1): 1-10. https://doi.org/10.1139/cjps-2024-0169

Palmieri D, Vitale S, Lima G, Di Pietro A, Turrà D. 2020. A bacterial endophyte exploits chemotropism of a fungal pathogen for plant colonization. Nat Commun. 11(1): 5264. https://doi.org/10.1038/s41467-020-18994-5.

Perera, I., Kisiala, A., Thompson, K. A., and Emery, R. J. N. 2025. Soil health improvements under cover crops are associated with enhanced soil content of cytokinins. Plant Biol. J. 27: 265–278. https://doi.org/10.1111/plb.13743.

Pierron, A., Alassane-Kpembi, I., and Oswald, I. P. 2016. Impact of two mycotoxins deoxynivalenol and fumonisin on pig intestinal health. Porcine Health Management 2:1–8. https://doi.org/10.1186/s40813-016-0041-2.

Quazi, S. A. J., Meon, S., Jaafar, H., and Ahmad, Z. A. B. M. 2015. The role of phytohormones in relation to bakanae disease development and symptoms expression. Physiological and Molecular Plant Pathology 90:27–38. https://doi.org/10.1016/j.pmpp.2015.02.001.

Rispail, N., and Di Pietro, A. 2010. The two-component histidine kinase FoSln1 mediates osmosensing and is required for pathogenicity in *Fusarium oxysporum*. Mol. Plant Pathol. 11:395–407. https://doi.org/10.1111/j.1364-3703.2010.00612.x

Schouten, A., Van Den Berg, G., Edel-Hermann, V., Steinberg, C., Gautheron, N., Alabouvette, C., De Vos, C. H. (Ric), Lemanceau, P., and Raaijmakers, J. M. 2004. Defense Responses of *Fusarium oxysporum* to 2,4-Diacetylphloroglucinol, a Broad-Spectrum Antibiotic Produced by Pseudomonas fluorescens. MPMI 17:1201–1211. https://doi.org/10.1094/MPMI.2004.17.11.1201.

Senthilkumar, M., and Krishnamoorthy, R. 2017. Isolation and Characterization of Tomato Leaf Phyllosphere Methylobacterium and Their Effect on Plant Growth. Int.J.Curr.Microbiol.App.Sci 6:2121–2136. https://doi.org/10.20546/ijcmas.2017.611.250.

Sharaf, E. F., and Farrag, A. A. 2004. Induced Resistance in Tomato Plants by IAA against *Fusarium oxysporum lycopersici*. Polish Journal of Microbiology 53:111–116.

Shephard, G. S. 2008. Impact of mycotoxins on human health in developing countries. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 25:146–151. https://doi.org/10.1080/02652030701567442.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., Dreyfus, B. 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacteriol. 183(1):214-20. https://doi.org/10.1128/JB.183.1.214-220.2001.

Sørensen, J. L., Benfield, A. H., Wollenberg, R. D., Westphal, K., Wimmer, R., Nielsen, M. R., Nielsen, K. F., Carere, J., Covarelli, L., Beccari, G., Powell, J., Yamashino, T., Kogler, H., Sondergaard, T. E., and Gardiner, D. M. 2018. The cereal pathogen *Fusarium pseudograminearum* produces a new class of active cytokinins during infection. Molecular Plant Pathology 19:1140–1154. https://doi.org/10.1111/mpp.12593.

Trdá, L., Barešová, M., Šašek, V., Nováková, M., Zahajská, L., Dobrev, P. I., Motyka, V., and Burketová, L. 2017. Cytokinin Metabolism of Pathogenic Fungus *Leptosphaeria maculans* Involves Isopentenyltransferase, Adenosine Kinase and Cytokinin Oxidase/Dehydrogenase. Front. Microbiol. 8:1374. https://doi.org/10.3389/fmicb.2017.01374.

Udomkun, P., Wiredu, A. N., Nagle, M., Bandyopadhyay, R., Müller, J., and Vanlauwe, B. 2017. Mycotoxins in Sub-Saharan Africa: Present situation, socioeconomic impact, awareness, and outlook. Food Control 72:110–122. https://doi.org/10.1016/j.foodcont.2016.07.039.

Uemura, E. V. G., Barbosa, M. D. S., Simionatto, S., Al-Harrasi, A., Al-Hatmi, A. M. S., and Rossato, L. 2022. Onychomycosis Caused by *Fusarium* Species. JoF 8:360. https://doi.org/10.3390/jof8040360.

Van Staden, J., and Nicholson, R. I. D. 1989. Cytokinins and mango flower malformation II. The cytokinin complement produced by *Fusarium moniliforme* and the ability of the fungus to incorporate [8-14C] adenine into cytokinins. Physiological and Molecular Plant Pathology 35:423–431. https://doi.org/10.1016/0885-5765(89)90061-1.

Ventorino, V., Sannino, F., Piccolo, A., Cafaro, V., Carotenuto, R., and Pepe, O. 2014. *Methylobacterium populi* VP2: Plant Growth-Promoting Bacterium Isolated from a Highly Polluted Environment for Polycyclic Aromatic Hydrocarbon (PAH) Biodegradation. The Scientific World Journal 2014:1–11. https://doi.org/10.1155/2014/931793.

Walsh, T. J., and Groll, A. H. 1999. Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. Transplant Infectious Dis 1:247–261. https://doi.org/10.1034/j.1399-3062.1999.010404.x.

Wheeler, M. S., Mcginnis, M. R., Schell, W. A., and Walker, D. H. 1981. *Fusarium* Infection in Burned Patients. American Journal of Clinical Pathology 75:304–311. https://doi.org/10.1093/ajcp/75.3.304.

Wielogorska, E., Mooney, M., Eskola, M., Ezekiel, C. N., Stranska, M., Krska, R., and Elliott, C. 2019. Occurrence and Human-Health Impacts of Mycotoxins in Somalia. Journal of Agricultural and Food Chemistry 67:2052–2060. https://doi.org/10.1021/acs.jafc.8b05141.

Wu, F., Groopman, J. D., and Pestka, J. J. 2014. Public health impacts of foodborne mycotoxins. Annual Review of Food Science and Technology 5:351–372. https://doi.org/10.1146/annurev-food-030713-092431.

Xu, J.-R. 2000. MAP kinases in fungal pathogens. Fungal Genetics and Biology, 31(3), 137–152. https://doi.org/10.1006/fgbi.2000.1237

Yoshida, S., Hiradate, S., Koitabashi, M., Kamo, T., and Tsushima, S. 2017. Phyllosphere *Methylobacterium* bacteria contain UVA-absorbing compounds.

Journal of Photochemistry and Photobiology 167:168–175. https://doi.org/10.1016/j.jphotobiol.2016.12.019.

Zain, M. E. 2011. Impact of mycotoxins on humans and animals. Journal of Saudi Chemical Society 15:129–144. https://doi.org/10.1016/j.jscs.2010.06.006.

CHAPTER 6

6.1. PREFACE

Title: Signal Interference: plant associated *Methylobacterium* spp. alter hormone virulence-factors of phytopathogenic *Fusarium.*

Authors: Daniel Palberg and R. J. Neil Emery

Reference: This chapter is currently under preparation for submission. The published version will appear different than presented here.

Contributions: Conceptualization, investigation, methodology, formal analysis, figure and table creation, and writing of the initial draft by D.P. Review of methodology, project supervision, and acquisition of funding by R.J.N.E.

CHAPTER 6

Signal Interference: plant associated *Methylobacterium* spp. alter hormone virulence-factors of phytopathogenic *Fusarium*

6.2. ABSTRACT

In this study, we investigate the hormone-mediated dynamics underlying antagonism between *Methylobacterium* spp. and phytopathogenic *Fusarium* species. We show that *Methylobacterium* strains with strong biological control (BC) potential produce a broader and more bioactive cytokinin profile in axenic cultures – including *trans*-Zeatin – compared to ineffective strains, while also producing greater levels of indole-3-acetic acid (IAA), salicylic acid (SA). In co-culture with *Fusarium*, the presence of BC candidate strains was associated with higher concentrations of jasmonic acid (JA) relative to co-cultures with non-BC strains. Differential hormonomics suggests that *Methylobacterium* may suppress fungal development by disrupting endogenous hormone signalling and triggering compensatory defense-associated pathways typically used to subvert plant immune responses. These findings highlight hormone interference as a key mechanism of microbial antagonism and support further investigation into secondary metabolites and other antifungal determinants involved in *Methylobacterium*-mediated biocontrol.

KEYWORDS: Phytohormones, pathogen suppression, *Fusarium* spp., *Methylobacterium* spp., interkingdom signalling, chemical ecology, hormone flux

6.3. INTRODUCTION

Widely distributed, *Fusarium* species are prominent agents of plant disease and, as such, the genus ranks among the most extensively studied groups of fungi in the world. Despite a century of study, pathogenic strains continue to impart significant crop losses – with estimates reaching $5 billion USD annually (Johns et al. 2022; Wilson et al. 2018). Their success in this regard is owed largely to their ecological versatility - alternating between necrotrophic and saprotrophic lifestyles, as needed - and the abundance of their asexual spores, which enhance dispersal and longevity (Leslie and Xu, 2010).

Collectively, the genus *Fusarium* features species with a staggering host-range capable of affecting every agricultural group from cereals to ornamentals. Prominent examples include Fusarium head blight (FHB) of wheat and barley caused by *F. graminearum*, and bakanae disease ('Foolish Seedling Blight') of rice by *F. fujikuroi* (Crippin et al. 2019; Van Der Lee et al. 2015; Zhan et al. 2024). Outbreaks of FHB, including the 2012 epidemic in China, not only reduce yields but also contaminate harvested grains with mycotoxins rendering them unsafe for consumption and trade (Xu et al. 2015). Similarly, bakanae disease routinely leads to shortfalls in projected rice production in India, Japan, and Korea that range between 3-95% depending on the specific region and year (Bashyal et al. 2016; Gupta et al. 2015). But perhaps most notorious of crop diseases related to *Fusarium* is Panama disease. Caused by *F. oxysporum* f. sp. *cubense*, the vascular wilt so devastated banana cultivation during the 20th century, that the Gros Michel cultivar was replaced with the Cavendish, seemingly overnight (Ploetz 2015). The economic burden of disease caused by *Fusarium* is substantial and disproportionally affects smallholders and those in developing nations

who lack access to infrastructure, equipment, and products essential to disease management (Udomkun et al. 2017; Vitale et al. 2007; Windels, 2000).

Phytopathogenic fusaria make formidable opponents not only for their diverse infection strategies and survivability in soil or plant debris, but also their capacity to influence and interfere with host phytohormone levels and defense mechanisms. For example, some species of *Fusarium* stimulate host production of abscisic acid (ABA), a hormone classically associated with abiotic stress responses, including regulation of stomatal closure, seed dormancy, and drought tolerance (Hu and Bidochka, 2021). Specifically, *F. solani*, has been shown to stimulate elevated ABA accumulation in host tissues during the infection of bean plants (*Phaseolus vulgaris*), triggering stomatal closure and upregulation of ABA-responsive genes. Notably, exogenous application of ABA further enhanced *Fusarium* colonization, suggesting that the pathogen may co-opt ABA signalling to facilitate infection (Hu and Bidochka, 2021). Similarly, in flax (*Linum usitatissimum*) infected with *F. oxysporum*, transcriptomic evidence indicated redirection of the terpenoid pathway toward ABA biosynthesis, correlating with cell-wall fortification and defense gene activation during early infection stages (Boba et al. 2020). However, while ABA may transiently contribute to defense priming, it often suppresses core immune pathways – such as interfering in signal cascades which activate salicylic acid (SA) mediated defenses – and is generally regarded as a negative regulator of pathogen resistance in plants (Buhrow et al. 2016; Cauchon et al. 2017; Qi et al. 2016; Ton et al. 2009).

Certain *Fusarium* isolates employ a similar tactic with indole-3-acetic acid (IAA), a primary auxin involved in plant cell elongation, division, and differentiation.

During invasion, phytopathogen disruption of host IAA pathways result in an overproduction endogenously, leading to organ hypertrophy and increased vascular tissue proliferation (Kidd et al. 2011; Sharaf and Farrag 2004; Tsavkelova et al. 2012). Ultimately, these alterations enable deeper colonization by disrupting plant cell defenses and increasing nutrient availability at the site of infection. Hormonal manipulation is a sophisticated facet in the infection process employed by the fusaria which enables them to evade host detection, establish infection more effectively, and redirect host nutrient resources for their own benefit.

Perhaps the most well-documented case of phytohormone production, *de novo,* by *Fusarium* though, is the saturation of rice seedlings with gibberellins (GAs), mainly GA_3 and GA_4 from *F. fujikuroi* which stimulates uncontrolled seedling growth, rapid depletion of nutrients, and the diminished crop stand characteristic of Bakanae disease (Avalos et al. 2007; Cen et al. 2020; Hori, 1898; Michniewicz, 1989; Spadaro 2017; Troncoso et al. 2009 Wiemann et al. 2013).

Despite decades of intense research, controlling diseases caused by *Fusarium* species remains a major challenge, worsened largely by the proliferation of monocropping and the use of susceptible high-yield hybrids (Ekwomadu and Mwanza, 2023). The effects of disease outbreaks caused by *Fusarium* are experienced worse particularly in agrarian societies where starch-dense crops are not only dietary staples domestically, but important export commodities as well (Beukes et al. 2017; Pradhan et al. 2025). Current strategies for the management of *Fusarium* are generally limited to quarantine, destruction of infected plants, use of synthetic fungicides, and development of resistant cultivars (Rampersad 2020). Naturally, each strategy is accompanied with

its own range of drawbacks including, but not limited to, cost and logistical complexity. For example, while broadly considered the first line of defense, fungicides offer variable efficacy based on a constellation of factors including: local weather, host compatibility, stage of the disease cycle, developmental maturity of the host, and pathogen susceptibility (Rampersad 2020).

The growing incidence of resistance to commonly used fungicidal compounds is also a major factor contributing to a downturn in product development. As resistance evolves, the commercial lifespan of new formulations diminishes significantly, often undermining their profit potential. This challenge is well illustrated by the case of phenamacril, a fungicide designed to target *Fusarium graminearum* by disrupting the function of myosin-5; a molecular motor protein responsible for the transportation of cargo along actin filaments, considered essential for moving resources needed for growth and reproduction. Despite early success however, *Fusarium* populations quickly developed resistance, as specific mutations in the *FaMyo*5 gene allowed evasion of phenamacril (Zheng et al. 2015; Zhou et al. 2020). Similar trends have started to emerge in other fungicide families including the QoI (quinone outside inhibitor) class, particularly in Brazil where field populations of *F. graminearum* have already shown reduced sensitivity to multiple individuals belonging to the QoI family (Machado et al. 2017).

Simultaneously, regulatory agencies worldwide have tightened requirements for product registration, including extensive data on environmental persistence and toxicity to humans and wildlife. These regulations often require dozens of studies per active ingredient, substantially increasing the time and cost needed to bring a new product to

market. In the U.S., for example, the estimated cost to develop and register a new fungicide now approaches $300 million, with development timelines often extending beyond a decade in some cases (Beckerman et al. 2023). In addition to the increased cost of development and unpredictability of approval, public scrutiny of synthetic chemicals has shifted consumer expectations, and further deterred corporate investment in discovery pipelines. These factors coalesce in a development bottleneck: as resistance expands, the incentive to invest in new chemistries declines, and a stagnation in innovation ensues (Beckerman et al. 2023).

Lower-cost strategies at present largely consist of reformulating existing products, though investment in biological control (BC) as an alternative, is increasing. BC makes use of non-phytopathogenic bacteria, fungi, and actinomycetes, to suppress the proliferation of fungal pathogens by applying competitive pressure for nutrients and space, the production of antifungal metabolites, fungal parasitism, and induction of plant immune responses. Strains of *Bacillus*, *Pseudomonas*, *Trichoderma*, and *Streptomyces* have already been widely studied for their ability to supress the growth of pathogenic *Fusarium* through metabolites (e.g., iturins, phenazines) and lytic enzymes (e.g., chitinases, glucanases) (Köhl et al. 2019; Solórzano et al. 2025).

Unlike fungicides alone, BC offers greater long-term durability by suppressing fungal growth through multiple, often adaptive mechanisms, in alignment with the core principles of integrated pest management (IPM). While consistent field efficacy remains a challenge – owing to environmental variability, inconsistent host colonization, and interactions with native microbiota – ongoing advances in microbial ecology are refining strain selection and application methods. Importantly, emerging evidence

continues to highlight the role that phytohormones may play in the infective strategies of certain *Fusarium* species, particularly *F. graminearum* and *F. fujikuroi*, where hormone dynamics are better characterized. Exploring hormone signalling at the pathogen-antagonist interface, then, may offer valuable insights into microbial modes of action and aid in the identification of novel targets for signal interference or pathway disruption. While not necessarily central to infection in all cases, hormone cross-talk is likely one of several pillars influencing disease outcomes – warranting further investigation, particularly as our understanding of the interkingdom potential of phytohormones continues to expand (Akhtar et al. 2020; Anand et al. 2022; Aoki et al. 2020; Berger et al. 2020).

An emerging candidate for BC, the genus *Methylobacterium* first garnered attention largely for their diverse plant growth-promoting traits. The *Methylobacterium* are gram-negative, rod-shaped, facultative methylotrophs, widely distributed in terrestrial and aquatic ecosystems and are particularly abundant in the phyllosphere of plants (Green 2006). Several species exhibit exceptional tolerance to abiotic stress, including desiccation, freezing, and UV radiation – traits which collectively, help facilitate their colonization of aerial plant surfaces (Dourado et al. 2015; Holland 1997a; Kamo et al. 2018). Their competitive advantage as symbionts is further supported by their ability to metabolize methanol – emitted through the stoma during cell wall remodeling – as a primary carbon source, and the ability of some isolates to produce uniquely high levels of phytohormones (Ivanova et al. 2000, 2001; Palberg et al. 2022). These traits, at least in part, help explain the consistent presence and dominance of *Methylobacterium* in the phyllosphere of a wide range of crops under both natural and managed conditions.

Beyond enhancing productivity, *Methylobacterium* strains also deter fungal phytopathogens, likely some mix of enzymatic activity towards fungal tissues, nutrient competition, or by triggering induced systemic resistance (ISR) in the host plant (Poorniammal et al. 2009). However, to-date an understanding of any interplay between phytohormone producing *Methylobacterium* and pathogenic *Fusarium* remains wholly unexplored, despite the established role of phytohormones in the infection process of *Fusarium* spp. – most notably in *F. fujikuroi* infections of rice (bakanae disease), where gibberellin production is a key determinant of virulence (Yabuta, 1935).

Given their frequent association with plants and a well-documented capacity to produce phytohormones, *Methylobacterium* isolates are likely to influence interkingdom signalling networks within the phytobiome. While not all *Fusarium* species may possess the full complement of machinery required to detect or respond to hormone cues, some are known to engage with or otherwise manipulate host-derived hormonal signals during infection (Vrábka et al. 2019). As such, the potential for *Methylobacterium* to modulate phytohormone levels warrants closer investigation, particularly in the context of their interactions with hormone-sensitive pathogens like *Fusarium*, as well as with the host plant.

This study presents the first comprehensive and systematic investigation into the phytohormone crosstalk between a phylogenetically diverse collection of *Methylobacterium* isolates and three phytopathogenic species of *Fusarium*. The bacterial strains included in this work were specifically selected based on prior BC performance (Chapter 5), and consist of both highly suppressive and minimally suppressive isolates as determined through co-culture assays with their corresponding

Fusarium species. Conducted under both rich and nutrient-minimum conditions, this study uniquely integrates the analysis of 32 phytohormones in spent broth and cellular tissues, while also examining the potential of *Methylobacterium* strains to interfere with or distort chemical signals of fungal origin. The breadth and rigor of this approach mark a significant advancement in the understanding of microbial phytohormone dynamics and their broader role in shaping plant–symbiont–pathogen interactions.

6.4. MATERIALS AND METHODS

Materials and Media Preparation

Growth media for isolations and preservation of stocks included tryptic soy broth (TSB) and potato dextrose broth (PDB) (Fisher Scientific). In all cases, solid medium was prepared using agar (Fisher Bioreagents, BP1423-500) at a rate of 1.6% w/v, and uniform volume of 20 mL in single-use petri dishes (FisherBrand™, 100 x 15 mm).

Selective minimum nutrient media for the propagation of *Methylobacterium* isolates was prepared in accordance with the DSMZ recipe (DSMZ Index #125): KNO_3 1.00 g; $MgSO_4 \cdot 7H_2O$ - 0.2 g; $CaCl_2 \cdot 2H_2O$ - 0.02 g; Na_2HPO_4 0.23 g; NaH_2PO_4 - 0.07 g; $FeSO_4 \cdot 7H_2O$ - 1.00 mg; $CuSO_4 \cdot 5H_2O$ – 5.00 µg; H_3BO_3 – 10.00 µg; $MnSO_4 \cdot 5H_2O$ – 10.00 µg; ZnSO4 • 7H2O – 70.00 µg; MoO_3 – 10.00 µg; H_2O - 1,000 mL; CH_3OH - 5 mL; pH 6.80-6.88). Use of DSMZ-125 was restricted to the maintenance of *Methylobacterium* stocks, as the presence of methanol in the final preparation proved lethal to fungal isolates (data not shown).

For co-culture experiments evaluating phytohormone dynamics under nutrient-minimum conditions, a variation of Hoagland's complete nutrient hydroponic solution was used, with modifications to support fungal and bacterial growth. Use of this medium – used successfully in Chapter 5 – was essential to eliminating bias of growth effectors or background metabolites present in nutrient-rich medium like TSB or Murashige and Skoog (MS) and mimicking the limited nutrient environment of the soil and plant surface.

The base solution, Hoagland's complete nutrient broth (HCNB) was prepared by adding macronutrients at the following rates: 6 mL of 1M $NH_4H_2PO_4$, 4 mL of 1M KNO_3, 2 mL of 1M $Ca(NO_3)_2$, and 1 mL of 1M $MgSO_4$, per liter of deionized water. Subsequently, 1 mL of micronutrient stock (2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2 \cdot 4H_2O$, 0.22 g/L $ZnSO_4 \cdot 7H_2O$, 0.08 g/L $CuSO_4 \cdot 5H_2O$, 0.02 g/L $NaMoO_4 \cdot H_2O$) and 1 mL of iron supplement (10.4 g/L EDTA, 7.8 g/L FeSO4 • $7H_2O$, 56.0 g/L KOH) were added. A modified version of the broth (MHCNB) was made by supplementing D-glucose (BioShop) at a rate of 10 g/L as a carbon source. In all cases, the broth was heated to 45 ^oC, and the pH was adjusted to 6.90 ± 0.1 using sodium hydroxide (1M NaOH). 100 mL of broth was transferred into 250 mL Erlenmeyer flasks and autoclaved at 121 ^oC for 20 minutes.

Isolate Selection and Propagation

Preparation and manipulation of all bacterial and fungal cultures were conducted aseptically. Freeze-dried cultures of *Methylobacterium* spp. were obtained from four microbe collections: the Belgian Coordinated Collections of Microorganisms (BCCM/LMG) and the National Institute of Technology and Evaluation's (NITE) Biological Resource Center (NBRC). Strains were originally collected from different biological (living plants) and non-biological sources. Information on strain taxonomy, origin, and known characteristics is provided in Table 6.1. Freeze-dried bacterial strains were revived in 50 mL of nutrient rich R2A broth (VWR, Mississauga, Canada) at 27 ^oC in a rotary incubator (120 RPM). After 5 days, a sterile loop was used to inoculate plates containing DSMZ 125 selective medium, from which single colonies would be selected to inoculate flasks containing 50 mL of DSMZ 125 broth. This series of passages was necessary to ensure potential contaminants were removed and an aliquot

of the final broth was used to develop cryogenic stocks (~ 10^8 CFU/mL) in 15% (v/v) glycerol, subsequently maintained at -80 °C.

Revival of *Methylobacterium* strains was achieved by streaking cryogenic stocks on nutrient-rich tryptic soy agar (TSA). After 5 days of incubation at 26 ºC, single colonies were extracted from each plate and used to inoculate 50 mL of tryptic soy broth (TSB) liquid growth media in 250 mL glass Erlenmeyer flasks and maintained in a rotary incubator for 7 days (27 ºC and 110-120 RPM). When *Methylobacterium* cultures reached the late exponential/early stationary phase after approximately 7 days (OD_{600} = 0.6 – 1.2, depending on strain) selective nutrient minimum agar plates (DSMZ 125) were inoculated with 100 µL of diluted (10^{-5}) aliquots.

Transfer to a minimum nutrient media ensured high selectivity for *Methylobacterium* and minimal contamination risk and after 5-7 days of incubation at 27 ºC (inverted, darkness), single colonies were used for subsequent experiments. DSMZ 125 agar plates were maintained at 4 ºC for and used as stock plates and retained for a maximum of 21 days.

Live cultures of *Fusarium* spp. were obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH) (Gage Research Institute, University of Toronto, Canada). Information on fungal isolate origin and known characteristics are provided in Table 2. Fungal isolates were maintained in potato dextrose agar (PDA) after transfer from transport container, at 27 ºC in darkness. After 10 days, a spore suspension was developed by plate flood and subsequently used to produce stocks preserved at -80 ºC in 20% glycerol (v/v) (~ 10^6 conidia/mL). Revival of isolates were achieved by transferring 50 µL of cryogenic spore suspensions to plates containing

TSA, as needed. Fungal stock plates were maintained in darkness at 26 ºC for a maximum of 10 days before being discarded.

Culture Conditions and Harvest

Methylobacterium impact on the growth and hormone dynamics of *Fusarium* was evaluated in TSB and modified MHCNB. Specific *Methylobacterium* isolates were selected for inclusion in this experiment based on performance in biocontrol experiments carried out previously (Chapter 5), where the three bacterial strains with the greatest fungal growth suppression were co-cultured with their respective species of *Fusarium*. This was followed by a replication of the experiment, involving bacterial strains that produced little or no biocontrol against their respective fungal species.

The experimental framework included 5 conditions per fungal strain: (i) *Fusarium* monoculture, (ii) *Fusarium* challenged by an effective BC candidate, (ii) *Fusarium* challenged by an ineffective bacterial isolate, and monocultures of both the (iv) BC candidate, and the (v) ineffective isolate. Each framework was reproduced in TSB and MHCNB, with four biological replicates in each, and carried out identically for each of the three selected fungal pathogens. Replicates of uninoculated controls were included in each framework and both broth types and handled identically to the test cohort.

Running stocks of *Methylobacterium* were created by inoculating Erlenmeyer flasks containing 100 mL of either TSB or MHCNB with single colonies selected from refrigerated stock plates by loop transfer. After the cell density of each culture had reached approximately 10^7 CFU/mL (3-5 and 10-12 days for TSB and MHCNB,

respectively), 20 mL aliquots were transferred to sterile conical flasks and centrifuged at 4,600 RCF for 20 minutes. The supernatant was subsequently discarded, and the pellet in each flask was resuspended in phosphate buffered saline (pH 6.9) and centrifuged again for 20 minutes at 4,600 RCF. After discarding the supernatant, the pellet in each tube was again resuspended in 10 mL of either TSB or MHCNB. An aliquot of 1 mL was removed from each tube and further diluted as needed to verify cell density by haemocytometer, and the double count method at 1,000 x magnification. Based on the results of total cell density in the washed bacterial suspensions, additional diluent of either TSB or MHCNB was added to each tube to achieve a final concentration of 10^6 CFU/mL. This procedure was necessary to cleanse the running stocks of excess culture media, but also to achieve a uniform inoculum density in preparation for transfer to test flasks.

Of the five flasks included in each iteration of the experiment, four were initially inoculated with *Methylobacterium*: a bacterial monoculture for the BC candidate and ineffective strain, and a duplicate set which would be inoculated with Fusarium afterwards. Each experimental flask, containing 50 mL of either TSB or MHCNB was inoculated with 1 mL of washed running stock (10^6 CFU/mL) from a single bacterial strain. After each of the experimental flasks had been inoculated, a 100 µL aliquot was removed from each washed running stock, serially diluted (10^{-4}), transferred to TSA plates, and spread by sterile loop. Plates were incubated and examined after 3-4 days to ensure accuracy in the intended inoculum density and cell viability after all washing steps. Meanwhile, experimental flasks were incubated in darkness at 27 ^oC and agitated at 120 RPM on an orbital shaker for 48 hours. Experimental flasks (fungal monoculture, and both co-culture challenges) were inoculated with their respective *Fusarium* strains

using a 2 mm agar plug taken from the mycelial leading edge of a stock plate. Flasks were incubated for an additional 10 days in darkness at 27 ºC and 120 RPM.

Following the final incubation period, flasks were decanted into sterile conical flasks, then centrifuged at 2 ºC for 30 minutes at 4,200 RCF (Beckman Coulter Allegra X-12R). The supernatant was extracted, immediately filter sterilized (PES, 0.22 µm, 25 mm) and frozen overnight at -20 ºC in 10 mL fractions. Pelleted fungal and bacterial tissues were resuspended in cold phosphate buffered saline (PBS) and centrifuged again for 20 minutes at 2 ºC and 4,200 RCF. This process was repeated for a total of three washes, with the resuspended biomass transferred to a new, pre-weighed conical tube prior to the final centrifugation step. Supernatant from all washing steps were discarded and the solid biomass was frozen overnight at -20 ºC. Spent broth and biomass samples were further frozen at -80 ºC for 24 hours before being lyophilized over 72 hours (Labcono Model: 7753020 at -56ºC and 0.28 mBar). Tubes containing culture biomass were subsequently reweighed to determine the total pellet dry weight.

This process was replicated in its entirety, four times per media type, and repeated for each species of *Fusarium* for a total of 24 rounds or 144 individual flasks.

Solid Phase Extraction (SPE) and Phytohormone Isolation

Phytohormones were extracted sequentially using a modified protocol adapted from previously published (Kisiala et al. 2019) multi-extraction methods for 39 cytokinins (CKs) and acidic hormones including abscisic acid (ABA), gibberellins (GA_1, GA_4, GA_7, GA_9, GA_{20}), indole-3-acetic acid (IAA), jasmonic acid (JA), and salicylic acid (SA) (Table S6.1.). Lyophilized 10 mL fractions of spent broth were

reconstituted in 2 mL of ice-cold 50% ACN, while homogenization of biomass into a powder using a clean and baked (350 ºC for 6 hours) mortar and pestle was first necessary to blend fungal and bacterial tissues obtained from co-cultures. Once homogeneous, 300 mg of biomass powder was transferred to 2 mL microfuge tubes (Eppendorf), resuspended in 2 mL of ice-cold 50% ACN and further disrupted mechanically by two zirconium oxide (ZirO) beads at 4 °C and 25 Hz for 10 minutes (Retsch 300 ball mill, Haan, Germany).

To both the broth and biomass samples, labelled internal standards (IS) were added during reconstitution to achieve the following final content per sample: 60.1 ng of ABA-d_5 (PBI, Saskatoon, Canada), 10 ng each of IAA-d_6 and SA-d_5 (OLChelmm, Olomouc, Czech Republic), and 20 ng of each GA. Additionally, 10 ng of labelled CK standards were included, covering isoprenoid, aromatic, methylthiolated, and glucoside forms (see Table S6.1. for complete inventory). In each case, internal standards were added at a volume rate of 10 µL per sample, and all standards were resuspended and maintained in HPLC-grade methanol as the primary diluent.

Both reconstituted broth and biomass samples were vortexed vigorously at 4 ºC and stored at −20 °C overnight for passive extraction. After thawing, samples were centrifuged at 4,600 RCF for 10 min (ThermoScientific Sorvall ST 16), and supernatants were collected. Method blanks consisting of empty 2 mL microfuge tubes containing clean ZirO beads, were prepared in triplicate. Media blanks of TSB and MHCNB were prepared contemporaneously with each cycle of the experiment (n = 4).

HLB SPE Processing

Supernatants and blanks were centrifuged again at 4,600 RCF for 5 min and loaded onto hydrophilic-lipophilic balance (HLB) SPE cartridges (VIOLET™ 200 mg/6 mL, 40 µm; Canadian Life Sciences, Peterborough, ON, Canada) preconditioned with methanol and ultrapure water (18.2 MΩ-cm). After equilibration with 50% ACN, samples were loaded, followed by 2 mL of 30% ACN. The total elution volume for each sample was divided into equal 1 mL aliquots for (a) CK analysis and (b) derivatization of acidic phytohormones in 5 mL collection tubes. All tubes were then dried overnight in a vacuum centrifuge (Thermo Savant UVS 400a).

Derivatization of Acidic Phytohormones

Acidic hormones were derivatized to enhance chromatographic separation, improve ionization efficiency, and increase detection sensitivity during HPLC-HRMS/MS analysis, thereby enabling more accurate quantification. The dried acidic phytohormone fraction was reconstituted with 75 µL 1-propanol, 20 µL high purity water H_2O, 5 µL of 500 mM bromocholine (in 70% ACN), and 1 µL triethylamine (Fisher Scientific). After vortexing, collection tubes were incubated at 80 °C for 130 minutes, cooled on ice for 60 minutes, then subsequently dried again in a vacuum concentrator overnight (Kisiala et al. 2019).

Sequential Elution of Cytokinin Fractions

Desiccated CK supernatant samples were reconstituted in 1 mL of 1 M formic acid, vortexed, and centrifuged at 4,600 RCF for 5 min. Samples were loaded onto mixed-mode cation exchange (MCX) SPE cartridges (IRIS™ MCX 200 mg/6 mL, Canadian Life Sciences), preconditioned with methanol and 1 M formic acid. CK

nucleotides were eluted first using 0.35 M ammonium hydroxide (NH_4OH), and remaining CK forms (free base, methylthiolated, glucoside, and riboside) were eluted with 0.35 M NH_4OH in 60% methanol. All eluted fractions were collected in 5 mL tubes and dried in a vacuum centrifuge overnight.

To improve the detection of CK nucleotides by mass spectrometry, dephosphorylation was carried out. Dried nucleotide fractions were resuspended in 1.0 mL of 0.1 M ethanolamine and vortexed. Then, 12 µL of phosphatase enzyme (New England BioLabs Ltd.; 5,000 unit/mL) was added, followed by incubation overnight at 37 °C. Samples were again dried under vacuum overnight, and redissolved in 1.5 mL ultrapure water, vortexed, and centrifuged at 4,600 RCF for 10 min.

C18 SPE cartridges (6 cc, 500 mg; Canadian Life Sciences) were preconditioned with methanol and ultrapure water. Samples were loaded by gravity and CKs were eluted with 1.25 mL of 80% MeOH, and dried overnight. All dried fractions from each sample (acidic hormones, CK nucleotides, and remaining CK forms) were resuspended in 300 µL 0.08% AcOH in 5% ACN, vortexed, centrifuged at 4,600 RCF for 10 min, and transferred to 2 mL vials with glass inserts (300 µL) at −20 °C until analysis (Kisiala et al. 2019).

Ultra-High Pressure High-Resolution Tandem Mass Spectrometry (HPLC-HRMS/MS)

Phytohormone quantification was performed using a Thermo Q Exactive Orbitrap mass spectrometer with a HESI-II source coupled to a Dionex Ultimate 3000 HPLC system (Thermo Fisher, San Jose, USA). Chromatographic separation, LC

settings, and instrument parameters were set in accordance with previously validated methods, with minor modification for available resources (Kisiala et al. 2019). CKs were separated using a Kinetex C18 column (2.1 mm × 50 mm, 2.6 μm; Phenomenex, Torrance, CA) at 22 °C, with elution controlled via Chromeleon 6.8 software. The mobile phases were high purity water H_2O + 0.08% acetic acid (A) and ACN + 0.08% acetic acid (B), at a flow rate of 0.5 mL/min. The gradient profile was: 5% B for 0.5 min; linear increase to 45% B by 4.5 min; increase to 95% B by 6.5 min; held for 1 min, then returned to initial conditions for a total run time of 8.2 min. Injection volume was 25 μL.

The analytical run was performed using instrument parameters based on previously optimized settings for small molecule analysis (Kisiała et al. 2019). Briefly, the HESI-II auxiliary gas heater and capillary temperatures were set to 450 °C and 300 °C, respectively. The spray voltage was 3.9 kV, with sheath, auxiliary, and sweep gas flow rates set to 30, 8, and 0 arbitrary units, respectively. The S-lens RF level was maintained at 60, and data acquisition was conducted in positive ion mode using time-scheduled parallel reaction monitoring (PRM) events at 35,000 resolution, with an AGC target of 3×10^6, and IT of 128 ms. The precursor isolation window was maintained at *m/z* 1.2, and the normalized collision energy (NCE) was individually optimized per compound to retain ~10% of the unfragmented precursor (Table S6.1.).

Statistical Analysis and Figure Curation

All statistical analyses were performed using Python (v3.11) with the statsmodels package. For each *Fusarium* species and culture medium (rich and minimum), hormone concentrations were compared across five culture groups, in both

supernatant and bulk biomass separately: (i) *Fusarium* monoculture, (ii) *Fusarium* challenged by an effective BC candidate, (ii) *Fusarium* challenged by an ineffective bacterial isolate, and monocultures of both the (iv) BC candidate, and the (v) ineffective isolate. Each condition was represented by four independent biological replicates.

A one-way analysis of variance (ANOVA) was first conducted for each hormone to test for significant differences in mean concentrations across the five groups. Hormones with ANOVA p-values < 0.05 were subjected to Tukey's Honest Significant Difference (HSD) post-hoc test to identify statistically distinct pairwise comparisons. Statistical significance was inferred when adjusted p-values were < 0.05 and confidence intervals (CIs) excluded zero. Hormone concentration data were log-transformed where necessary to improve normality and homoscedasticity, though results remained consistent in both raw and transformed forms. To account for any baseline signal, hormone levels detected in sterile media controls were subtracted from all culture measurements, such that all reported concentrations reflect only hormone production above background. While background levels in tryptic soy broth (TSB) were low, those in MHCNB were undetectable, enhancing sensitivity and reducing signal interference – an issue occasionally encountered with cultures grown in TSB. This hierarchical approach allowed detection of both global and pairwise differences in hormone profiles attributable to microbial interactions. Hormone concentrations in the broth of axenic and co-cultures were normalized to total biomass (grams dry weight) per flask to account for intra-treatment variability in growth and enabling accurate comparisons of biosynthetic activity independent of yield. All hormone concentrations (pmol/g or nmol/g) in spent broth and biomass are reported as mean and the standard error of the mean (± SEM).

6.5. RESULTS

Phytohormone profiles of Fusarium axenic cultures

Baseline hormone expression was assessed in fungal monocultures of *Fusarium graminearum* (UAMH 3329), *F. oxysporum* (UAMH 9013), and *F. fujikuroi* (UAMH 9877) in both TSB and MHCNB to characterize constitutive hormone production and general responses to a defined nutrient source. Z-score–normalized phytohormone profiles (Figure 6.13) revealed both shared and divergent trends across the three *Fusarium* isolates and media types. While most hormones followed a species- and medium-dependent pattern of hormone secretion in filtered broth, several key contrasts emerged. Specifically, *F. graminearum* (UAMH 3329) exhibited broad elevations in overall hormone concentrations across all classes in TSB relative to MHCNB, suggesting a more hormonally active phenotype in a fastitious medium. *F. oxysporum* f. sp. *cubense* (UAMH 9013) also showed moderate increases in certain CKs in TSB. In contrast however, *F. fujikuroi* (UAMH 9877) displayed generally lower relative production of most hormones, especially auxins and CKs; however, this pattern was countered by a striking and consistent elevation of jasmonic acid (JA) and gibberellins (GA_4 and GA_7), particularly in MHCNB (Figure 6.3., Figure 6.13, and Table S6.7). This suggests a potentially distinct signaling or stress-response phenotype in *F. fujikuroi* that diverges from the broader hormonal suppression observed in its profile.

Hormone concentrations, corrected for background levels using both media controls and method blanks, were found to be higher in the spent broth of TSB cultures than in that of MHCNB, with freebase CK forms showing the most pronounced differences. Specifically, *t*Z concentrations in MHCNB filtered broth decreased by 82.7% in *F. graminearum* (15.86 ± 4.55 to 2.75 ± 0.12 pmol/mL), 76.5% in *F.*

oxysporum cultures (3.41 ± 0.26 to 0.80 ± 0.06 pmol/mL), and 97.8% in *F. fujikuroi* (4.01 ± 0.22 to 0.09 ± 0.03 pmol/mL) relative to identical cultures in TSB, normalized for total biomass (Table S6.2-4). Similarly, *cZ* content decreased in MHCNB broth by 81.6% in *F. graminearum* (760.11 ± 23.92 to 140.19 ± 16.24 pmol/mL), 65.4% in *F. oxysporum* (207.39 ± 14.94 to 71.78 ± 18.01 pmol/mL), and 94.9% in *F. fujikuroi* (0.89 ± 0.09 to 0.05 ± 0.01 pmol/mL) compared to TSB (Table S6.2-4). Interestingly, dihydrozeatin (DZ) became undetectable in the broth of all fungal controls cultured in MHCNB, and isopentenyladenine (iP) decreased by 63.7% in *F. graminearum* (53.76 ± 1.32 to 19.53 ± 1.01 pmol/mL), 96.2% in *F. oxysporum* (42.21 ± 1.80 to 1.60 ± 0.28 pmol/mL), and was absent in MHCNB cultures containing *F. fujikuroi* (Table S6.2-4). When cultured in TSB, harvested biomass of *F. graminearum* exhibited the highest levels of intracellular *c*Z (1.18 ± 0.02 nmol/gDW)(Table S6.11), followed by *F. fujikuroi* (440.85 ± 21.74 pmol/gDW)(Table S6.13), and then *F. oxysporum* which fell below detectable limits (Table S6.12). Similarly, iP was most abundant in *F. graminearum* tissues (226.49 ± 27.21 pmol/gDW), while *F. oxysporum* and *F. fujikuroi* showed much lower levels (14.32 and 40.85 pmol/gDW, respectively). *t*Z was led by *F. fujikuroi* (61.33 ± 33.55 pmol/gDW) – albeit with large variability, followed by *F. graminearum* (27.39 ± 7.01 pmol/gDW) and *F. oxysporum* (11.45 ± 1.14 pmol/gDW) (Table S6.11-13). In MHCNB, cytokinins were consistently reduced in fungal tissues across species: *F. graminearum* and *F. fujikuroi* showed *c*Z at just 14.9 and 26.85 pmol/gDW, respectively, while strict nutrient conditions appear to have stimulated intracellular *c*Z in F. oxysporum (7.22 ± 0.94 pmol/gDW). iP dropped below detectable limits in cultures of *F. graminearum*, while *F. oxysporum* and *F. fujikuroi* retained modest levels (5.57 and 8.81 pmol/gDW, respectively).

When cultured in MHCNB, broth concentrations of ABA decreased for all fungal monocultures relative to identical experiments in TSB (Table S6.5-7): In *F. graminearum* controls, ABA levels dropped from 232.87 pmol/mL in TSB to undetectable levels in MHCNB, while *F. fujikuroi* exhibited a 98.9% decline (666.19 ± 55.54 to 7.41 ± 3.51 pmol/mL). *F. oxysporum* did not produce detectable levels of ABA in either medium. IAA levels in broth also declined in a similar trend: 99.2% reduction in MHCNB cultures containing *F. graminearum* (17.18 ± 1.97 to 0.13 ± 0.01 nmol/mL) (Table S6.5), 88.6% in *F. oxysporum* cultures (4.40 ± 0.24 to 0.50 ± 0.03 nmol/mL) (Table S6.6), and 98.0% in *F. fujikuroi* cultures (15.41 ± 2.34 to 0.31 ± 0.07 pmol/mL) (Table S6.7). In TSB, IAA content in fungal tissues were highest for *F. oxysporum* (27.79 ± 17.82 nmol/gDW) (Table S6.15), followed by *F. graminearum* (23.32 ± 10.17 nmol/gDW) (Table S6.14) and *F. fujikuroi* (6.56 ± 0.13 nmol/gDW) (Table S6.16). In MHCNB, IAA content in biomass declined significantly with less variability between replicates: 2.43 ± 0.04 nmol/gDW (*F. oxysporum*), 1.24 ± 0.02 nmol/gDW (*F. graminearum*), and 2.38 ± 0.08 nmol/gDW (*F. fujikuroi*). ABA levels could not be reliably detected in fungal tissues and are not reported (Tables S6.14-16).

In general, broth samples contained higher quantities of JA in MHCNB relative to TSB (Figure 6.13): *F. graminearum* did not produce detectable quantities of JA in TSB but accumulated 15.93 nmol/mL in MHCNB. Similarly, *F. fujikori* increased JA secretion by over an order of magnitude in MHCNB (1.70 ± 0.26 μmol/mL) relative to TSB (150.98 ± 20.41 pmol/mL) (Table S6.7). However, JA remained undetectable in the broth of all cultures containing *F. oxysporum*. Endogenous SA and JA signals from fungal biomass samples exhibited poor peak resolution and low signal intensity and reliable quantification was not possible.

In spent broth, gibberellin expression was only reliably detectable in cultures of *F. fujikuroi*. Broth levels of GA_4 increased in axenic MHCNB cultures containing *F. fujikuroi* (1.14 ± 0.05 to 2.49 ± 0.08 nmol/mL) relative to TSB, while GA_7 secretion decreased slightly (3.5%). GA_1, GA_9, GA_{20} were undetectable in culture broth and tissues across all species, irrespective of media type. In *F. fujikuroi* tissues cultured in TSB, GA_4 was detected at 9.24 ± 4.3 nmol/mL, while identical axenic cultures in MHCNB, tissue concentrations of GA_4 were significantly elevated 1.01 ± 0.22 μmol/mL – suggesting specific regulation of gibberellin biosynthesis under restricted nutrient sources (Table S6.16). GA_4 and GA_7 were not detected in the tissue of axenic cultures of *F. oxysporum* and *F. graminearum*, irrespective of growth medium.

Interestingly, when cultured in TSB, *F. oxysporum* appeared to accumulate *c*ZR and iPR intracellularly (67.37 and 24.47 pmol/gDW, respectively), which was seemingly impaired when cultured in MHCNB (15.59 and 9.69 pmol/gDW, respectively). Riboside forms showed identical shifts in *F. graminearum* tissues between TSB (*c*ZR: 985.70 pmol/gDW, iPR: 191.96 pmol/gDW) and MHCNB (*c*ZR: 477.46 pmol/gDW, iPR: 5.80 pmol/gDW), while *F. fujikuroi* departed from this trend and accumulated more of these compounds in MHCNB (cZR: 740.76 pmol/gDW, iPR: 1223.43pmol/gDW) than TSB (cZR: 649.10 pmol/gDW, iPR: 46.58 pmol/gDW) – suggesting species-level differences in cytokinin transport, conjugation, and accumulation strategies.

Methylthiolated cytokinin derivatives, MeSZ and MeSiP, were consistently depleted in MHCNB relative to TSB, dropping below detectable limits for all three species. Riboside forms, MeSZR and MeSiPR were undetectable in both TSB and

MHCNB across all species. In fungal biomass, signals from methylthiolated cytokinins exhibited poor peak integrity and low signal intensity, and reliable quantification was not possible.

Differential phytohormone profile of axenic Methylobacterium cultures

An examination of the stand-alone bacterial controls in rich medium revealed notable differences between strains known to be effective inhibitors of fungal growth (LMG 6083, NBRC 103128, and LMG 23582) and those that are ineffective (NBRC 103129, NBRC 107716, and LMG 6379) – as classified based on previous *in vitro* biocontrol experiments (Chapter 5). Among the BC strains, total cytokinin production was consistently higher (Figure 6.14). When cultured in TSB, average broth concentrations of *c*Z were greater for BC strains (13.5 vs. 2.4 pmol/mL), and the average isopentenyladenine (iP) content in broth was almost sixteen times higher (139.17 vs. 8.83 pmol/mL). Interestingly, dihydrozeatin (DZ) was detected exclusively in the broth of BC bacteria, while being entirely absent in the group of non-BC isolates.

In contrast, average IAA levels were consistently ($p < 0.05$) lower in BC strains (50.51 pmol/mL) compared to non-BC individuals (64.18 pmol/mL), suggesting that non-BC bacteria may inadvertently support fungal growth through higher auxin production (Figures 6.1-3 and Tables S6.5-7). Methylthiolated CK forms however, showed more mixed results. MeSZ was substantially higher in BC strains (520.15 vs. 158.73 pmol/mL), while methyl-isopentenyladenine MeSiP and its riboside form (MeSiPR) were generally lower in BS isolates relative to their non-BC counterparts (Figures 6.7-9 and Tables S6.8-10).

Notably, classical plant stress- and defense-associated hormones – including ABA, SA, and JA – and the gibberellins GA_1, GA_4, GA_7, GA_9, and GA_{20} were undetectable in axenic cultures of all strains, irrespective of the growth medium, and consistent with the findings of previous work (Palberg et al. 2022).

Hormone shifts in F. graminearum-Methylobacterium co-cultures

In nutrient-rich TSB, co-culturing *F. graminearum* with the BC candidate *M. organophilum* (LMG 6083) resulted in a reduction to overall (mixed) biomass (8.2%), relative to axenic fungal cultures. This decline was accompanied by significant hormonal changes in the spent broth of co-cultures relative to fungal monoculture, normalized against total biomass (Figure 6.10): (*t*Z decreased by 8.81 pmol/mL (55.5%), *c*Z by 318.72 pmol/mL (41.9%), and iP by 35.99 pmol/mL (66.9%) (Figure 6.1.). ABA also decreased notably by 92.71 pmol/mL (39.8%), relative to *F. graminearum* monoculture (Figure 6.4.). In contrast, both DZ and IAA increased in co-culture with *M. organophilum* by 51.0% and 11.7%, respectively, while the stress-related hormone SA, spiked from 3.93 ± 0.55 nmol/mL in monoculture to 34.96 ± 0.57 nmol/mL in co-culture (Figure 6.1 and Figure 6.4.). Some of the methylthiolated CKs also experienced substantial increases: 2-methylthiol zeatin (MeSZ) rose from 0.79 ± 0.26 to 4.04 ± 0.21 pmol/mL, and 2-methylthio-N^6-isopentenyladenine (MeSiP) from 0.14 ± 0.02 to 2.36 ± 0.82 pmol/mL (Figure 6.7.). In TSB, GA_1, GA_4, GA_7, GA_9, GA_{20}, JA, 2-methylthio-*trans/cis*-Zeatin riboside (MeSZR), and methylthio-isopentenyladenine-riboside (MeSiPR) were not detected (Figure 6.7).

Shifts in hormone levels intracellularly were also detected: relative to axenic cultures of *F. graminearum*, mixed biomass of co-cultures containing the BC candidate

M. organophilum had less *t*Z (1.18 to 0.71 nmol/gDW) *cZ* (1.18 to 0.71 nmol/gDW), and iP (226.5 to 47.50 pmol/gDW) (Table S6.14). Salicylic acid (SA) was also substantially increased in the co-culture biomass relative to fungal monoculture from 8.21 to 25.80 μmol/gDW and ABA, initially 231.1 pmol/gDW present in biomass of fungal monoculture, became undetectable in the co-culture biomass (Table S6.11). Average concentrations of *cis*-Zeatin riboside (*c*ZR) in fungal control biomass dropped precipitously in the recovered biomass of the co-culture with the BC candidate *M. organophilum* (985.70 ± 96.34 to 272.17 ± 73.52 pmol/gDW) along with iP (191.96 ± 64.34 to 65.27 ± 21.11 pmol/gDW) (Table S6.14).

In the nutrient-limited medium, MHCNB, co-culturing with the BC candidate *M. organophilum* led to a small increase to total dry weight biomass of 20.7 mg (16.8%), but more substantial hormonal shifts in the broth: the average concentration of *t*Z increased by 413% (2.75 ± 0.12 to 14.12 ± 0.67 pmol/mL), and *c*Z increased by a similar 496% though to a much greater absolute concentration (140.19 ± 16.24 to 836.47 ± 94.15 pmol/mL) (Figure 6.1.). IAA increased from 136.83 ± 13.03 to 368.85 ± 19.57 pmol/mL (169.6%), while SA appeared at 360.47 nmol/mL and JA more than tripled (15.93 ± 6.38 to 54.26 ± 3.28 nmol/mL) (Figure 6.4.).

In co-culture biomass, cytokinin freebase forms were increased relative to the fungal monoculture, most notably iP which was previously absent in both fungal and bacterial controls, was detected (8.08 ± 0.88 pmol/gDW) (Table S6.11). Undetectable in bacterial biomass, ABA content in fungal biomass (968.23 ± 124.30 pmol/gDW) declined in the biomass of co-culture with the BC candidate (238.19 ± 24.94 pmol/gDW) (Table S6.14). Similarly absent in the pellet of *M. organophilum* axenic

cultures, *c*ZR content in fungal biomass dropped in co-culture (477.46 ± 11.05 to 280.50 ± 39.64 pmol/gDW), while iPR climbed sharply (5.80 ± 0.91 to 112.82 ± 32.01 pmol/gDW).

Co-cultures of nutrient-rich TSB containing *F. graminearum* and *M. extorquens* (NBRC 103129) – a non-BC candidate – resulted in a moderate increase in dry weight biomass (4.9 mg), relative to *F. graminearum* monoculture. Hormone responses showed similar trends seen in co-cultures with the BC candidate, though generally to a lesser extent: concentrations of *t*Z in filtered broth declined by 34.5%, *cis*-Zeatin by 28.0%, iP by 60.4%, and ABA by 55.2% (Figure 6.1. and 6.4.). Meanwhile, despite IAA and SA increasing (24% and 350% compared to fungal control, respectively), the total yield remained less than half that measured in the co-culture containing the BC candidate, despite similar total biomass.

Co-culture biomass (*F. graminearum* and *M. extorquens*) revealed similar declines in intracellular cytokinins relative to controls, though not as drastic: *c*Z (0.82 ± 0.03 nmol/gDW) and iP (70.96 ± 4.25 pmol/gDW). IAA increased significantly (35.54 ± 13.92 nmol•gDW) in co-culture biomass, exceeding that of identical cultures containing the BC candidate *M. organophilum*. ABA content in *M. extorquens* co-culture mixed biomass increased to 1.16 ± 0.63 nmol/gDW, contrasting against the depletion seen in co-culture biomass with *M. organophilum*. JA, undetectable in fungal and bacterial monocultures, was highly induced at 625.88 nmol/gDW.

Hormone shifts in F. oxysporum f. sp. cubense- Methylobacterium co-cultures

When co-cultured with the BC candidate *M. thiocyanatum* (NBRC 103128), total dry biomass decreased by 65.8 mg (16.0%) compared to fungal monocultures. In TSB, hormonal changes in filtered broth included slight reduction in *t*Z (3.41 ± 0.26 to 2.67 ± 0.11 pmol/mL), and sharper drops in *c*Z (207.39 ± 14.94 to 71.43 ± 7.20 pmol/mL), and iP (42.21 ± 18.05 to 7.25 ± 3.12 pmol/mL) (Figure 6.2. and Table S6.3). In contrast, DZ increased by 30.5%, ABA emerged at 117.82 pmol/mL (previously absent in axenic fungal cultures), and IAA more than doubled (4.40 ± 0.24 to 9.37 ± 1.10 nmol/mL) (Figure 6.5. and Table S6.6). Methylthiolated cytokinins were also detected at slightly higher concentrations in the broth of co-cultures with the BC candidate: MeSZ increased from 0.55 ± 0.13 to 1.57 ± 0.6 pmol/mL and MeSiP from 0.06 ± 0.01 to 5.50 ± 3.59 pmol/mL (Figure 6.8. and Table S6.9). In harvested biomass, *cis*-Zeatin – previously not detected in the biomass of axenic *F. oxysporum* culture – increased to 10.01 ± 2.39 pmol/gDW in co-culture with the BC candidate, along with increases to intracellular iP (14.32 ± 1.44 to 36.79 ± 2.58 pmol/gDW).

In MHCNB, co-cultures of *F. oxysporum* and the BC candidate (*M. thiocyanatum*) hormone levels in filtered broth shifted in less extreme ways (Figure 6.11). The concentration of *t*Z and *c*Z declined by 46.4% and 47.0%, respectively, and iP decreased by 70.6% (1.60 ± 0.28 to 0.47 ± 0.22 pmol/mL) (Figure 6.2.). IAA increased slightly (503.23 ± 34.03 to 545.59 ± 19.21 pmol/mL) (Figure 6.5.), and methylthiolated cytokinins such as MeSZ and MeSiP appeared where they were absent in axenic fungal controls (Figure 6.8.). Results of previous work (Chapter 2) support production of these isoforms by *Methylobacterium*, where they were found in highest abundance (4.5 – 54.3 pmol/mL) second only to *t*Z in filtered broth (0.45 – 82.16

pmol/mL). In the mixed biomass of co-culture with the BC candidate, moderate increases in *c*Z (7.22 ± 0.94 to 14.91 ±0.28 pmol/gDW) and iP (8.81 ± 0.44 to 16.09 ±0.69 pmol/gDW) were detected, relative to fungal monoculture (Table S6.12). While undetected in the biomass of axenic bacterial controls, *c*ZR content in axenic fungal biomass (15.59 ± 2.35 pmol/gDW) increased drastically in co-culture tissues (240.16 ± 23.58 pmol/gDW) with iPR following a similar pattern in axenic (9.69 ± 0.99 pmol/gDW) and co-culture biomass (46.47 ± 5.63 pmol/gDW).

In nutrient-rich TSB, hormone shifts in co-cultures containing *F. oxysporum* and *M. oxalidis* (NBRC 107715) – the non-BC bacterial strain – were also pronounced, though with greater variability between replicates: *t*Z dropped by 61.3% (3.41 ± 0.26 to 1.32 ± 0.36 pmol/mL), cisZ by 74.0% (207.39 ± 14.94 to 53.83 ± 16.85 pmol/mL), and iP by 84.7% (42.21 ± 18.05 to 6.46 ± 2.99 pmol/mL) (Figure 6.2.). IAA surged more than five-fold to 23.39 ± 2.90 nmol/mL and ABA rose to 140.71 ± 26.81 pmol/mL (Figure 6.5.). MeSZ and MeSiP content also increased compared to fungal monoculture (Figure 6.8.). In MHCNB, IAA concentration in broth of co-cultures was 852.40 ± 18.06 pmol/mL (greater than co-cultures with *M. thiocyanatum*), while *cis*Z diminished (8.33 ± 0.45 pmol/mL). In analysis of tissues, biomass recovered from MHCNB revealed greater changes between bacterial strains. Importantly, along with fluctuations to similar changes to *t*Z, *c*Z, and iP – *c*ZR content in co-culture biomass with *M. thiocyanatum* (BC candidate) was 7-fold less than the rise detected in co-cultures with *M. oxalidis* (non-BC isolate) (32.42 ± 3.35 pmol/gDW) – a nearly identical pattern was also observed with iPR content in co-cultures containing non-BC bacterium (17.28 ± 4.11 pmol/gDW).

SA, JA, GA_1, GA_7, GA_9, GA_{20}, MeSZR, and MeSiPR were not successfully detected in all treatments and culture combinations containing *F. oxysporum*.

Hormone shifts in F. fujikuroi-Methylobacterium co-cultures

In nutrient-rich medium, co-cultures of *F. fujikuroi* (UAMH 9877) and the BC agent *M. oryzae* (LMG 23582) (Figure 6.12) – determined previously (Chapter 5) – had decreased total biomass dry weight by compared to fungal controls (20.4%). This reduction was accompanied by a drop in *t*Z (from 4.01 ± 2.23 to 0.55 ± 0.50 pmol/mL), *c*Z (0.89 ± 0.09 to 0.51 ± 0.12 pmol/mL), and DZ (1.02 ± 0.33 to 0.64 ± 0.18 pmol/mL) in filtred broth (Figure 6.3.). In contrast, broth content of iP increased modestly from 0.03 ± 0.05 to 0.18 ± 0.02 pmol/mL, and ABA rose by approximately 20% (666.19 ± 55.54 to 799.10 ± 71.69 pmol/mL). Notably, JA levels in spent broth doubled (150.98 ± 20.41 to 331.36 ± 30.25 nmol/mL), while concentrations of IAA declined by about 21% (15.41 ± 2.34 to 12.15 ± 0.89 nmol/mL) (Figure 6.6.). GA_4 and GA_7 decreased by 48.6% and 73.9%, respectively, and methylthiolated forms such as MeSZ and MeSiP dropped to undetectable levels (Figure 6.9.). Compared to axenic controls, mixed biomass recovered from co-culture with the BC isolate contained higher levels of iP (40.85 ± 1.50 to 166.32 ± 15.13 pmol/mL), IAA (6.56 ± 0.13 to 27.23 ± 0.85 nmol/mL), and GA_4 (9.24 ± 4.35 to 107.79 ± 4.49 nmol/mL). In contrast, these same co-cultures contained lower levels of intracellular *c*Z (440.88 ± 21.74 to 16.12 ± 9.45 pmol/mL), *c*ZR (649.10 ± 24.49 to 453.64 ± 17.66 pmol/mL), and iPR (46.58 ± 11.34 to 40.14 ± 16.60 pmol/mL).

In nutrient-minimum MHCNB, co-culture with the BC candidate (*M. oryzae*) experienced a dramatic dry weight biomass reduction of 91.9%, accompanied by shifts

in detectable hormones in the broth. For example, *trans*-Zeatin increased from 0.09 ± 0.03 pmol/mL in fungal control (axenic) flasks to 0.95 ± 0.16 pmol/mL in challenged co-cultures – contrasting sharply with the *t*Z content in *M. oryzae* monocultures (588.07 ± 18.59 pmol/mL) (Figure 6.3. Table S6.4). Concentrations of *c*Z in co-culture broth also increased moderately (0.05 ± 0.01 to 1.58 ± 0.57 pmol/mL), while larger increases in ABA (7.41 ± 3.52 to 36.03 ± 12.95 pmol/mL), JA (1.96 ± 0.26 to 12.94 ± 2.15 μmol/mL), and IAA (0.31 ± 0.07 to 2.99 ± 0.74 nmol/mL) were detected (Figure 6.6.). The average concentration of SA rose to 137.08 ± 10.41 nmol/mL (197% increase from axenic fungal control), along with increases to GA_4 (2.49 ± 0.08 nmol/mL) and GA_7 (57.33 ± 3.93 pmol/mL). In biomass from co-cultures, higher levels of iP (5.57 ± 0.95 to 17.42 ± 3.95 pmol/mL), IAA (2.38 ± 0.08 to 11.92 ± 0.05 nmol/mL), and GA_4 (1.01 ± 0.20 to 1.81 ± 0.14 μmol/mL) – though the magnitude of transitions were weaker than identical experiments in TSB, the absolute values were elevated (Table S6.16).

Co-culturing *F. fukikuroi* with the non-BC species *M. radiotolerans* (LMG 6379) in nutrient-rich medium reduced biomass dry weight by only 5.9% relative to fungal controls. Hormone responses followed similar directions as TSB cultures containing the BC candidate, except with much greater shifts in freebase fractions, most notably *t*Z (4.01 ± 2.23 to 1.19 ± 0.92 pmol/mL). In nutrient-rich TSB, broth content of iP and JA increased (0.20 ± 0.16 pmol/mL and 221.18 ± 29.47 nmol/mL, respectively). IAA experienced a decline to 11.22 ± 1.54 nmol/mL (27.2% lower than fungal monoculture), along with ABA which experienced a 40% decrease (400.16 ± 20.27 pmol/mL) – in stark contrast to ABA dynamics in flasks containing the BC candidate. When compared to axenic controls in TSB, biomass of *M. radiotolerans* contained less

iP (28.67 ± 5.32 pmol/mL) than the *M. oryzae* challenge (166.32 ± 15.13 pmol/mL) and *F. fukikuroi* controls (40.85 ± 1.50 pmol/mL), while IAA levels also increased in *M. radiotolerans* co-cultures (31.66 ± 1.20 nmol/mL) – surpassing the content in challenges with *M. oryzae*. Content of GA_4 in biomass (449.99 ± 35.99 nmol/mL) also greatly surpassed the levels in culture tissues from the *M. oryzae* challenge (107.79 ± 4.49 nmol/mL) – while being entirely undetectable in both bacterial monocultures. In contrast, co-cultures contained significantly higher levels of *c*Z (191.97 ± 38.42 pmol/mL) compared to challenges with *M. oryzae*, and similar outcomes were observed with *c*ZR and iPR. Interestingly, when replicated in MHCNB, the directionality of hormone shifts observed between axenic and co-culture conditions remained consistent with those in TSB; however, the magnitude of these changes was markedly amplified.

GA_1, GA_9, GA_{20} were undetectable in all treatments and culture combinations containing *F. fujikuroi*.

6.6. DISCUSSION

CK-FB, GA, and IAA shifts in co-culture

Phytohormones play a significant role in shaping the interactions at the antagonist-pathogen-host axis. Hormone signals can impact fungal physiology directly by altering cell structure, metabolism, or reproduction, and through indirect signalling pathways which prime the defenses of the host-plant.

Fungal systems, including species belonging to *Fusarium*, exhibit significant capabilities to synthesize and respond to phytohormones traditionally associated with plant physiology (Sørensen et al. 2018; Vrábka et al. 2019). For example, CKs have been identified across diverse fungal species, including *Fusarium pseudograminearum* and other prominent pathogens like *Ustilago maydis*, where CKs were found to be important to virulence and for symptom expression including tumor formation (Morrison et al. 2017; Chanclud and Morel, 2016). Other fungal taxa also demonstrate sensitivity and production of hormones. For instance, *Magnaporthe oryzae*, the rice blast pathogen, shows sensitivity to CKs and auxins, influencing virulence and infection strategy (Chanclud and Morel, 2016). Similarly, ethylene (ET) and ABA have been detected in arbuscular mycorrhizal fungi like *Rhizophagus irregularis*, where these hormones influence the regulation of mycorrhizal symbiosis (Pozo et al. 2015).

Fungi not only produce hormones to influence host interactions but also exhibit responses to exogenous hormones. For example, CKs have been shown to affect fungal cell division, sporulation, and morphological differentiation in various fungal species (Gupta et al., 2021; Nuzhnaya et al., 2024). Similarly, auxins can influence fungal mycelial extension, branching patterns, and hyphal morphology, potentially impacting

fungal growth dynamics, competitive interactions, and ecological adaptability (Prusty et al. 2004).

A recent in-depth study by Grich et al. (2024) significantly advances our understanding of fungal-derived CKs, using *Pleurotus ostreatus* as a model organism. Their work not only conclusively demonstrated endogenous CK biosynthesis by *P. ostreatus*, but definitive proof of a hormetic response to exogenous treatment with N^6-benzyladenine (BAP), Kinetin (KIN), iP, and *t*Z – characterized as a stimulatory effect at low concentrations and inhibitory at higher levels – through effects on mycelial morphology, hyphal branching, biomass accumulation, and growth rates (Grich et al. 2024).

Among fungal phytohormones though, GAs perhaps hold the most substantial historical and biological importance. The family of GAs were first identified in the context of Bakanae ("foolish seedling") disease in rice caused by *F. fujikuroi* (teleomorph: *Gibberella fujikuroi*). Study of the disease – characterized by excessive internodal elongation, chlorosis, and reduced vigor of seedlings – resulted in the isolation of a growth-promoting factor secreted by the pathogen, after which it was named (Tudzynski, 2005; Hedden and Sponsel, 2015). In Bakanae disease, the virulence of *F. fujikuroi* is directly linked to its prolific secretion of GAs (mainly GA_3) which disrupt hormonal balance in the host (Hori, 1898; Michniewicz, 1989; Troncoso et al. 2009; Wiemann et al. 2013).

Fungal manipulation of phytohormones highlights their complex and dynamic roles in interkingdom signalling, hormone regulation, and cross-talk – factors that are

increasingly important considerations in the development of next-generation BC strategies. Uniquely, our work highlights key shifts in hormone accumulations between phytopathogenic species of *Fusarium*, and *Methylobacterium* strains with contrasting capacity to disrupt fungal growth *in vitro*. A central finding was the general distinction in phytohormone patterns between BC candidates and bacterial strains which impart no measurable effect on fungal proliferation in previous studies (non-BC). Broadly, BC candidate strains consistently produced higher levels of isopentenyladenine (iP), and methylthiolated cytokinins in the broth of axenic cultures (iP: 14 – 321 pmol/mL and MeSZ: 46 – 2,410 pmol/mL), compared to non-BC strains (iP: 0 – 27 pmol/mL and MeSZ: 6 – 243 pmol/mL) (Figure 6.14). Interestingly, while studies examining the effects of CKs on phytopathogenic *Fusarium* remain limited, recent work has indicated that relatively low doses (1-100 μM) of the synthetic CK 6-benzylaminopurine (6-BAP) directly interferes with fungal cell division and morphology, resulting in suppression of mycelial growth of *F. oxysporum* f. sp. *lycopersici* (Gupta et al. 2021). Importantly, the absence of inherent cytotoxicity or lethality following exogenous exposure to these hormones was universal in the findings by other researchers, and consistent with the outcomes of our own work. In contrast to CK dynamics, average auxin production (IAA) across all three strains was slightly higher in the broth of axenic cultures containing non-BC isolates (64.18 pmol/mL) than BC candidates (50.51 pmol/mL), though this ratio reversed significantly under strict nutrient conditions, where secretion of IAA by non-BC strains plummeted in MHCNB (Figure 6.14). In fact, only BC candidates produced IAA in MHCNB.

The precise effects of exogenous phytohormones on fusaria, though, remains poorly understood and complex overall. Illustratively, when applied to *F. oxysporum* f.

sp. *lycopersici*, IAA decreased the rate of spore germination universally *in vitro*, while comparable concentrations (< 75 μM) initially promoted growth of the chickpea pathogen *F. delphinoides*, which was later inhibited with higher doses (≥ 500 μM) (Kulkarni et al. 2013; Sharaf and Farrag 2004). In fact, these variations in response may help explain variations in our previous work – mainly, why the growth supressing effects of bacterial broth amendments were greatly enhanced in MHCNB and diminished in TSB. Previous work has determined that *Methylobacterium* isolates produce IAA in nutrient-limited broth only when supplemented with L-tryptophan (Palberg et al. 2022). Though MHCNB is devoid of tryptophan, the presence of glucose enables endogenous L-tryptophan synthesis using phosphoenolpyruvate (PEP) and ertythrose-4-phosphate – terminal products of glycolysis – as precursors, validated by the presence of the *trpA* through *trpG* genes in sequenced species (Grossi et al. 2020; Marx et al. 2012; Peyraud et al. 2011). IAA production by *Methylobacterium* then, may explain differences between bacterial strains and dependant on growth media content type: amplified effects on fungal growth may be attributed to higher production of IAA by BC candidates in MHCNB, relative to ineffective bacterial isolates, and that the antagonistic effects of exogenous IAA in similar fusaria – disruption to hyphal elongation or branching, causing unstructured growth which impacts colony expansion – is surmountable in more nutrient-diverse growth media.

Methylthiolated Cytokinins

Methylthiolated cytokinin forms (MeSZ, MeSiP) were generally higher in the broth of axenic bacterial cultures, and only BC candidates produced MeSiP in MHCNB. Our work aligns with the growing body of evidence which suggests that 2MeS-CKs are key players in pathogen-host-symbiont interactions and are typically of bacterial origin.

For example, bacterially derived 2MeS-CKs have been implicated in regulating seed dormancy in ryegrass (*Lolium rigidum*), where removal of bacterial symbionts prevented seeds from breaking dormancy (Goggin et al. 2015).

The involvement of 2MeS-CKs in host–pathogen dynamics has also been observed. Elevated levels of 2MeS-CKs, iP, and *c*Z were detected in *Arabidopsis thaliana* plants infected with a virulent strain of *Rhodococcus fascians*, compared to infections by non-pathogenic strains, despite both producing a similar cytokinin profile (Pertry et al. 2009). The authors also propose that 2MeS-CKs, are less cytotoxic than classical CKs, and may persist longer in host tissues – suggesting that pathogenic strains may employ the 2MeS-CKs to maintain access to the host (Pertry et al. 2009). While evidence of 2MeS-CK production by *Fusarium* has not been confirmed in independent studies, these modified CKs may be present in host tissues during infection – as found in the pathosystem between *Ustilago maydis* and maize (Morrison et al. 2015a; Gibb et al. 2020).

These recent insights reveal an underexplored but potentially important mechanism through which microbial associates, including *Methylobacterium*, could interrupt interkingdom signalling in pathosystems. CKs, particularly those derived via tRNA degradation pathways (e.g., *cis*-Zeatin, 2MeS-CKs), have been documented in various microbial taxa, including *Methylobacterium* spp., and are known to affect plant developmental processes and stress responses (Gibb et al. 2020; Jorge et al. 2019; Palberg et al. 2022).

Given our observations of delayed *Fusarium* germination and impaired growth in media amended with *Methylobacterium* spent broth (Chapter 5), the potential role of bacterially derived methylthiolated cytokinins (2MeS-CKs) in disrupting fungal signalling becomes increasingly compelling. These modified CK forms may not only interfere with the ability for *Fusarium* to perceive host-derived CK cues but could also saturate or inhibit signalling cascades that are critical for chemotropism, host detection, and virulence expression. Although the functional role of 2MeS-CKs in fungal systems remains largely unexplored, we propose that these compounds may serve as focal points of hormonal interference – either as targets of fungal detoxification or as ligands misaligned with fungal developmental expectations.

Notably, if 2MeS-CKs are elicited in host tissues during infection (or are selectively produced by *Fusarium* exclusively during colonization of a vulnerable host) as seen in the *U. maydis*–maize pathosystem, then the early or ectopic presence of these compounds – by antagonistic *Methylobacterium* – could disrupt the developmental logic of the pathogen. *Fusarium* may interpret this premature or context-inappropriate signal as a cue of host immune activation or an unfavourable environment, triggering repression of growth or activation of detoxification responses. The poor performance of *Fusarium* in co-cultures with MeS-CK-producing bacteria may therefore reflect not only direct antagonism, but also a form of hormonal signal spoofing, whereby the fungus interprets its surroundings as immunologically hostile or developmentally inappropriate, resulting in a fungistatic effect. Though little published work exists concerning the role of 2MeS-CKs, we posit that these compounds may be targets of fungal detoxification or suppression and highlight the possibility for a competitive

hormone signalling axis where these hormones – or their intermediates and precursors – are contested during microbial competition.

Immune silencing and SA-JA signal antagonism

Across all fungal species, the trend was clear: BC bacteria more consistently triggered higher concentrations of SA and JA in spent broth of co-cultures than non-BC strains, while uniquely spiking gibberellin content in cultures containing *F. fujikuroi* (Figure 6.10 and Figure 6.12). This trend was particularly amplified with the use of MHCNB, especially for *F. graminearum* and *F. fujikuroi*. Both SA and JA are central components in plant defense signalling but their role in microbial interactions is being continually elucidated. In our work, we found SA was undetectable in the broth of fungal monocultures irrespective of growth medium but appeared in co-cultures with *Methylobacterium* – particularly BC candidate isolates (Figs 6.1 – 6.3). This finding suggests that the detectable quantities of SA are of bacterial origin and is consistent with existing literature which indicates filamentous fungi, including the fusaria, do not possess either the full isochorismate or phenylpropanoid pathway architecture necessary for SA biosynthesis (Soanes et al. 2008). Instead, they often produce isochorismatase effectors that degrade plant-derived SA precursors and salicylate hydroxylases that catabolize SA itself – consistent with an overall strategy of SA detoxification rather than synthesis (Hao et al. 2019; Rocheleau et al. 2019; Soanes et al. 2008). In contrast, many genera of bacteria retain the cellular machinery for SA biosynthesis, and plant-associated isolates such as *Pseudomonas* spp., likely employ SA as a strategy to thwart fungal competitors or prime plant immune responses (Audenaert et al. 2002; Mishra and Baek 2021; Wu et al. 2008). Importantly, SA production by

Methylobacterium species *in vitro* is further supported by existing species-level research (Ehinmitan et al. 2024).

Oppositely, JA was consistently detected at baseline levels in fungal monocultures, absent in axenic bacterial cultures, and dramatically elevated in co-culture – again, particularly with BC candidates (Figure 6.10 and Figure 6.12). Elevated broth content of JA in co-culture is likely a multifaceted adaptive response by *Fusarium*. Broadly, the genus is known to possess the complete enzymatic machinery required for JA synthesis through the octadecanoid pathway, and secretion of JA and related oxylipins is known to be induced by abiotic and biotic stressors, including microbial antagonism, and nutrient limitation (Brodhun and Feussner 2011; Eng et al. 2021; Masimbula et al. 2019; Miersch et al. 1992; Oliw and Hamberg 2017). Aside from being involved in enhancing stress resilience, directing shifts in developmental priorities, and marshalling chemical defenses in response to competition, *Fusarium* may also upregulate JA secretion as part of a generalized stress response to exogenous sources of SA.

In plants, JA and SA represent two major and often antagonistic trunks of immune signalling. In the canonical model, SA is generally associated with defense against biotrophic pathogens, while JA is linked to attack by necrotrophic pathogens and herbivorous insects (Yang et al. 2019). In most species of dicotyledonous plants, the crosstalk between these pathways enables a fine-tuned response tailored to the susceptibility of the invader. As hemibiotrophs, the infection process associated with many fusaria is typified uniquely by an initial biotrophic phase of behaviour wherein the pathogen is able to penetrate host tissues without setting off a cascade of quarantine

responses like programmed cell death (PDC) (Pietro et al. 2003). A central factor in the virulence of many species of *Fusarium*, is their ability to silence an immune response while establishing infection biotrophically through exploitation of SA-JA pathway antagonism in the host (Makandar et al. 2010; Qi et al. 2016; Trail 2009). Many phytopathogenic fungi – including *F. graminearum* – produce JA or JA-mimicking oxylipins, to tilt the host immune balance toward JA and indirectly weaken SA-associated defenses (Qi et al. 2016; Yang et al. 2019).

The elevated broth concentrations of SA and IAA observed in co-cultures likely form a two-pronged defensive property of *Methylobacterium* candidates which could be used in an integrated crop protection strategy. As a central regulator of plant defenses, exogenous sources of SA are known to activate systemic acquired resistance (SAR): setting off a cascade of processes which include the accumulation of pathogenesis-related proteins, oxidative bursts, and reinforced cell walls. Bacterial-derived SA could reduce host susceptibility by stimulating SAR and increasing the hostility of the phytobiome towards pathogenic fungi (Ali et al. 2018; Pieterse et al. 2014). Although *Fusarium* appears to respond to the presence of bacterial antagonism by increasing JA production, this reaction may be insufficient when SA is persistently elevated through bacterial secretion because of the insusceptibility of bacterial biosynthesis to the presence of JA.

CK and auxin production by *Methylobacterium* may also contribute additional protective effects: the combination of hormones likely establishes a microenvironment that interrupts fungal sensing and progress through the biotrophic phase. Despite clear activation of JA biosynthesis in response to bacterial antagonism, *Fusarium* isolates

may be unable to override the combined immunostimulatory effects of bacterially derived hormones on the host, the direct interference with fungal signalling and environmental receptivity, the emulation of a defensive or incompatible host, or trigger prolonged compensatory stress responses in *Fusarium* which exhaust nutrient reserves.

Signal interference as a facet of biological control

While this study focused on profiling a defined set of hormones, it is critical to acknowledge that the dynamics of antagonist-pathogen interactions are shaped by a broad and far more chemically complex landscape. The elevated levels of IAA, SA, and the accompanying surge in JA observed in our work, points to the existence of a central hormone axis imbedded in the reception of microbial competition. These responses, however, likely represent only a fraction of the total metabolic exchange occurring between partners, and though the current work did not resolve these attributes, we suspect that many contribute to the observed outcomes. Differences in the secretion of secondary metabolites, effectors, and nutrient challenges exacted on the fungal pathogen may represent some of the hidden differences between BC candidates and ineffective bacterial isolates, given their similar hormone expression in axenic cultures.

These response nuances were observed in our work, as each species of *Fusarium* species demonstrated a unique pattern of hormonal response to co-culture, suggesting that their sensitivities are both species- and context-specific. For example, broth content of *c*Z surges while *t*Z and iP fall when *F. graminearum* is confronted with the BC candidate (LMG 6083) relative to co-cultures containing the paired ineffective isolate (non-BC), yet this characteristic is reversed when *F. oxysporum* and *F. fujikuroi* are challenged by their respective BC candidates. In the case of *F. fujikuroi*, the presence of

the BC candidate (LMG 23582) causes an accumulation of GA_4 in the broth relative to the non-BC strain, yet changes to gibberellin content are insignificant between the challenge and control conditions for *F. graminearum* and *F. oxysporum*.

While our study provides detailed insights into hormonal fluctuations between *Fusarium* and *Methylobacterium* isolates under axenic and co-culture conditions, it is crucial to acknowledge that these findings represent only a partial view of the complex antagonist-pathogen-host interplay. Hormone dynamics observed *in vitro* may differ significantly from those occurring *in planta*, where the presence of plant tissues themselves introduces numerous additional variables, such as host-derived hormone secretion, secondary metabolite production, and involvement of host immune responses – all of which could profoundly alter the hormonal interplay between the antagonist and pathogen observed in our simplified system. Plant surfaces and tissues provide distinct microenvironments, nutrient conditions, and stress signals that influence bacterial and fungal behavior and may shift hormone production differently than observed in broth cultures. For example, the secretion of hormones *in vitro* such as SA or IAA by *Methylobacterium* may vary significantly when in contact with host tissues, as colonization patterns, metabolic activity, and gene expression may be altered significantly whilst in competition with resident microbiota.

How precisely, then, *Methylobacterium* fit into disease suppression remains undetermined but the reputation of the genus as prominent, ubiquitous, and evolutionarily-conserved natural endophytes is unequivocal (Corpe and Rheem 1989; Green 2006; Holland 1997a, 1997b; Kutschera 2007). The total scope of their activity in the phytobiome which results in the growth-promoting and stress-alleviating effects

in their hosts, is likely vastly underestimated and probably extends well beyond their current station as nutritional mutualists. The results of our work, however, support the hypothesis that hormone signalling is a shared target of microbial interactions, with implications for fungal development, signalling plasticity, and community-level metabolic regulation. In addition to secreted hormones by bacterial antagonists, we posit that additional strain-specific interactions, driven by unquantified metabolites or other signalling molecules, play a role in determining the overall success in disrupting fungal physiology.

Future studies involving a representative host will be critical to providing insights into the true efficacy of bacterial candidates as biocontrol agents *in situ*. Exploring hormonal dynamics within the host context will allow for a more holistic understanding of tripartite interactions, ensuring that potential biocontrol strategies are evaluated under realistic conditions. Such experiments should leverage semi-targeted metabolomics and transcriptomic profiling to assess hormone exchanges at the plant-microbe interface to capture the full spectrum of interkingdom communication.

6.7. CONCLUSION

Through hormone profiling of axenic and co-cultures of *Methylobacterium* and *Fusarium*, we reveal that bacterial candidates with BC potential consistently modulate levels of key signalling molecules – particularly JA and to a lesser extent, the CKs – with measurable consequences on fungal morphology. Notably, 2MeS-CKs, emerged as potential disruptors of fungal developmental signalling, possibly through mimicking of host cues. Furthermore, the observation that *Fusarium* species increase JA secretion in response to antagonistic bacterial strains – possibly in response to heightened levels of SA and IAA in the broth.

Our findings support the hypothesis that the BC potential of *Methylobacterium* may involve hormone interference or spoofing, in addition to classical modes of fungal inhibition including resource competition and substrate saturation. These mechanisms likely interact with broader microbial community dynamics and resource signalling, forming a chemically complex competitive landscape in which hormone perception and interpretation by *Fusarium* is both strain- and context-dependent. While *in vitro* systems offer controlled insight into these dynamics, validating these interactions in planta remains essential. Future investigations should incorporate host models and leverage multi-omics approaches to capture the full breadth of metabolic and transcriptional crosstalk at the pathogen–antagonist–host interface.

6.8. FIGURES

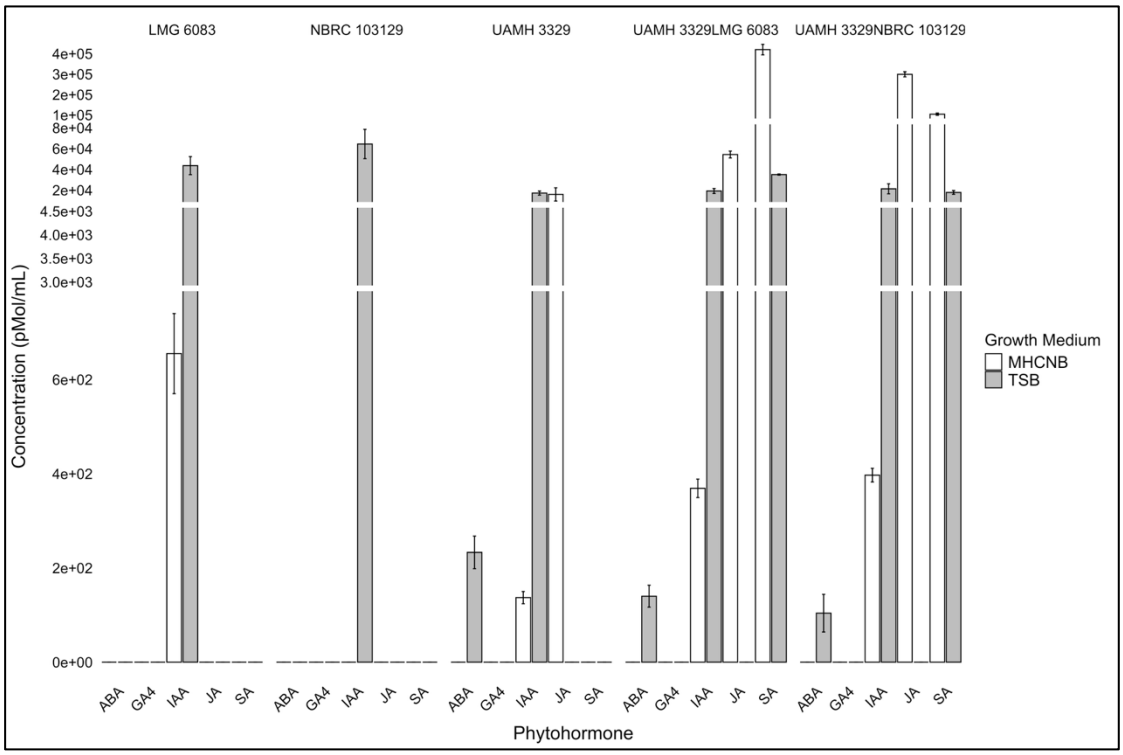

Figure 6.1: Average (n = 4) concentrations of acidic phytohormones (pmol/mL) in filtered culture broth, across axenic and co-culture conditions involving BC candidate *Methylobacterium organophilum* (LMG 6083), non-BC candidate *M. extorquens* (NBRC 103129), and phytopathogen *F. graminearum* (UAMH 3329). Cultures were grown in tryptic soy broth (TSB) or modified Hoagland's complete nutrient broth (MHCNB). Hormones were assessed under five experimental conditions: (i) *Fusarium* monoculture, (ii) *Fusarium* BC challenge, (iii) *Fusarium* non-BC challenge, and monocultures of both the (iv) BC candidate and (v) non-BC isolate. Error bars represent the standard error of the mean (± SEM).

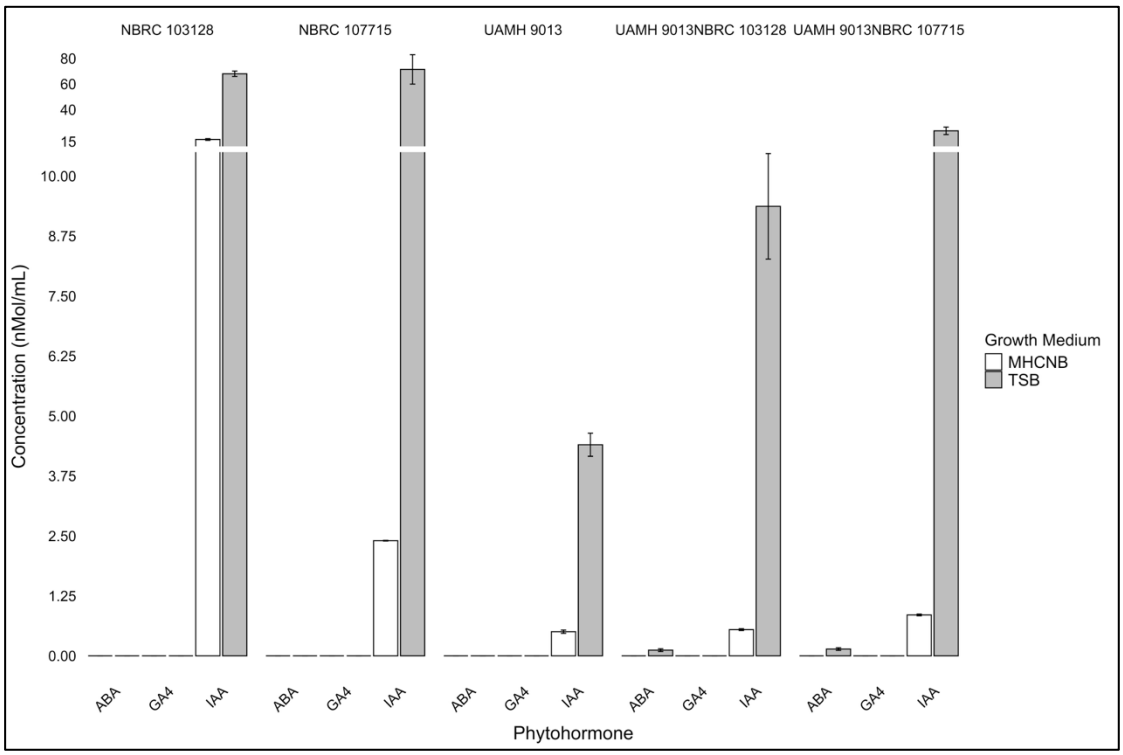

Figure 6.2: Average (n = 4) concentrations of detectable acidic phytohormones (nmol/mL) in filtered culture broth, across axenic and co-culture conditions involving BC candidate *M. thiocyanatum* (NBRC 103128), non-BC candidate *M. oxalidis* (NBRC 107715), and phytopathogen *F. oxysporum* f. sp. *cubense* (UAMH 9013). Cultures were grown in tryptic soy broth (TSB) or modified Hoagland's complete nutrient broth (MHCNB). Hormones were assessed under five experimental conditions: (i) *Fusarium* monoculture, (ii) *Fusarium* BC challenge, (iii) *Fusarium* non-BC challenge, and monocultures of both the (iv) BC candidate and (v) non-BC isolate. Error bars represent the standard error of the mean (± SEM).

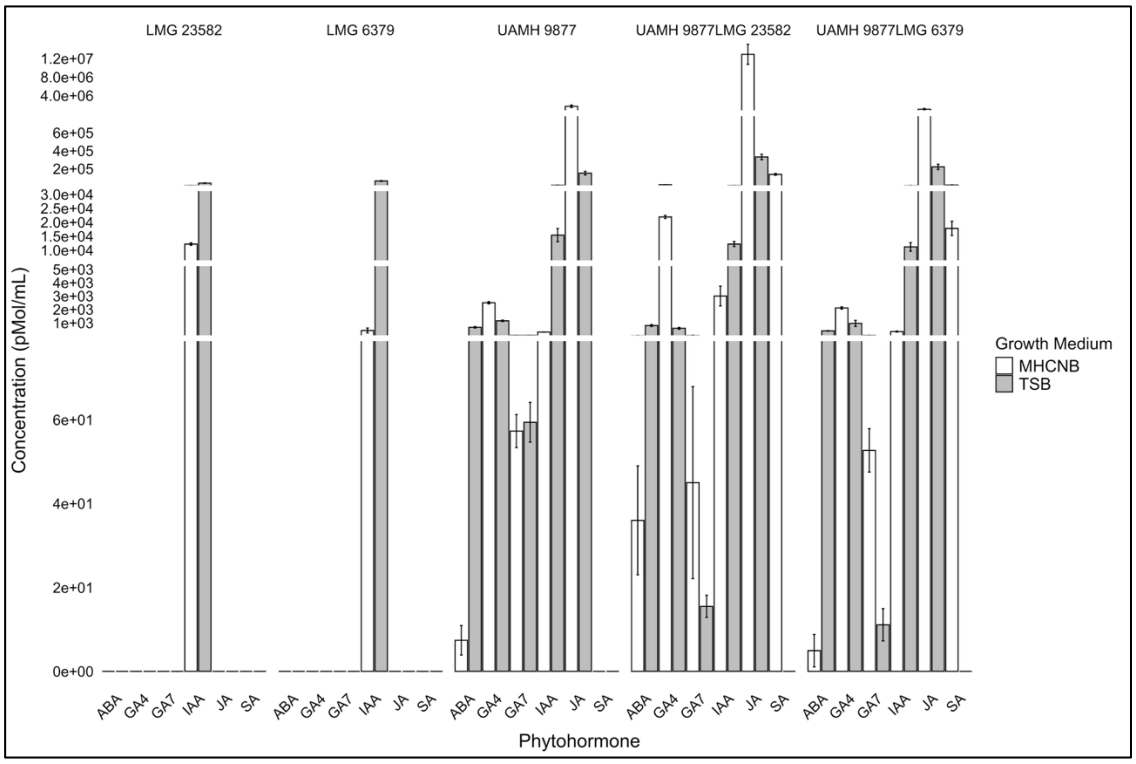

Figure 6.3: Average (n = 4) concentrations of acidic phytohormones (pmol/mL) in filtered culture broth, across axenic and co-culture conditions involving BC candidate *M. oryzae* (LMG 23582), non-BC candidate *M. radiotolerans* (LMG 6379), and phytopathogen *F. fujikuroi* (UAMH 9877). Cultures were grown in tryptic soy broth (TSB) or modified Hoagland's complete nutrient broth (MHCNB). Hormones were assessed under five experimental conditions: (i) *Fusarium* monoculture, (ii) *Fusarium* BC challenge, (iii) *Fusarium* non-BC challenge, and monocultures of both the (iv) BC candidate and (v) non-BC isolate. Error bars represent the standard error of the mean (± SEM).

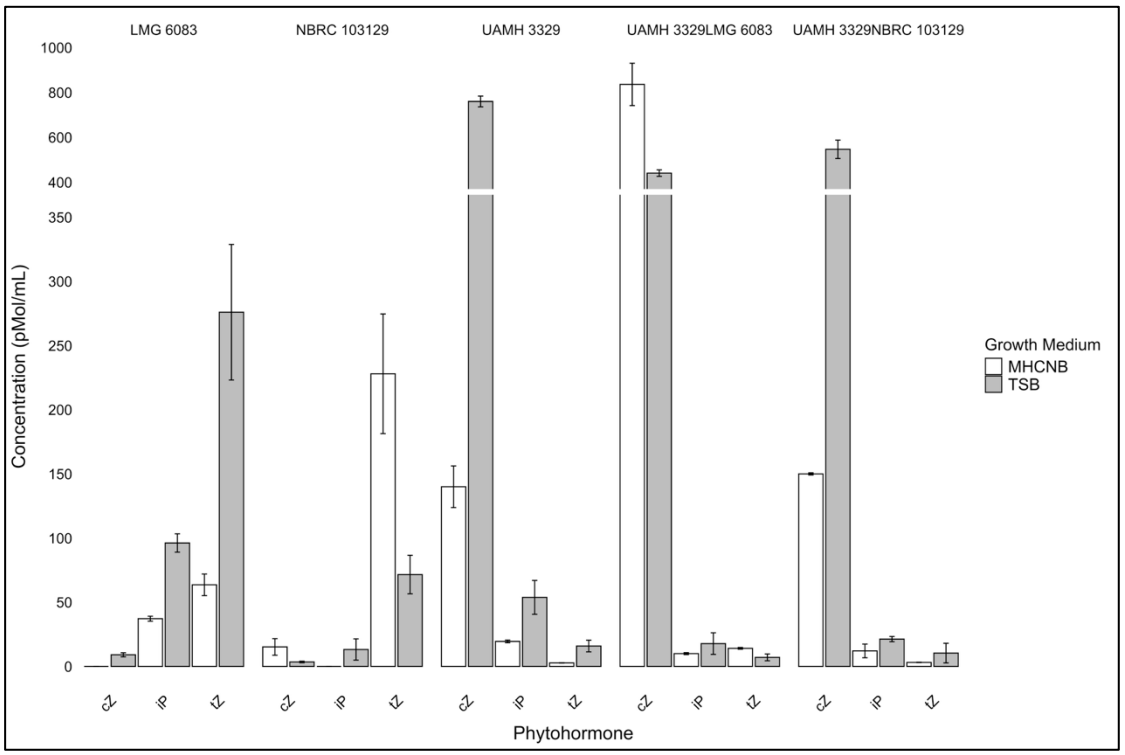

Figure 6.4: Average (n = 4) concentrations of detectable freebase phytohormones (pmol/mL) in filtered culture broth, across axenic and co-culture conditions involving BC candidate *M. organophilum* (LMG 6083), non-BC candidate *M. extorquens* (NBRC 103129), and phytopathogen *F. graminearum* (UAMH 3329). Cultures were grown in tryptic soy broth (TSB) or modified Hoagland's complete nutrient broth (MHCNB). Hormones were assessed under five experimental conditions: (i) *Fusarium* monoculture, (ii) *Fusarium* BC challenge, (iii) *Fusarium* non-BC challenge, and monocultures of both the (iv) BC candidate and (v) non-BC isolate. Error bars represent the standard error of the mean (± SEM).

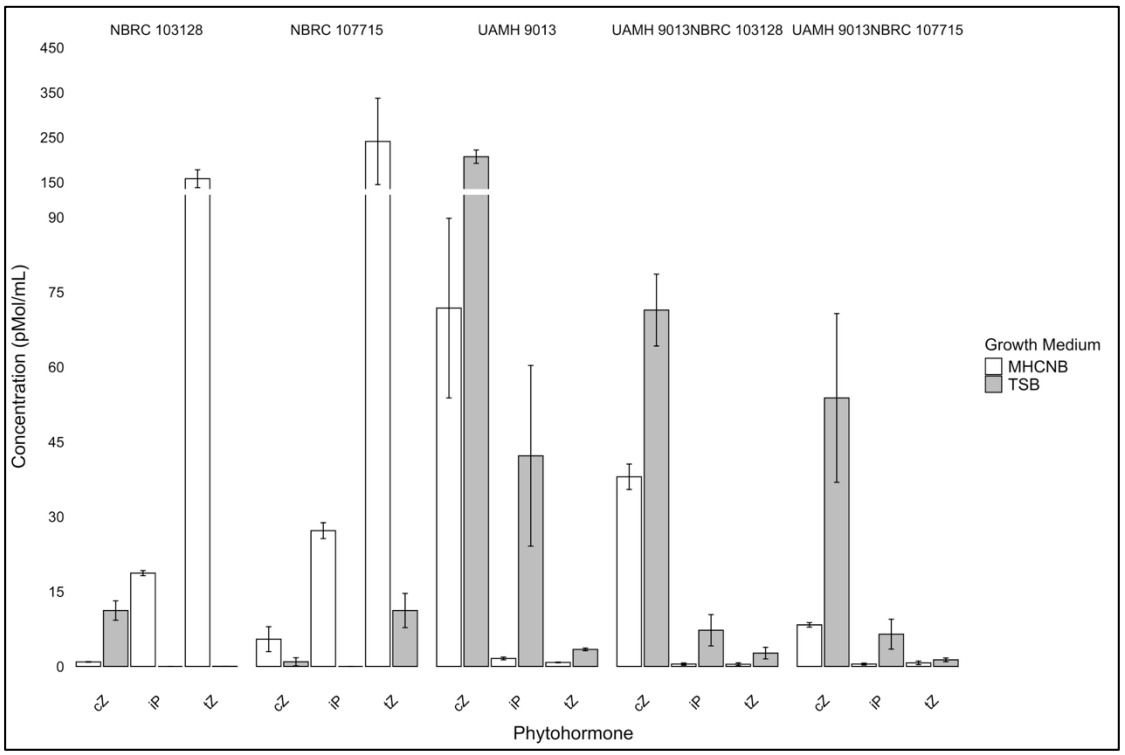

Figure 6.5: Average (n = 4) concentrations of detectable freebase phytohormones (pmol/mL) in filtered culture broth, across axenic and co-culture conditions involving BC candidate *M. thiocyanatum* (NBRC 103128), non-BC candidate *M. oxalidis* (NBRC 107715), and phytopathogen *F. oxysporum* f. sp. *cubense* (UAMH 9013). Cultures were grown in tryptic soy broth (TSB) or modified Hoagland's complete nutrient broth (MHCNB). Hormones were assessed under five experimental conditions: (i) *Fusarium* monoculture, (ii) *Fusarium* BC challenge, (iii) *Fusarium* non-BC challenge, and monocultures of both the (iv) BC candidate and (v) non-BC isolate. Error bars represent the standard error of the mean (± SEM).

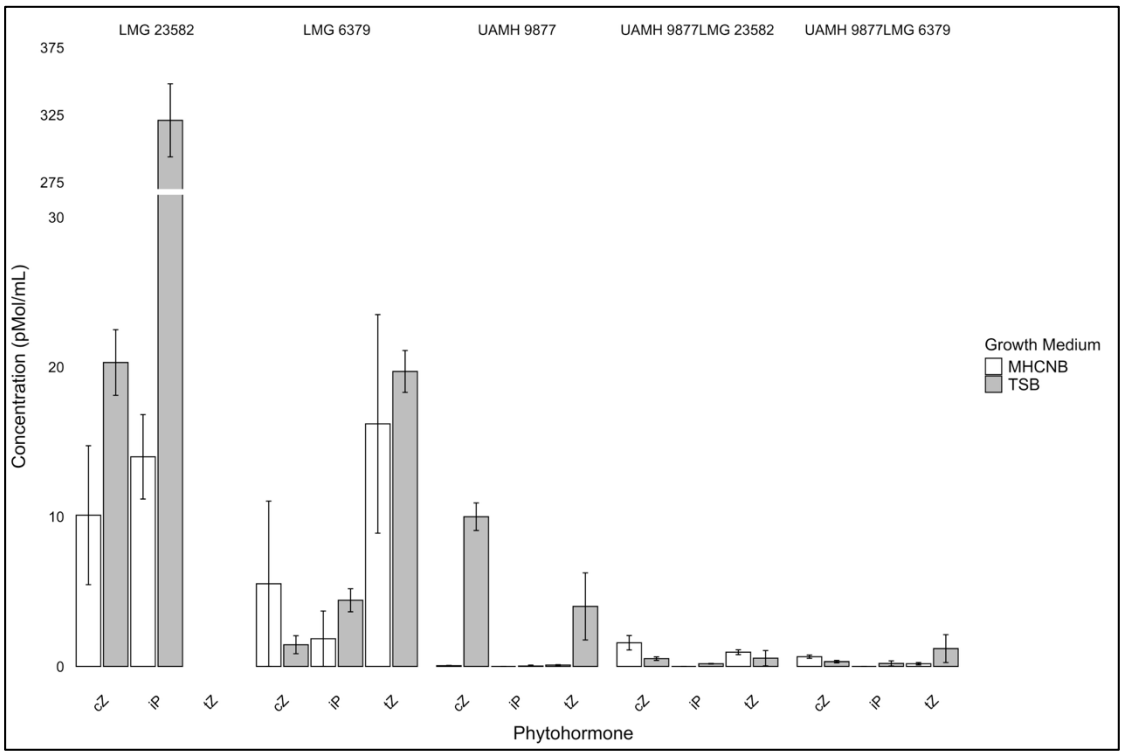

Figure 6.6: Average (n = 4) concentrations of detectable freebase phytohormones (pmol/mL) in filtered culture broth, across axenic and co-culture conditions involving BC candidate *M. oryzae* (LMG 23582), non-BC candidate *M. radiotolerans* (LMG 6379), and phytopathogen *F. fujikuroi* (UAMH 9877). Cultures were grown in tryptic soy broth (TSB) or modified Hoagland's complete nutrient broth (MHCNB). Hormones were assessed under five experimental conditions: (i) *Fusarium* monoculture, (ii) *Fusarium* BC challenge, (iii) *Fusarium* non-BC challenge, and monocultures of both the (iv) BC candidate and (v) non-BC isolate. Error bars represent the standard error of the mean (± SEM).

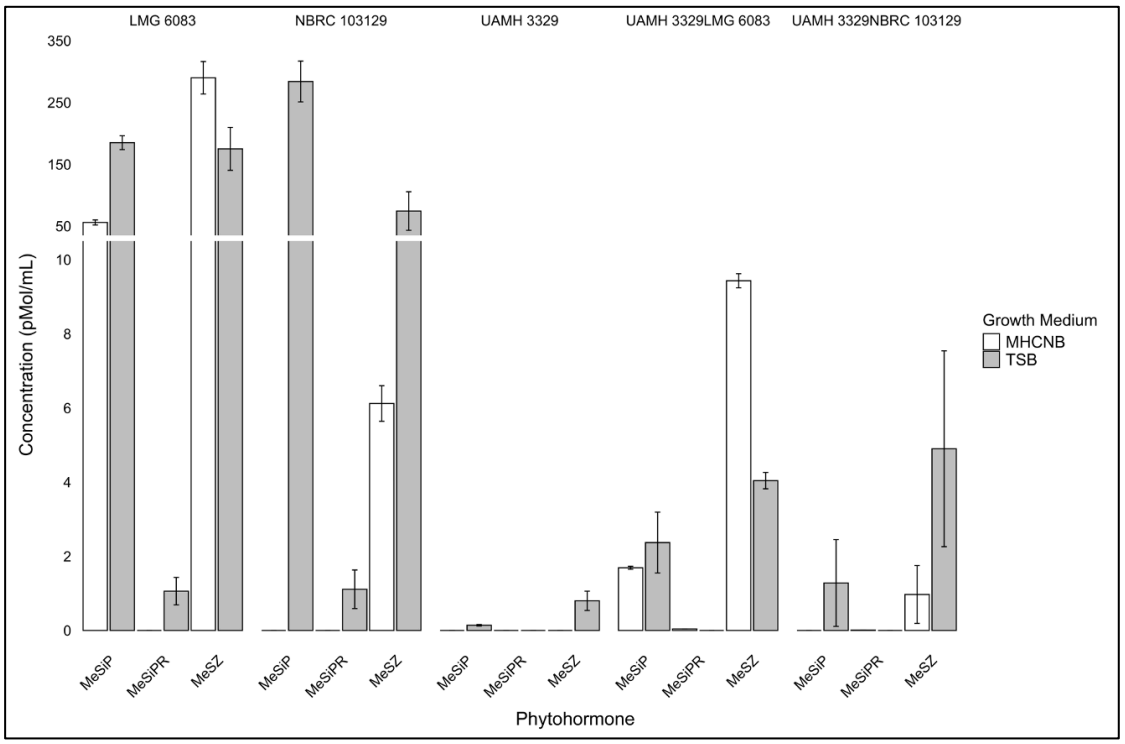

Figure 6.7: Average (n = 4) detectable methylthiolated freebase and riboside cytokinin forms in the broth of axenic cultures of *M. organophilum* (LMG 6083), *M. extorquens* (NBRC 103129), *F. graminearum* (UAMH 3329), and co-culture challenges in both tryptic soy broth (TSB) and modified Hoagland's complete nutrient broth (MHCNB), with error bars indicating the standard error of the mean (± SEM).

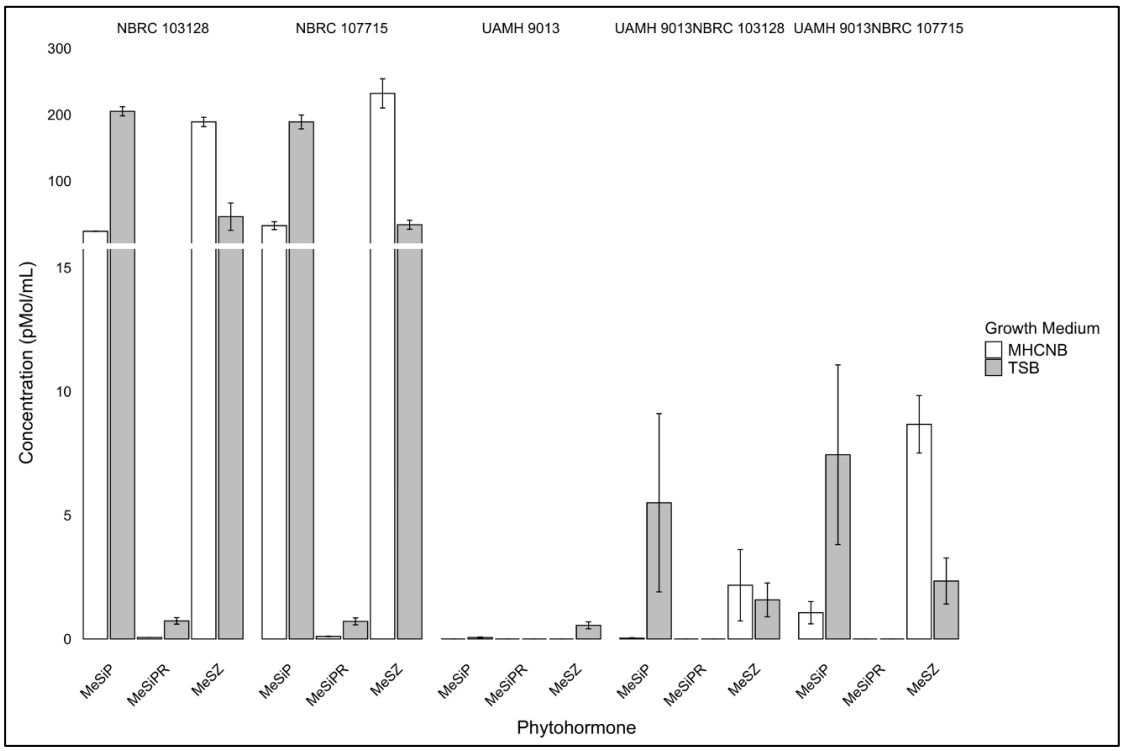

Figure 6.8: Average (n = 4) detectable methylthiolated freebase and riboside cytokinin forms in the broth of axenic cultures of *M. thiocyanatum* (NBRC 103128), *M. oxalidis* (NBRC 107715), *F. oxysporum* (UAMH 9013), and co-culture challenges in both tryptic soy broth (TSB) and modified Hoagland's complete nutrient broth (MHCNB), with error bars indicating the standard error of the mean (± SEM).

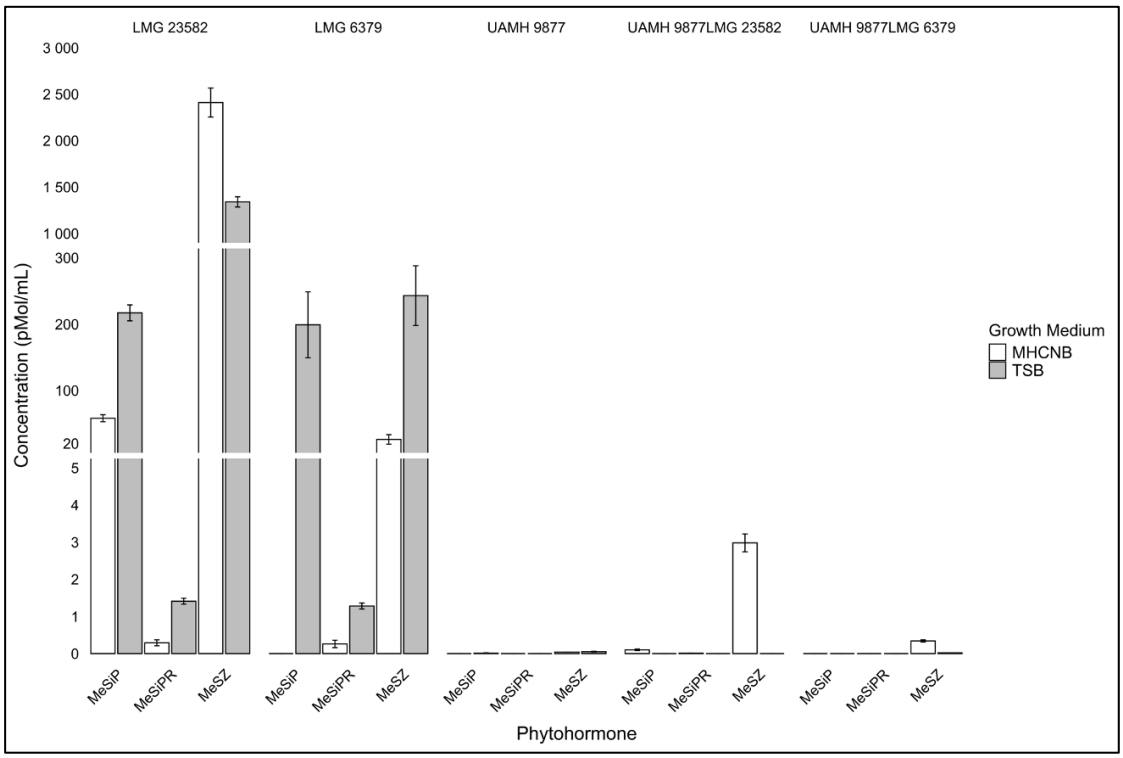

Figure 6.9: Average (n = 4) detectable methylthiolated freebase and riboside cytokinin forms in the broth of axenic cultures of *M. oryzae* (LMG 23582), *M. radiotolerans* (LMG 6379), *F. fujikuroi* (UAMH 9877), and co-culture challenges in both tryptic soy broth (TSB) and modified Hoagland's complete nutrient broth (MHCNB), with error bars indicating the standard error of the mean (± SEM).

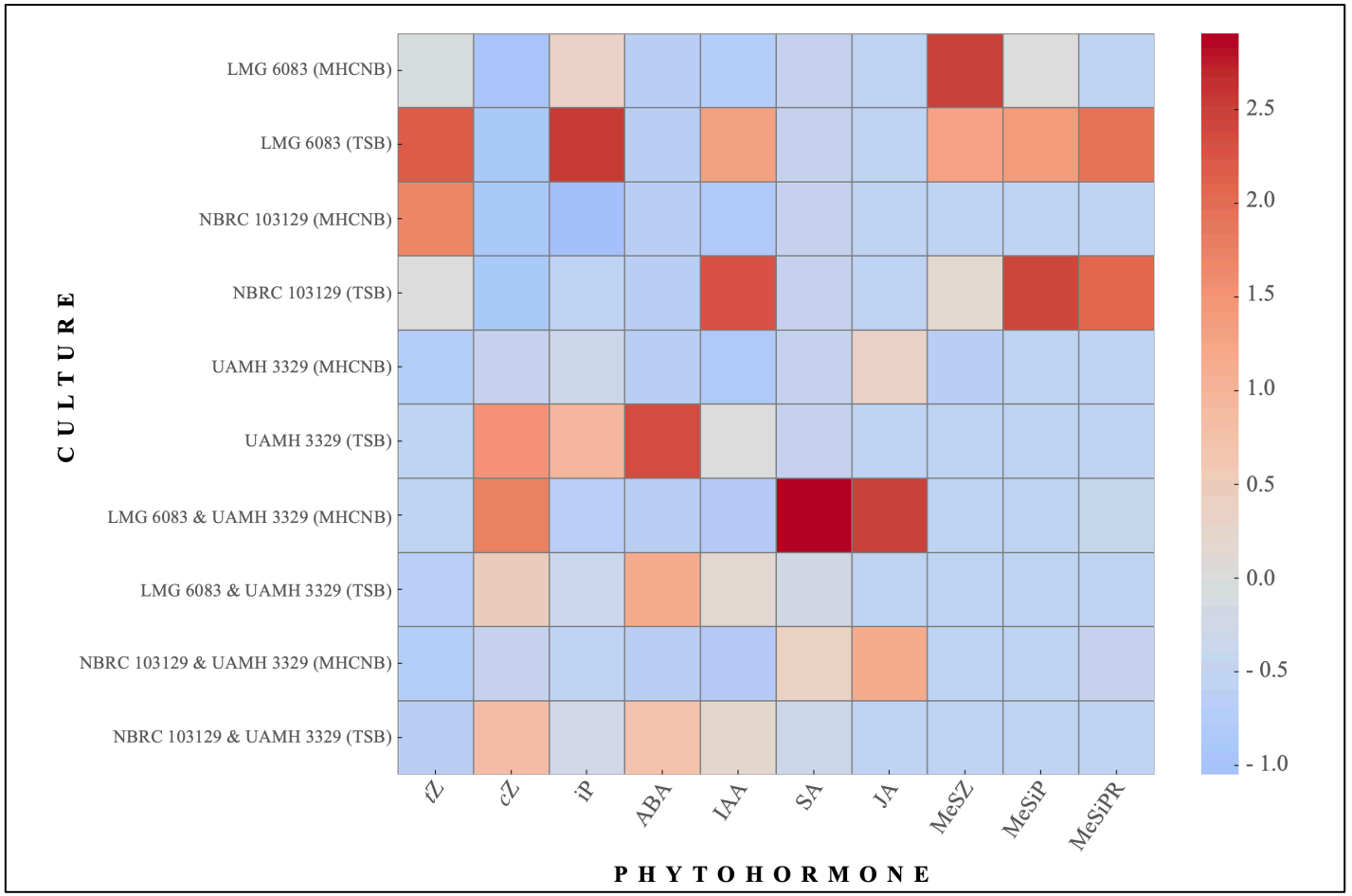

Figure 6.10. Z-score–normalized heatmap of average phytohormone production in filtered broth of BC candidate *M. organophilum* (LMG 6083), non-BC candidate *M. extorquens* (NBRC 103129), and phytopathogen *F. graminearum* (UAMH 3329) in both axenic and co-cultures. Mean concentrations ($n = 4$) were extracted for each culture–medium combination and normalized independently per hormone to account for variability in absolute abundance and eliminate a high-abundance bias.

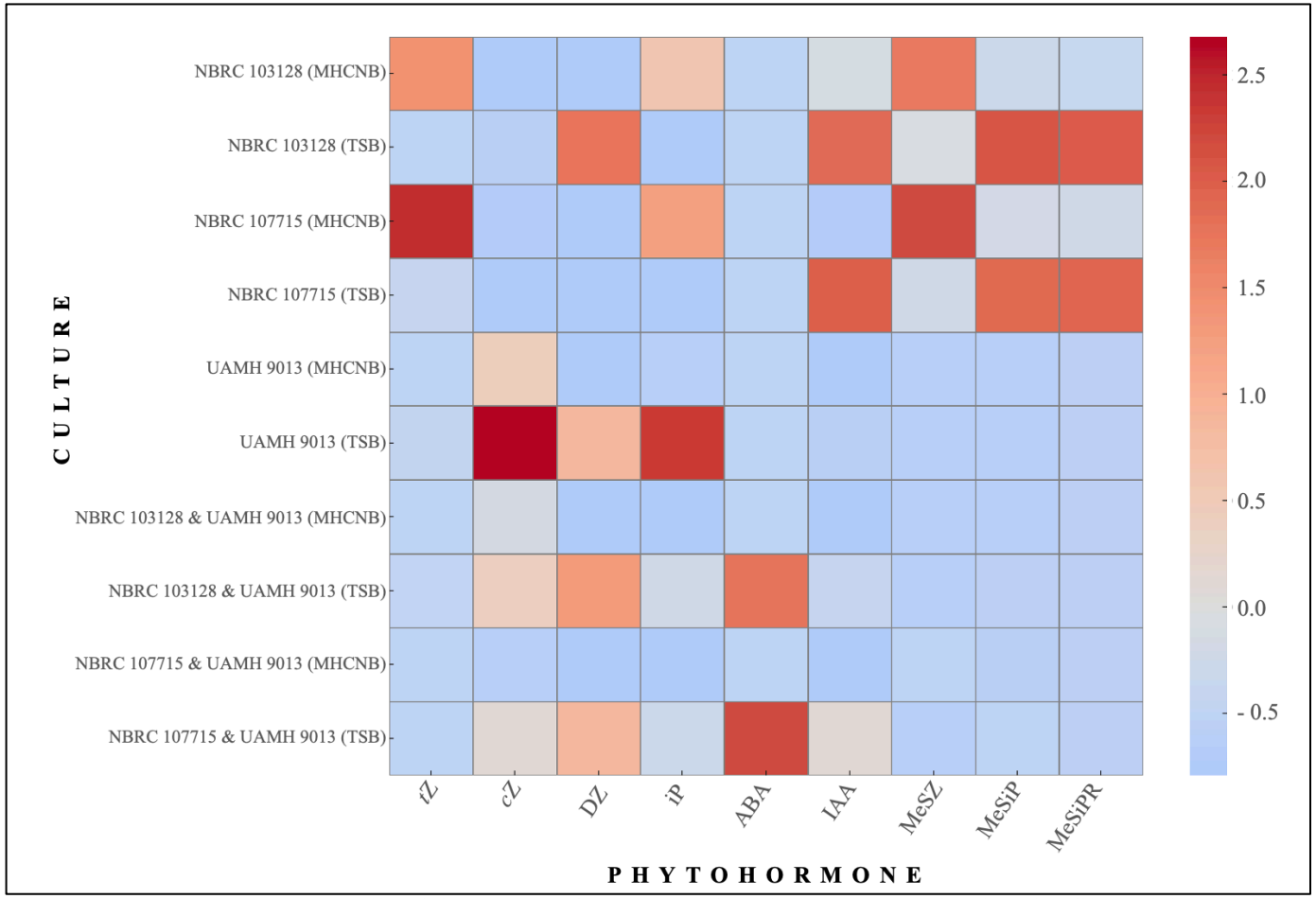

Figure 6.11. Z-score–normalized heatmap of average phytohormone production in filtered broth of BC candidate *M. thiocyanatum* (NBRC 103128), non-BC candidate *M. oxalidis* (NBRC 107715), and phytopathogen *F. oxysporum* f. sp. *cubense* (UAMH 9013) in both axenic and co-cultures. Mean concentrations ($n = 4$) were extracted for each culture–medium combination and normalized independently per hormone to account for variability in absolute abundance and eliminate a high-abundance bias.

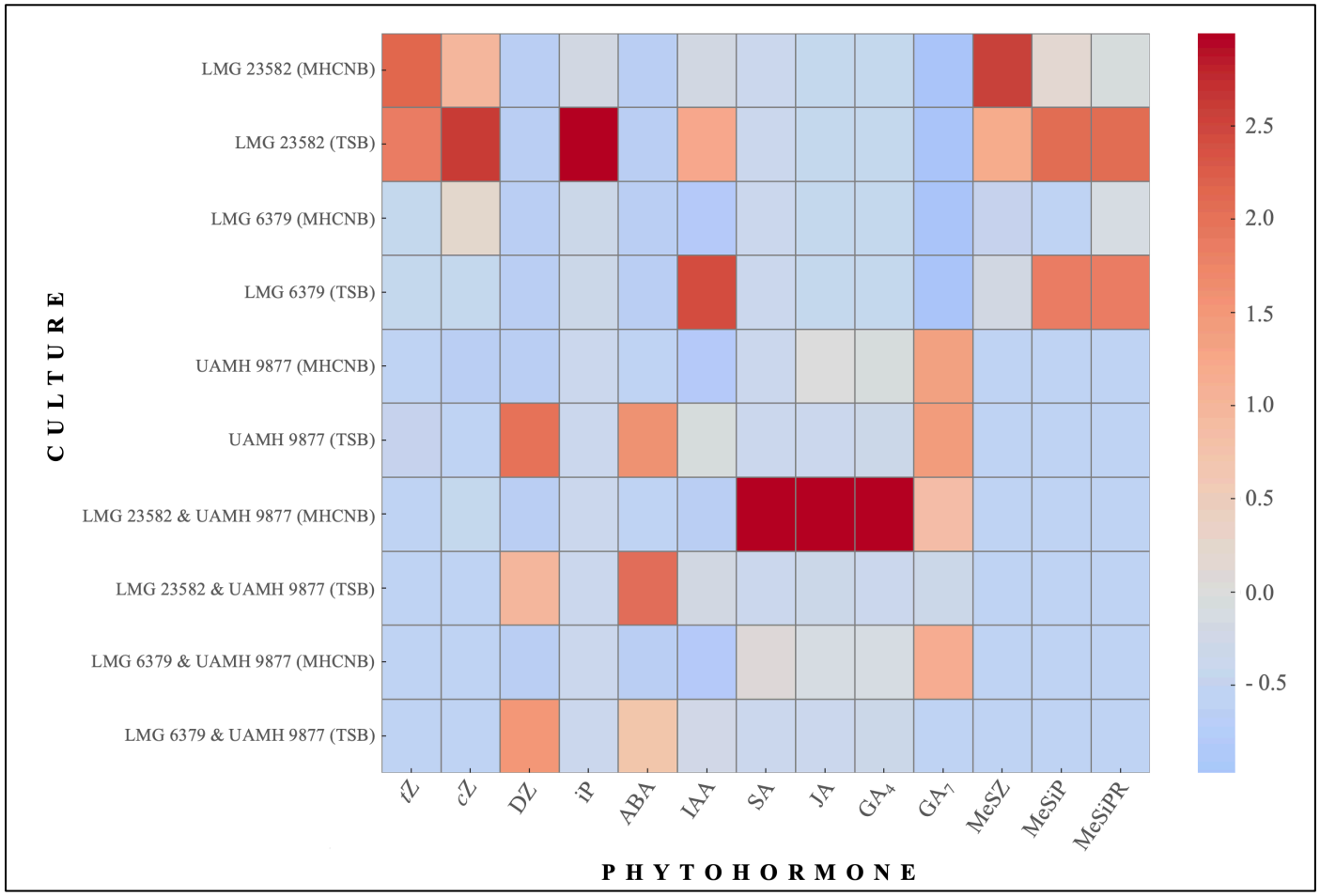

Figure 6.12. Z-score–normalized heatmap of average phytohormone production in filtered broth of BC candidate *M. oryzae* (LMG 23582), non-BC candidate *M. radiotolerans* (LMG 6379), and phytopathogen *F. fujikuroi* (UAMH 9877) in both axenic and co-cultures. Mean concentrations ($n = 4$) were extracted for each culture–medium combination and normalized independently per hormone to account for variability in absolute abundance and eliminate a high-abundance bias.

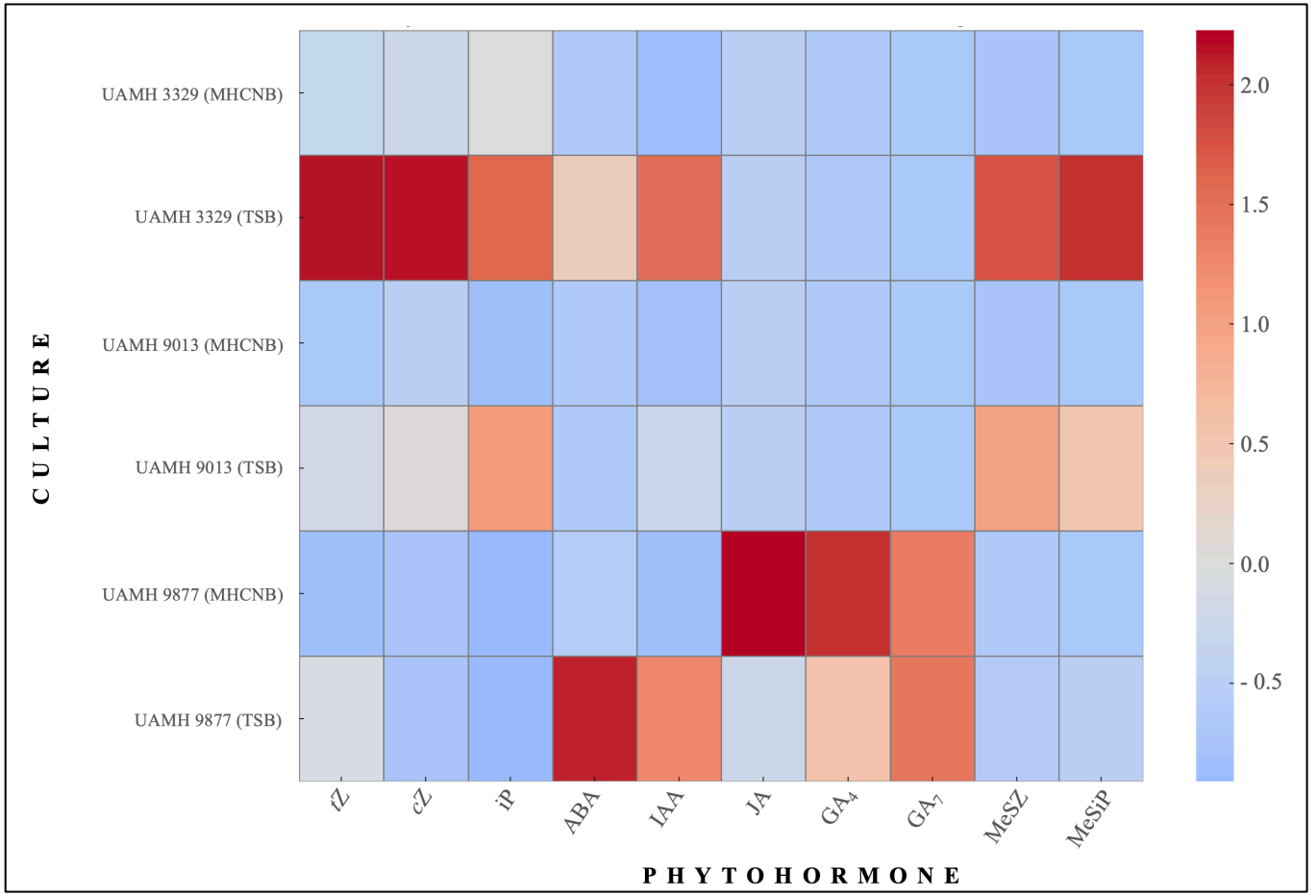

Figure 6.13. Z-score–normalized heatmap of phytohormone concentrations (pmol/mL) in filtered broth from axenic cultures of three fungal species: *F. graminearum* (UAMH 3329), *F. oxysporum* (UAMH 9013), and *F. fujikuroi* (UAMH 9877), reared in tryptic soy broth (TSB) or modified Hoagland's complete nutrient broth (MHCNB). Mean values (n = 4) for each hormone were normalized independently using z-score transformation to eliminate absolute abundance bias. Hormones with no detectable values across conditions (SA, MeSiPR) or limited representation (DZ) were excluded.

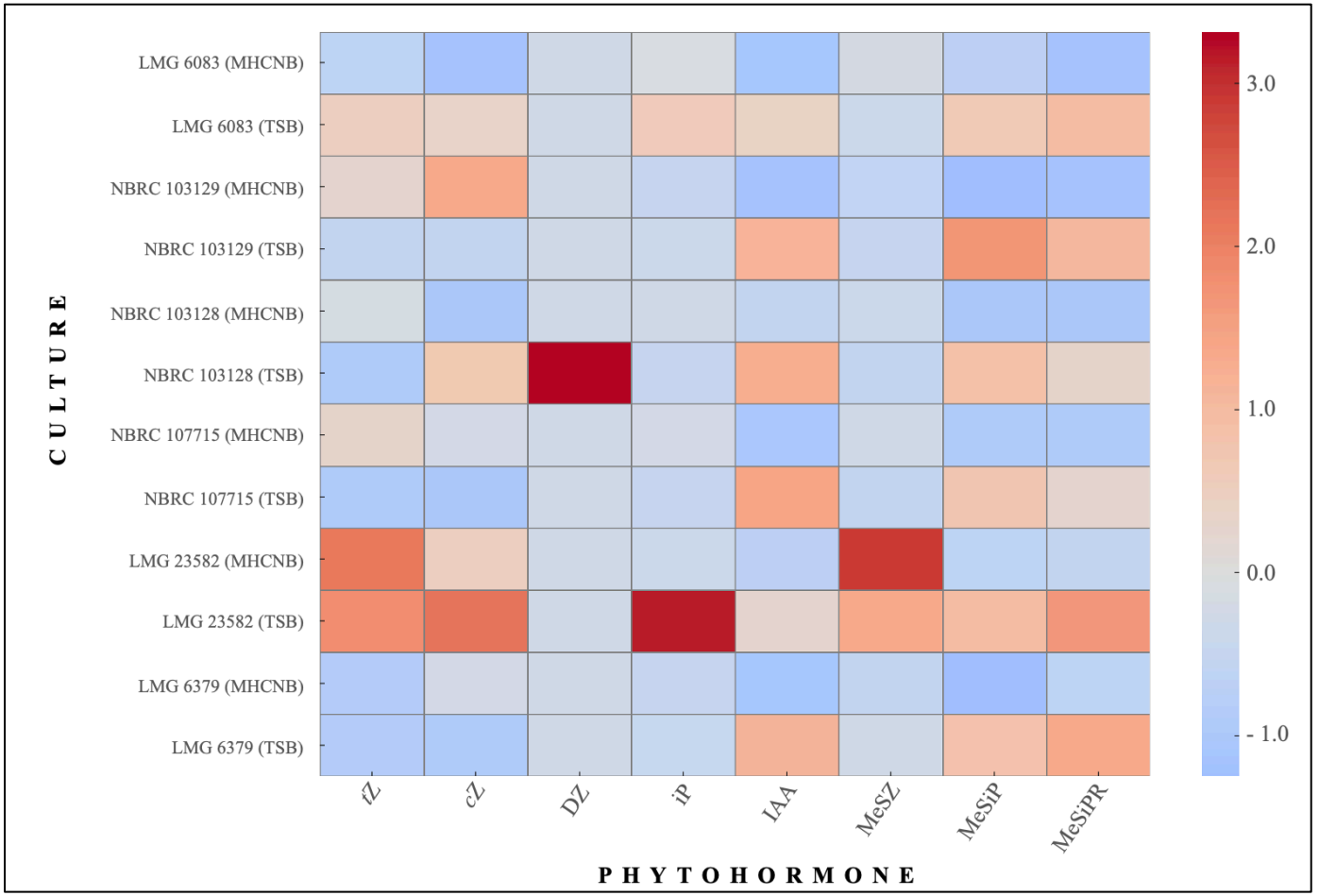

Figure 6.14. Z-score–normalized heatmap of phytohormone concentrations (pmol/mL) in filtered broth from axenic cultures of six *Methylobacterium* isolates grown in tryptic soy broth (TSB) or modified Hoagland's complete nutrient broth (MHCNB); including BC candidate strains (LMG 6083, NBRC 103128, LMG 23582) and non-BC isolates (NBRC 103129, NBRC 107716, LMG 6379). Mean hormone concentrations (n = 4) were independently normalized using z-score transformation to eliminate absolute abundance bias. The heatmap reveals media-dependent and strain-specific variation in hormone production, with specific compounds (iP, MeSZ) particularly enriched in BC candidates.

Table 6.1. Inventory of *Methylobaterium* strains examined for biocontrol of *Fusarium*.

Species	Strain	Isolation Source
M. extorquens	NBRC 103129	Soil-litter close to *Eucalyptus* sp.
M. oxalidis	NBRC 107715 (*T*)	*Oxalis corniculata* - phyllosphere
M. organophilum	LMG 6083 (*T*)	Lake water, lake sediment
M. oryzae	LMG 23582 (*T*)	*Oryzae sativa* cv Nam-Pyeoung
M. radiotolerans	LMG 6379	Forest soil
M. thiocyanatum	NBRC 103128	Soil-litter close to *Mesenbryanthemum* sp.

(T) Indicates "type strain" cultures that were descended from a strain designated as the nomenclatural type.

Table 6.2. Inventory of *Fusarium* spp. isolates.

Species	Strain	Isolation Source
F. graminearum	UAMH 3329	Undocumented.
F. oxysporum f. sp. cubense	UAMH 9013	Rhizome of banana plant (*Musae* sp.) showing symptoms of Panama disease. Jamaica, 1997.
F. fujikuroi	UAMH 9877	Infected sugar cane (*Saccharum officinarum*). Taiwan, 2000.

Table 6.3. Catalogue of *Methylobacterium* and *Fusarium* challenges.

Fungus	**Bacteria**	**Biocontrol Candidacy**
F. graminearum	*M. organophilum*	Candidate
	M. extorquens	Ineffective
F. oxysporum f. sp. cubense	*M. thiocyanatum*	Candidate
	M. oxalidis	Ineffective
F. fujikuori	*M. oryzae*	Candidate
	M. radiotolerans	Ineffective

6.9. SUPPLEMENTARY MATERIALS

Table S6.1. Phytohormones, included in the HPLC-MS/MS method, including their corresponding isotoptically labelled form or analogue.

Analyte	Abbreviation	Corresponding Labeled Analyte
trans-zeatin-7-glucoside	*t*Z7G	*t*Z7G-d_5
trans-zeatin-O-glucoside	*t*ZOG	*t*ZOG-d_5
trans-zeatin	*t*Z	*t*Z-d_5
dihydrozeatin	DZ	DZ-d_3
cis-zeatin-O-glucoside	*c*ZOG	*t*ZOG-d_5
dihydrozeatin-O-glucoside	DZOG	DZOG-d_7
trans-zeatin-9-glucoside	*t*Z9G	*t*Z9G-d_5
dihydrozeatin-9-glucoside	DZ9G	DZ9G-d_3
cis-zeatin	*c*Z	*t*Z-d_5
cis-zeatin-9-glucoside	*c*Z9G	*t*Z9G-d_5
isopentenyladenine-7-glucoside	iP7G	iP7G-d_6
trans-zeatin-O-glucoside riboside	*t*ZROG	*t*ZROG-d_5
kinetin	KIN	KIN-$^{15}N_4$
dihydrozeatin-O-glucoside riboside	DZROG	DZROG-d_7
cis-zeatin-O-glucoside riboside	*c*ZROG	*t*ZROG-d_5
dihydrozeatin riboside	DZR	DZR-d_3
trans-zeatin-riboside	*t*ZR	*t*ZR-d_5
cis-zeatin-riboside	*c*ZR	*t*ZR-d_5
isopentenyladenine	iP	iP-d_6
isopentenyladenine-9-glucoside	iP9G	iP7G-d_6
ortho-topolin oT	oT	oT-$^{13}C_5$
benzyladenine	BA	BA-d_7
isopentenyladenosine	iPR	iPR-d_6
2-methylthio-*trans/cis*-zeatin	MeSZ	MeSZ-d_5
2-methylthio-*trans/cis*-zeatin riboside	MeSZR	MeSZR-d_5
6-benzylaminopurine riboside	BAR	BAR-d_7
2-methylthio-6-isopentyladenosine	MeSiPR	MeSiPR-d_6
2-methylthio-6-isopentyladenine	MeSiP	MeSiP-d_6
Abscisic acid	ABA	ABA-d_6
Salicylic acid	SA	SA-d_6
Jasmonic acid	JA	ABA-d_6
Indole-3-acetic acid	IAA	IAA-d_5
Gibberellin 1	GA_1	GA_1-d_2
Gibberellin 3	GA_3	GA_3-d_2
Gibberellin 4	GA_4	GA_4-d_2
Gibberellin 7	GA_7	GA_7-d_2
Gibberellin 9	GA_9	GA_7-d_2
Gibberellin 20	GA_{20}	GA_7-d_2

Table S6.2. Concentrations of freebase phytohormones detected in culture broth of *F. graminearum* (UAMH 3329), *M. organophilum* (LMG 6083), and *M. extorquens* (NBRC 103129) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (pmol/mL)			
		trans-Zeatin (*tZ*)	*cis*-Zeatin (*cZ*)	Dihydrozeatin (DZ)	Isopentenyl adenine (iP)
LMG 6083	MHCNB	63.6 ± 8.4^{a}	n.d.	n.d.	37.2 ± 1.9^{a}
	TSB	276.0 ± 52.8^{a}	9.08 ± 1.5^{a}	n.d.	96.2 ± 7.1^{a}
NBRC 103129	MHCNB	228.0 ± 46.6^{b}	15.2 ± 6.4^{a}	n.d.	n.d.
	TSB	71.6 ± 15.0^{b}	3.4 ± 0.6^{a}	n.d.	13.2 ± 8.2^{b}
UAMH 3329	MHCNB	2.7 ± 0.1^{c}	140.0 ± 16.2^{b}	n.d.	19.5 ± 1.0^{b}
	TSB	15.9 ± 4.5^{b}	760.0 ± 23.9^{b}	n.d.	53.8 ± 13.2^{a}
UAMH 3329 and LMG 6083	MHCNB	14.1 ± 0.6^{a}	836.0 ± 94.2^{c}	n.d.	10.0 ± 0.7^{c}
	TSB	7.0 ± 2.6^{b}	441.0 ± 14.6^{a}	n.d.	17.8 ± 8.3^{b}
UAMH 3329 and NBRC 103129	MHCNB	3.1 ± 0.1^{c}	150.0 ± 0.8^{b}	n.d.	12.1 ± 5.3^{c}
	TSB	10.4 ± 7.6^{b}	547.0 ± 41.0^{a}	n.d.	21.3 ± 2.0^{b}

Table S6.3. Concentrations of freebase phytohormones detected in culture broth of *F. oxysporum* f. sp. *cubense* (UAMH 9013), *M. thiocyanatum* (NBRC 103128), and *M. oxalidis* (NBRC 107715) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (pmol/mL)			
		trans-Zeatin (*tZ*)	*cis*-Zeatin (*cZ*)	Dihydrozeatin (DZ)	Isopentenyl adenine (iP)
NBRC 103128	MHCNB	158.0 ± 19.9^{a}	0.9 ± 0.06^{a}	n.d.	18.7 ± 0.51^{a}
	TSB	0.03 ± 0^{a}	11.2 ± 1.94^{a}	0.7 ± 0.67^{a}	n.d.
NBRC 107715	MHCNB	241.0 ± 96.2^{a}	5.46 ± 2.5^{a}	n.d.	27.2 ± 1.59^{b}
	TSB	11.2 ± 3.4^{a}	0.93 ± 0.81^{a}	n.d.	n.d.
UAMH 9013	MHCNB	0.8 ± 0.07^{b}	71.8 ± 18^{b}	n.d.	1.6 ± 0.28^{c}
	TSB	3.41 ± 0.27^{b}	207 ± 14.9^{b}	0.45 ± 0.17^{a}	42.2 ± 18.1^{a}
UAMH 9013 and NBRC 103128	MHCNB	0.43 ± 0.28^{b}	38 ± 2.55^{c}	n.d.	0.47 ± 0.22^{c}
	TSB	2.67 ± 1.16^{b}	71.4 ± 7.2^{c}	0.58 ± 0.05^{b}	7.25 ± 3.13^{b}
UAMH 9013 and NBRC 107715	MHCNB	0.71 ± 0.36^{b}	8.33 ± 0.45^{a}	n.d.	0.47 ± 0.19^{c}
	TSB	1.32 ± 0.37^{b}	53.8 ± 16.9^{c}	0.45 ± 0.4^{a}	6.46 ± 3.0^{b}

Table S6.4. Concentrations of freebase phytohormones detected in culture broth of *F. fujikuroi* (UAMH 9877), *M. oryzae* (LMG 23582), and *M. radiotolerans* (LMG 6379) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (pmol/mL)			
		trans-Zeatin (*tZ*)	*cis*-Zeatin (*cZ*)	Dihydrozeatin (DZ)	Isopentenyl adenine (iP)
LMG 23582	MHCNB	588 ± 18.6^{a}	10.1 ± 4.64^{a}	n.d.	142.8 ± 2.0^{a}
	TSB	526 ± 24.4^{a}	20.3 ± 2.19^{a}	n.d.	321 ± 27.1^{a}
LMG 6379	MHCNB	16.2 ± 7.3^{b}	5.52 ± 5.52^{b}	n.d.	1.85 ± 1.85^{b}
	TSB	19.7 ± 1.4^{b}	1.45 ± 0.6^{b}	n.d.	4.42 ± 0.77^{b}
UAMH 9877	MHCNB	0.09 ± 0.03^{c}	0.05 ± 0.02^{c}	n.d.	n.d.
	TSB	4.01 ± 2.24^{c}	10.9 ± 2.0^{c}	$1.020.34^{a}$	0.03 ± 0.06^{c}
UAMH 9877 and LMG 23582	MHCNB	0.95 ± 0.16^{d}	1.58 ± 0.48^{d}	n.d.	n.d.
	TSB	0.55 ± 0.51^{c}	0.52 ± 0.12^{d}	$0.640.11^{b}$	0.18 ± 0.02^{d}
UAMH 9877 and LMG 6379	MHCNB	0.18 ± 0.08^{d}	0.65 ± 0.11^{e}	n.d.	n.d.
	TSB	1.19 ± 0.93^{c}	0.32 ± 0.08^{d}	0.83 ± 0.47^{a}	0.2 ± 0.17^{d}

Table S6.5. Concentrations of acidic phytohormones detected in culture broth of *F. graminearum* (UAMH 3329), *M. organophilum* (LMG 6083), and *M. extorquens* (NBRC 103129) axenic and co-cultures grown in MHCNB and TSB media. Values (nmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (nmol/mL)				
		IAA	*SA*	JA	GA_4	GA_7
LMG 6083	MHCNB	0.65 ± 0.08^{a}	n.d.	n.d.	n.d.	n.d.
	TSB	43.6 ± 8.69^{a}	n.d.	n.d.	n.d.	n.d.
NBRC 103129	MHCNB	n.d.	n.d.	n.d.	n.d.	n.d.
	TSB	64.4 ± 14.1^{a}	n.d.	n.d.	n.d.	n.d.
UAMH 3329	MHCNB	0.13 ± 0.01^{b}	n.d.	15.9 ± 6.3^{a}	n.d.	n.d.
	TSB	17.2 ± 1.9^{b}	n.d.	n.d.	n.d.	n.d.
UAMH 3329 and LMG 6083	MHCNB	0.36 ± 0.01^{a}	420.0 ± 25.7^{a}	54.3 ± 3.2^{a}	n.d.	n.d.
	TSB	19.2 ± 2.3^{b}	35.0 ± 0.5^{a}	n.d.	n.d.	n.d.
UAMH 3329 and NBRC 103129	MHCNB	0.39 ± 0.01^{a}	104.0 ± 5.5^{b}	299.0 ± 12.4^{b}	n.d.	n.d.
	TSB	21.3 ± 4.8^{b}	17.8 ± 1.8^{b}	n.d.	n.d.	n.d.

Table S6.6. Concentrations of acidic phytohormones detected in culture broth of of *F. oxysporum* f. sp. *cubense* (UAMH 9013), *M. thiocyanatum* (NBRC 103128), and *M. oxalidis* (NBRC 107715) axenic and co-cultures grown in MHCNB and TSB media. Values (nmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (nmol/mL)				
		IAA	*SA*	JA	GA_4	GA_7
NBRC 103128	MHCNB	16.6 ± 0.6^{a}	n.d.	n.d.	n.d.	n.d.
	TSB	68.0 ± 2.0^{a}	n.d.	n.d.	n.d.	n.d.
NBRC 107715	MHCNB	2.4 ± 0.07^{b}	n.d.	n.d.	n.d.	n.d.
	TSB	71.4 ± 11.5^{a}	n.d.	n.d.	n.d.	n.d.
UAMH 9013	MHCNB	0.50 ± 0.03^{c}	n.d.	n.d.	n.d.	n.d.
	TSB	4.40 ± 0.24^{b}	n.d.	n.d.	n.d.	n.d.
UAMH 9013 and NBRC 103128	MHCNB	0.54 ± 0.01^{c}	n.d.	n.d.	n.d.	n.d.
	TSB	9.37 ± 1.10^{b}	n.d.	n.d.	n.d.	n.d.
UAMH 9013 and NBRC 107715	MHCNB	0.85 ± 0.01^{c}	n.d.	n.d.	n.d.	n.d.
	TSB	23.4 ± 2.91^{c}	n.d.	n.d.	n.d.	n.d.

Table S6.7. Concentrations of acidic phytohormones detected in culture broth of *F. fujikuroi* (UAMH 9877), *M. oryzae* (LMG 23582), and *M. radiotolerans* (LMG 6379) axenic and co-cultures grown in MHCNB and TSB media. Values (nmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (nmol/mL)				
		IAA	*SA*	JA	GA_4	GA_7
LMG 23582	MHCNB	12.20 ± 0.45^{a}	n.d.	n.d.	n.d.	n.d.
	TSB	40.00 ± 3.05^{a}	n.d.	n.d.	n.d.	n.d.
LMG 6379	MHCNB	0.42 ± 0.12^{b}	n.d.	n.d.	n.d.	n.d.
	TSB	63.90 ± 4.10^{a}	n.d.	n.d.	n.d.	n.d.
UAMH 9877	MHCNB	0.31 ± 0.07^{b}	n.d.	$1{,}700.0 \pm 270.0^{a}$	2.50 ± 0.08^{a}	0.05 ± 0.003^{a}
	TSB	15.40 ± 2.35^{b}	n.d.	151.0 ± 20.4^{a}	1.15 ± 0.05^{a}	0.059 ± 0.004^{a}
UAMH 9877 and LMG 23582	MHCNB	3.00 ± 0.74^{c}	137.0 ± 10.40^{a}	$12{,}900.0 \pm 2{,}160.0^{b}$	21.9 ± 0.56^{b}	0.045 ± 0.022^{a}
	TSB	12.20 ± 0.89^{b}	n.d.	331.0 ± 30.2^{b}	0.59 ± 0.06^{b}	0.015 ± 0.002^{a}
UAMH 9877 and LMG 6379	MHCNB	0.34 ± 0.03^{b}	17.80 ± 2.55^{b}	$1{,}060.0 \pm 183.0^{a}$	2.11 ± 0.08^{a}	0.052 ± 0.005^{a}
	TSB	11.20 ± 1.55^{b}	n.d.	221.0 ± 29.5^{a}	0.95 ± 0.22^{a}	0.011 ± 0.003^{a}

Table S6.8. Concentrations of methylthiolated phytohormones detected in culture broth of *F. graminearum* (UAMH 3329), *M. organophilum* (LMG 6083), and *M. extorquens* (NBRC 103129) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (nmol/mL)		
		MeSZ	*MeSiP*	*MeSiPR*
LMG 6083	MHCNB	290.00 ± 26.30[a]	55.90 ± 4.10[a]	n.d.
	TSB	175.00 ± 34.70[a]	185.00 ± 11.20[a]	1.06 ± 0.37[a]
NBRC 103129	MHCNB	6.12 ± 0.48[b]	n.d.	n.d.
	TSB	74.40 ± 31.20[b]	284.00 ± 33.0[b]	1.11 ± 0.52[a]
UAMH 3329	MHCNB	n.d.	n.d.	n.d.
	TSB	0.80 ± 0.26[c]	0.14 ± 0.02[c]	n.d.
UAMH 3329 and LMG 6083	MHCNB	9.43 ± 0.19[b]	1.69 ± 0.04[b]	0.04 ± 0.00[a]
	TSB	4.04 ± 0.22[c]	2.37 ± 0.82[c]	n.d.
UAMH 3329 and NBRC 103129	MHCNB	0.97 ± 0.78[b]	n.d.	0.01 ± 0.00[a]
	TSB	4.90 ± 2.64[c]	1.28 ± 01.17[c]	n.d.

Table S6.9. Concentrations of methylthiolated phytohormones detected in culture broth of of *F. oxysporum* f. sp. *cubense* (UAMH 9013), *M. thiocyanatum* (NBRC 103128), and *M. oxalidis* (NBRC 107715) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (nmol/mL)		
		MeSZ	*MeSiP*	*MeSiPR*
NBRC 103128	MHCNB	189.00 ± 7.04[a]	24.10 ± 0.24[a]	0.06 ± 0.00[a]
	TSB	46.20 ± 20.60[a]	205.00 ± 6.87[a]	0.73 ± 0.13[a]
NBRC 107715	MHCNB	232.00 ± 22.0[b]	32.60 ± 5.89[b]	0.10 ± 0.01[a]
	TSB	34.00 ± 6.80[a]	189.00 ± 10.50[a]	0.71 ± 0.14[a]
UAMH 9013	MHCNB	n.d.	n.d.	n.d.
	TSB	0.55 ± 0.14[b]	0.06 ± 0.02[b]	n.d.
UAMH 9013 and NBRC 103128	MHCNB	2.17 ± 1.44[c]	0.03 ± 0.02[c]	n.d.
	TSB	1.58 ± 0.68[b]	5.50 ± 3.60[b]	n.d.
UAMH 9013 and NBRC 107715	MHCNB	8.67 ± 1.16[c]	1.06 ± 0.45[c]	n.d.
	TSB	2.34 ± 0.93[b]	7.44 ± 3.63[b]	n.d.

Table S6.10. Concentrations of methylthiolated phytohormones detected in culture broth of *F. fujikuroi* (UAMH 9877), *M. oryzae* (LMG 23582), and *M. radiotolerans* (LMG 6379) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/mL, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable. Letters indicate statistically significant differences between treatments as determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Comparisons were made within each hormone group and media type only: treatments sharing a letter are not significantly different.

Culture	Medium	Phytohormone Concentration (nmol/mL)		
		MeSZ	*MeSiP*	*MeSiPR*
LMG 23582	MHCNB	2,410.00 ± 156.0[a]	58.10 ± 5.29[a]	0.29 ± 0.08[a]
	TSB	1,340.00 ± 55.0[a]	217.00 ± 12.0[a]	1.41 ± 0.08[a]
LMG 6379	MHCNB	26.20 ± 7.16[b]	n.d.	0.26 ± 0.10[a]
	TSB	243.00 ± 45.0[b]	199.00 ± 49.70[a]	1.28 ± 0.08[a]
UAMH 9877	MHCNB	0.04 ± 0.01[c]	n.d.	n.d.
	TSB	0.05 ± 0.01[c]	0.01 ± 0.01[b]	n.d.
UAMH 9877 and LMG 23582	MHCNB	2.98 ± 0.24[d]	0.10 ± 0.02[b]	0.01 ± 0.00[b]
	TSB	n.d.	n.d.	n.d.
UAMH 9877 and LMG 6379	MHCNB	0.34 ± 0.03[e]	n.d.	n.d.
	TSB	0.02 ± 0.00[c]	n.d.	n.d.

Table S6.11. Concentrations of freebase phytohormones detected in harvested biomass of *F. graminearum* (UAMH 3329), *M. organophilum* (LMG 6083), and *M. extorquens* (NBRC 103129) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/gDW, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable.

		Phytohormone Concentration (pmol/mgDW)			
Culture	Medium	*trans*-Zeatin (*tZ*)	*cis*-Zeatin (*cZ*)	Dihydrozeatin (DZ)	Isopentenyl adenine (iP)
LMG 6083	MHCNB	27.07 ± 4.7	24.57 ± 7.30	n.d.	n.d.
	TSB	76.51 ± 27.78	n.d.	n.d.	n.d.
NBRC 103129	MHCNB	n.d.	14.11 ± 8.72	n.d.	n.d.
	TSB	154.06 ± 71.33	17.60 ± 6.31	n.d.	8.43 ± 4.41
UAMH 3329	MHCNB	16.97 ± 2.16	14.85 ± 1.01	n.d.	n.d.
	TSB	27.39 ± 7.00	1,180.37 ± 22.94	n.d.	226.49 ± 27.21
UAMH 3329 and LMG 6083	MHCNB	18.42 ± 5.43	81.46 ± 16.85	n.d.	8.08 ± 0.88
	TSB	7.52 ± 2.08	716.70 ± 144.61	n.d.	47.52 ± 9.14
UAMH 3329 and NBRC 103129	MHCNB	12.02 ± 4.77	96.65 ± 7.87	n.d.	n.d.
	TSB	16.23 ± 12.47	811.37 ± 32.96	n.d.	70.96 ± 4.25

Table S6.12. Concentrations of freebase phytohormones detected in harvested biomass of of *F. oxysporum* f. sp. *cubense* (UAMH 9013), *M. thiocyanatum* (NBRC 103128), and *M. oxalidis* (NBRC 107715) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/gDW, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable.

		Phytohormone Concentration (pmol/mgDW)			
Culture	Medium	*trans*-Zeatin (*tZ*)	*cis*-Zeatin (*cZ*)	Dihydrozeatin (DZ)	Isopentenyl adenine (iP)
NBRC 103128	MHCNB	50.13 ± 30.70	31.85 ± 9.06	n.d.	n.d.
	TSB	443.07 ± 135.88	32.72 ± 4.32	n.d.	n.d.
NBRC 107715	MHCNB	15.88 ± 0.25	15.46 ± 0.41	n.d.	n.d.
	TSB	168.69 ± 37.86	n.d.	n.d.	2.16 ± 1.22
UAMH 9013	MHCNB	7.58 ± 0.84	7.22 ± 0.94	n.d.	8.81 ± 0.44
	TSB	11.48 ± 1.11	n.d.	n.d.	14.32 ± 1.44
UAMH 9013 and NBRC 103128	MHCNB	45.34 ± 13.14	14.91 ± 0.26	n.d.	16.09 ± 0.69
	TSB	13.30 ± 3.00	10.00 ± 2.39	n.d.	36.79 ± 2.58
UAMH 9013 and NBRC 107715	MHCNB	6.14 ± 2.57	12.11 ± 0.57	n.d.	n.d.
	TSB	23.85 ± 6.54	47.56 ± 6.81	n.d.	20.16 ± 7.29

Table S6.13. Concentrations of freebase phytohormones detected in harvested biomass of *F. fujikuroi* (UAMH 9877), *M. oryzae* (LMG 23582), and *M. radiotolerans* (LMG 6379) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/gDW, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable.

		Phytohormone Concentration (pmol/mgDW)			
Culture	Medium	*trans*-Zeatin (*tZ*)	*cis*-Zeatin (*cZ*)	Dihydrozeatin (DZ)	Isopentenyl adenine (iP)
LMG 23582	MHCNB	14.28 ± 0.91	22.48 ± 6.70	n.d.	47.04 ± 1.32
	TSB	74.78 ± 39.66	6.65 ± 0.22	n.d.	31.26 ± 16.31
LMG 6379	MHCNB	34.57 ± 3.87	58.93 ± 13.49	n.d.	41.61 ± 14.90
	TSB	1,500.56 ± 242.68	174.49 ± 18.53	n.d.	n.d.
UAMH 9877	MHCNB	13.04 ± 6.40	26.85 ± 1.48	n.d.	5.57 ± 0.95
	TSB	61.33 ± 33.55	440.88 ± 21.74	n.d.	40.85 ± 1.50
UAMH 9877 and LMG 23582	MHCNB	20.84 ± 5.14	25.79 ± 8.55	n.d.	17.42 ± 3.95
	TSB	8.69 ± 3.98	16.12 ± 9.45	n.d.	166.32 ± 15.13
UAMH 9877 and LMG 6379	MHCNB	12.50 ± 1.51	12.50 ± 5.40	n.d.	4.10 ± 0.47
	TSB	52.70 ± 1.80	191.97 ± 38.42	n.d.	28.67 ± 5.32

Table S6.14. Concentrations of acidic phytohormones detected in harvested biomass of *F. graminearum* (UAMH 3329), *M. organophilum* (LMG 6083), and *M. extorquens* (NBRC 103129) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/gDW, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable.

Culture	Medium	Phytohormone Concentration (nmol/mgDW)		
		IAA	*SA*	JA
LMG 6083	MHCNB	668.50 ± 91.07	n.d.	n.d.
	TSB	5,984.62 ± 354.22	n.d.	n.d.
NBRC 103129	MHCNB	5,052.97 ± 334.78	n.d.	n.d.
	TSB	4,265.01 ± 439.11	n.d.	n.d.
UAMH 3329	MHCNB	1,242.26 ± 24.31	n.d.	n.d.
	TSB	23,320.69 ± 1,017.78	$8.20 \cdot 10^6 \pm 0.77 \cdot 10^6$	
UAMH 3329 and LMG 6083	MHCNB	7,831.26 ± 580.12	n.d.	n.d.
	TSB	27,989.55 ± 2,890.01	$8.20 \cdot 10^6 \pm 0.77 \cdot 10^6$	
UAMH 3329 and NBRC 103129	MHCNB	5,677.68 ± 276.62	n.d.	n.d.
	TSB	35,548.68 ± 13,927.92	$8.20 \cdot 10^6 \pm 0.77 \cdot 10^6$	

Table S6.15. Concentrations of acidic phytohormones detected in harvested biomass of of *F. oxysporum* f. sp. *cubense* (UAMH 9013), *M. thiocyanatum* (NBRC 103128), and *M. oxalidis* (NBRC 107715) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/gDW, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable.

Culture	Medium	Phytohormone Concentration (pmol/mgDW)			
		IAA	*SA*	JA	GA_4
NBRC 103128	MHCNB	794.94 ± 72.59	n.d.	n.d.	n.d.
	TSB	6,600.14 ± 822.39	n.d.	n.d.	n.d.
NBRC 107715	MHCNB	591.44 ± 100.81	n.d.	n.d.	n.d.
	TSB	7,625.00 ± 1,562.07	n.d.	n.d.	n.d.
UAMH 9013	MHCNB	2,438.34 ± 40.60	n.d.	n.d.	n.d.
	TSB	27,779.83 ± 1,782.69	n.d.	n.d.	n.d.
UAMH 9013 and NBRC 103128	MHCNB	7,054.48 ± 70.65	n.d.	n.d.	n.d.
	TSB	20,212.20 ± 5,545.09	n.d.	n.d.	n.d.
UAMH 9013 and NBRC 107715	MHCNB	7,883.02 ± 679.52	n.d.	n.d.	n.d.
	TSB	64,723.94 ± 2,034.80	n.d.	n.d.	n.d.

Table S6.16. Concentrations of acidic phytohormones detected in harvested biomass of *F. fujikuroi* (UAMH 9877), *M. oryzae* (LMG 23582), and *M. radiotolerans* (LMG 6379) axenic and co-cultures grown in MHCNB and TSB media. Values (pmol/gDW, mean ± standard deviation) represent net hormone concentrations with minimal background levels in base media formulations subtracted; "n.d." indicates non-detectable.

Culture	Medium	Phytohormone Concentration (nmol/mgDW)			
		IAA	*SA*	JA	GA_4
LMG 23582	MHCNB	$1.70 \cdot 10^3 \pm 0.16 \cdot 10^3$	n.d.	n.d.	n.d.
	TSB	$2.04 \cdot 10^3 \pm 0.20 \cdot 10^3$	n.d.	n.d.	n.d.
LMG 6379	MHCNB	$5.59 \cdot 10^3 \pm 1.07 \cdot 10^3$	n.d.	n.d.	n.d.
	TSB	$3.73 \cdot 10^3 \pm 0.22 \cdot 10^3$	n.d.	n.d.	n.d.
UAMH 9877	MHCNB	$2.38 \cdot 10^3 \pm 0.08 \cdot 10^3$	n.d.	$3.16 \cdot 10^7 \pm 0.28 \cdot 10^6$	$1.01 \cdot 10^6 \pm 0.22 \cdot 10^6$
	TSB	$6.56 \cdot 10^3 \pm 0.13 \cdot 10^3$	n.d.	$0.70 \cdot 10^6 \pm 0.29 \cdot 10^5$	$9.24 \cdot 10^3 \pm 4.35 \cdot 10^3$
UAMH 9877 and LMG 23582	MHCNB	$1.19 \cdot 10^4 \pm 0.05 \cdot 10^3$	n.d.	$1.36 \cdot 10^7 \pm 0.63 \cdot 10^6$	$1.79 \cdot 10^6 \pm 0.14 \cdot 10^6$
	TSB	$2.72 \cdot 10^4 \pm 0.81 \cdot 10^3$	n.d.	$2.60 \cdot 10^6 \pm 0.73 \cdot 10^5$	$1.07 \cdot 10^5 \pm 4.49 \cdot 10^3$
UAMH 9877 and LMG 6379	MHCNB	$3.76 \cdot 10^3 \pm 0.39 \cdot 10^3$	n.d.	$7.67 \cdot 10^6 \pm 0.87 \cdot 10^6$	$1.52 \cdot 10^6 \pm 0.07 \cdot 10^6$
	TSB	$3.16 \cdot 10^4 \pm 1.20 \cdot 10^3$	n.d.	$1.13 \cdot 10^6 \pm 0.77 \cdot 10^5$	$4.49 \cdot 10^5 \pm 3.59 \cdot 10^4$

6.10. REFERENCES

Akhtar SS, Mekureyaw MF, Pandey C, Roitsch T. 2020. Role of Cytokinins for Interactions of Plants With Microbial Pathogens and Pest Insects. Front Plant Sci. (10): 1777. https://doi.org/10.3389/fpls.2019.01777.

Ali, A., Shah, L., Rahman, S., Riaz, M. W., Yahya, M., Xu, Y. J., Liu, F., Si, W., Jiang, H., and Cheng, B. 2018. Plant defense mechanism and current understanding of salicylic acid and NPRs in activating SAR. Physiological and Molecular Plant Pathology 104:15–22. https://doi.org/10.1016/j.pmpp.2018.08.001.

Anand G, Gupta R, Marash I, Leibman-Markus M, Bar M. 2022. Cytokinin production and sensing in fungi. Microbiol Res. 262:127103. https://doi.org/10.1016/j.micres.2022.127103.

Aoki MM, Emery RJN, Anjard C, Brunetti CR, Huber RJ. 2020. Cytokinins in *Dictyostelia* - A Unique Model for Studying the Functions of Signaling Agents From Species to Kingdoms. Front Cell Dev Biol. 8:511. https://doi.org/10.3389/fcell.2020.00511.

Audenaert, K., Pattery, T., Cornelis, P., and Höfte, M. 2002. Induction of Systemic Resistance to Botrytis cinerea in Tomato by Pseudomonas aeruginosa 7NSK2: Role of Salicylic Acid, Pyochelin, and Pyocyanin. MPMI 15:1147–1156. https://doi.org/10.1094/MPMI.2002.15.11.1147.

Avalos, J., Cerda-Olmedo, E., Reyes, F., and Barrero, A. 2007. Gibberellins and Other Metabolites of Fusarium fujikuroi and Related Fungi. COC 11:721–737. https://doi.org/10.2174/138527207780598729.

Bashyal, B. M., Aggarwal, R., Sharma, S., Gupta, S., Rawat, K., Singh, D., Singh, A. K., and Gopala Krishnan, S. 2016. Occurrence, identification and pathogenicity of *Fusarium* species associated with bakanae disease of basmati rice in India. Eur J Plant Pathol 144:457–466. https://doi.org/10.1007/s10658-015-0783-8.

Beckerman, J., Palmer, C., Tedford, E., and Ypema, H. 2023. Fifty years of fungicide development, deployment, and future use. Phytopathology, 113(4), 694–706. https://doi.org/10.1094/PHYTO-10-22-0399-IA

Berger S, Van Wees SCM, Nybroe O, Großkinsky DK. 2020. Phytohormone Functions at the Plant-Microbe Interface and Beyond. Front Plant Sci. 11:386. https://doi.org/10.3389/fpls.2020.00386.

Beukes I, Rose LJ, Shephard GS, Flett BC, Viljoen A. 2017. Mycotoxigenic *Fusarium* species associated with grain crops in South Africa – A review. South African Journal of Science, 113(3/4), Art. #2016-0121. https://doi.org/10.17159/sajs.2017/20160121

Boba A, Kostyn K, Kozak B, Wojtasik W, Preisner M, Prescha A, Gola EM, Lysh D, Dudek B, Szopa J, Kulma A. 2020. *Fusarium oxysporum* infection activates the plastidial branch of the terpenoid biosynthesis pathway in flax, leading to

increased ABA synthesis. Planta. 251(2):50. doi: https://doi.org/10.1007/s00425-020-03339-9. PMID: 31950395.

Brodhun, F., and Feussner, I. 2011. Oxylipins in fungi. The FEBS Journal 278:1047–1063. https://doi.org/10.1111/j.1742-4658.2011.08027.x.

Buhrow, L. M., Cram, D., Tulpan, D., Foroud, N. A., and Loewen, M. C. 2016. Exogenous Abscisic Acid and Gibberellic Acid Elicit Opposing Effects on Fusarium graminearum Infection in Wheat. Phytopathology. 106:986–996. https://doi.org/10.1094/PHYTO-01-16-0033-R.

Cen, Y.-K., Lin, J.-G., Wang, Y.-L., Wang, J.-Y., Liu, Z.-Q., and Zheng, Y.-G. 2020. The Gibberellin Producer *Fusarium fujikuroi*: Methods and Technologies in the Current Toolkit. Front. Bioeng. Biotechnol. 8:232. https://doi.org/10.3389/fbioe.2020.00232.

Chanclud, E., and Morel, J.-B. 2016. Plant hormones: a fungal point of view. Mol. Plant Pathol. 17:1289–1297. https://doi.org/10.1111/mpp.12393

Corpe, W. A., and Rheem, S. 1989. Ecology of the methylotrophic bacteria on living leaf surfaces. FEMS Microbiology Letters 62:243–249. https://doi.org/10.1016/0378-1097(89)90248-6.

Crippin, T., Renaud, J. B., Sumarah, M. W., and David Miller, J. 2019. Comparing genotype and chemotype of *Fusarium graminearum* from cereals in Ontario, Canada. PLoS ONE. 14:1–18. https://doi.org/10.1371/journal.pone.0216735.

Dourado, M. N., Camargo Neves, A. A., Santos, D. 2015. Biotechnological and agronomic potential of endophytic pink-pigmented methylotrophic *Methylobacterium* spp. BioMed Research International. https://doi.org/10.1155/2015/909016.

Ehinmitan, E., Siamalube, B., Mamati, E., Ngumi, V., Juma, P., and Losenge, T. (2024). Evaluating Growth-Promotion and Drought Tolerance Properties of Endophytic Methylobacterium spp. from Semi-Arid Kenya Soil. Scope, 14(3), 924-940.

Ekwomadu, T. I., and Mwanza, M. 2023. Fusarium fungi pathogens, identification, adverse effects, disease management, and global food security: A review of the latest research. Agriculture (Basel). 13(9), 1810. https://doi.org/10.3390/agriculture13091810

Eng, F., Marin, J. 2021. Jasmonic acid biosynthesis by fungi: derivatives, first evidence on biochemical pathways and culture conditions for production. Peer J 9:e10873. https://doi.org/10.7717/peerj.10873.

Goggin, D. E., Emery, R. J. N., and Powles, S. B. 2015. A potential role for endogenous microflora in dormancy release, cytokinin metabolism and the response to fluridone in *Lolium rigidum* seeds. Annals of Botany. 115(2): 293–301. https://doi.org/10.1093/aob/mcu231

Green, P. N. 2006. Methylobacterium. ed. Stackebrandt E. Dworkin M., Falkow S., Rosenberg E., Schleifer KH. New York, NY: Springer US. https://doi.org/10.1007/0-387-30745-1_14.

Grossi, C. E. M., Fantino, E., Serral, F., Zawoznik, M. S., Fernandez Do Porto, D. A., and Ulloa, R. M. 2020. Methylobacterium sp. 2A Is a Plant Growth-Promoting Rhizobacteria That Has the Potential to Improve Potato Crop Yield Under Adverse Conditions. Front. Plant Sci. 11:71. https://doi.org/10.3389/fpls.2020.00071.

Gupta, R., Anand, G., Pizarro, L., Laor Bar-Yosef, D., Kovetz, N., Sela, N., Yehuda, T., Gazit, E., and Bar, M. 2021. Cytokinin Inhibits Fungal Development and Virulence by Targeting the Cytoskeleton and Cellular Trafficking. mBio 12:1–22. https://doi.org/10.1128/mBio.03068-20.

Gupta, A. K., Solanki, I. S., Bashyal, B. M., Singh, Y., and Srivastava, K. 2015. Bakanae of Rice: An emerging disease in Asia. J. Anim. Plant Sci. 25. ISSN: 1018-7081

Hao, G., Naumann, T. A., Vaughan, M. M., McCormick, S., Usgaard, T., Kelly, A., and Ward, T. J. 2019. Characterization of a *Fusarium graminearum* Salicylate Hydroxylase. Frontiers in microbiology, 9, 3219. https://doi.org/10.3389/fmicb.2018.03219

Hedden, P., and Sponsel, V. 2015. A century of gibberellin research. J. Plant Growth Regul. 34:740–760. https://doi.org/10.1007/s00344-015-9546-1

Holland, M. A. 1997a. Methylobacterium and plants. Recent Research Developments in Plant Physiology 207–213.

Holland, M. A. 1997b. Occam's Razor Applied to Hormonology. Plant Physiology 115:865–868. https://doi.org/10.1104/pp.115.3.865

Hori, S. (1898). Some observations on bakanae disease of the rice plant. Memoirs of Tokyo Agricultural Experiment Station, 12, 110–119.

Hu, S., and Bidochka, M. J. 2021. Abscisic acid implicated in differential plant responses of *Phaseolus vulgaris* during endophytic colonization by Metarhizium and pathogenic colonization by Fusarium. Scientific Reports. (11)11327. https://doi.org/10.1038/s41598-021-90232-4

Ivanova, E. G., Doronina, N. V., Shepelyakovskaya, A. O., Laman, A. G., Brovko, F. A., and Trotsenko, Y. A. 2000. Facultative and obligate aerobic *Methylobacteria* synthesize cytokinins. Microbiology 69:646–651. https://doi.org/10.1023/A:1026693805653.

Ivanova, E. G., Doronina, N. V., and Trotsenko, Y. A. 2001. Aerobic *Methylobacteria* are capable of synthesizing auxins. Microbiology 70:392–397. https://doi.org/10.1023/A:1010469708107.

Johns, L.E., Bebber, D.P., Gurr, S.J., and Brown, N. A. 2022. Emerging health threat and cost of *Fusarium* mycotoxins in European wheat. Nat Food 3, 1014–1019. https://doi.org/10.1038/s43016-022-00655-z

Kamo, T., Hiradate, S., Suzuki, K., Fujita, I., Yamaki, S., Yoneda, T., Koitabashi, M., and Yoshida, S. 2018. Methylobamine, a UVA-absorbing compound from the plant-associated bacteria *Methylobacterium* sp. Natural Product Communications 13:141–143. https://doi.org/10.1177/1934578x1801300208.

Kidd, B. N., Kadoo, N. Y., Dombrecht, B., Tekeoglu, M., Gardiner, D. M., Thatcher, L. F., Aitken, E. A. B., Schenk, P. M., Manners, J. M., and Kazan, K. 2011. Auxin Signalling and Transport Promote Susceptibility to the Root-Infecting Fungal Pathogen *Fusarium oxysporum* in Arabidopsis. MPMI 24:733–748. https://doi.org/10.1094/MPMI-08-10-0194.

Kisiala, A.; Kambhampati, S.; Stock, N.L.; Aoki, M.; Emery, R.J.N. 2019. Quantification of Cytokinins Using High-Resolution Accurate- Mass Orbitrap Mass Spectrometry and Parallel Reaction Monitoring (PRM). Anal Chem. 91(23):15049-15056. doi: 10.1021/acs.analchem.9b03728.

Köhl, J., Kolnaar, R., and Ravensberg, W. J. 2019. Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Frontiers in Plant Science 10:845. https://doi.org/10.3389/fpls.2019.00845.

Kulkarni, G. B., Sanjeevkumar, S., Kirankumar, B., Santoshkumar, M., and Karegoudar, T. B. 2013. Indole-3-Acetic Acid Biosynthesis in *Fusarium delphinoides* Strain GPK, a Causal Agent of Wilt in Chickpea. Appl Biochem Biotechnol. 169:1292–1305. https://doi.org/10.1007/s12010-012-0037-6.

Kutschera, U. 2007. Plant-associated *Methylobacteria* as co-evolved phytosymbionts: A hypothesis. Plant Signalling and Behavior 2:74–78. https://doi.org/10.4161/psb.2.2.4073.

Leslie, J. F., and Xu, J.-R. 2010. *Fusarium* genetics and pathogenicity. In: Borkovich, K. A., and Ebbole, D. J., eds. Cellular and Molecular Biology of Filamentous Fungi. American Society for Microbiology Press, Washington, DC. pp. 607–609.

Machado, F. J. de A., Fernandes, J. M. C., Bergstrom, G. C., Del Ponte, E. 2017. Sensitivity of *Fusarium graminearum* from wheat to QoI fungicides in Brazil. Tropical Plant Pathology. 42: 95–99. https://doi.org/10.1007/s40858-016-0126-6

Makandar, R., Nalam, V., Chaturvedi, R., Jeannotte, R., Sparks, A. A., and Shah, J. 2010. Involvement of Salicylate and Jasmonate Signalling Pathways in *Arabidopsis* Interaction with *Fusarium graminearum*. MPMI 23:861–870. https://doi.org/10.1094/MPMI-23-7-0861.

Marx, C. J., Bringel, F., Chistoserdova, L., Moulin, L., Farhan Ul Haque, M., Fleischman, D. E., Gruffaz, C., Jourand, P., Knief, C., Lee, M.-C., Muller, E. E.

L., Nadalig, T., Peyraud, R., Roselli, S., Russ, L., Goodwin, L. A., Ivanova, N., Kyrpides, N., Lajus, A., Land, M. L., Medigue, C., Mikhailova, N., Nolan, M., Woyke, T., Stolyar, S., Vorholt, J. A., and Vuilleumier, S. 2012. Complete Genome Sequences of Six Strains of the Genus *Methylobacterium*. Journal of Bacteriology. 194: 4746–4748. https://doi.org/10.1128/JB.01009-12.

Masimbula, R., Oki, K., Shibata, H., Osawa, H., Kondo, N., Takahashi, K., and Matsuura, H. 2019. Ability of plant pathogenic fungi *Gibberella fujikuroi* and *Fusarium* commune to react with airborne methyl jasmonate. Bioscience, Biotechnology, and Biochemistry 83: 1650–1654. https://doi.org/10.1080/09168451.2019.1617108.

Michniewicz, M. 1989. Growth regulators formed by Fusaria: Their significance for fungus growth, sporulation and pathogenicity towards the host plant. In J. Chelkowski (Ed.), *Fusarium*: Mycotoxins, taxonomy, pathogenicity (Vol. 2, Topics in Secondary Metabolism, pp. 227–241). Elsevier. https://doi.org/10.1016/B978-0-444-87468-9.50018-3

Miersch, O., Brückner, B., Schmidt, J., and Sembdner, G. 1992. Cyclopentane fatty acids from *Gibberella fujikuroi*. Phytochemistry 31: 3835–3837. https://doi.org/10.1016/S0031-9422(00)97537-X.

Mishra, A., and Baek, K.-H. 2021. Salicylic Acid Biosynthesis and Metabolism: A Divergent Pathway for Plants and Bacteria. Biomolecules 11: 705. https://doi.org/10.3390/biom11050705.

Morrison, E. N., Emery, R. J. N., and Saville, B. J. 2017. Phytohormone involvement in the *Ustilago maydis-Zea mays* pathosystem: relationships between cytokinins and symptoms. Mol. Plant Pathol. 18:829–842.

Nuzhnaya, T. V., Sorokan, A. V., Burkhanova, G. F., Maksimov, I. V., and Veselova, S. V. 2024. The role of cytokinins and abscisic acid in the growth, development and virulence of the pathogenic fungus *Stagonospora nodorum* (Berk.). Biomolecules. 14(5): 517. https://doi.org/10.3390/biom14050517

Oliw, E. H., and Hamberg, M. 2017. An allene oxide and 12-oxophytodienoic acid are key intermediates in jasmonic acid biosynthesis by *Fusarium oxysporum*. Journal of Lipid Research. 58: 1670–1680. https://doi.org/10.1194/jlr.M077305.

Palberg, D., Kisiała, A., Jorge, G. L., and Emery, R. J. N. 2022. A survey of *Methylobacterium* species and strains reveals widespread production and varying profiles of cytokinin phytohormones. BMC Microbiology. 22: 1–17. https://doi.org/10.1186/s12866-022-02454-9.

Pertry I, Václavíková K, Depuydt S, Galuszka P, Spíchal L, Temmerman W, Stes E, Schmülling T, Kakimoto T, Van Montagu MC, Strnad M, Holsters M, Tarkowski P, Vereecke D. 2009. Identification of *Rhodococcus fascians* cytokinins and their *modus operandi* to reshape the plant. Proc Natl Acad Sci USA. 106(3): 929-34. https://doi.org/10.1073/pnas.0811683106.

Peyraud, R., Schneider, K., Kiefer, P., Massou, S., Vorholt, J. A., and Portais, J.-C. 2011. Genome-scale reconstruction and system level investigation of the metabolic network of *Methylobacterium extorquens* AM1. BMC Syst Biol 5: 189. https://doi.org/10.1186/1752-0509-5-189.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. 2014. Induced Systemic Resistance by Beneficial Microbes. Annu. Rev. Phytopathol. 52: 347–375. https://doi.org/10.1146/annurev-phyto-082712-102340.

Pietro, A. D., Madrid, M. 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. Molecular Plant Pathology 4: 315–325. https://doi.org/10.1046/j.1364-3703.2003.00180.x.

Ploetz, R. C. 2015. Fusarium Wilt of Banana. Phytopathology. 105: 1512–1521. https://doi.org/10.1094/PHYTO-04-15-0101-RVW.

Poorniammal, R., Sundaram, S. P., and Kumutha, K. 2009. *In-vitro* Biocontrol Activity of *Methylobacterium Extorquens* Against Fungal Pathogens. International Journal of Plant Protection 2: 59–62. https://doi.org/10.13140/2.1.3086.0163.

Pozo, M. J., López-Ráez, J. A., Azcón-Aguilar, C., and García-Garrido, J. M. 2015. Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. New Phytol. 205:1431–1436. https://doi.org/10.1111/nph.13252

Pradhan, A., Mishra, S. 2025. *Fusarium* wilt of cotton: A growing threat to global cotton production. Vigyan Varta, 6(3), 48–51. E-ISSN: 2582-9467

Prusty R, Grisafi P, Fink GR. 2004. The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A. 101(12):4153-7. https://doi.org/10.1073/pnas.0400659101.

Qi, P.-F., Zhang, Y.-Z., Liu, C.-H., Chen, Q., Guo, Z.-R., Wang, Y., Xu, B.-J., Jiang, Y.-F., Zheng, T., Gong, X., Luo, C.-H., Wu, W., Kong, L., Deng, M., Ma, J., Lan, X.-J., Jiang, Q.-T., Wei, Y.-M., Wang, J.-R., and Zheng, Y.-L. (2019). Functional Analysis of FgNahG Clarifies the Contribution of Salicylic Acid to Wheat (*Triticum aestivum*) Resistance against *Fusarium* Head Blight. Toxins. 11(2), 59. https://doi.org/10.3390/toxins11020059

Qi, P.-F., Balcerzak, M., Rocheleau, H., Leung, W., Wei, Y.-M., Zheng, Y.-L., and Ouellet, T. 2016. Jasmonic acid and abscisic acid play important roles in host–pathogen interaction between *Fusarium graminearum* and wheat during the early stages of *Fusarium* head blight. Physiological and Molecular Plant Pathology. 93: 39–48. https://doi.org/10.1016/j.pmpp.2015.12.004.

Rampersad, S. 2020. Pathogenomics and Management of *Fusarium* Diseases in Plants. Pathogens. 9: 340. https://doi.org/10.3390/pathogens9050340.

Rocheleau H, Al-Harthi R, Ouellet T. 2019. Degradation of salicylic acid by *Fusarium graminearum.* Fungal Biol. 123(1):77-86. https://doi.org/10.1016/j.funbio.2018.11.002.

Sharaf, E. F., and Farrag, A. A. 2004. Induced Resistance in Tomato Plants by IAA against *Fusarium oxysporum lycopersici*. Polish Journal of Microbiology 53:111–116.

Soanes DM, Alam I, Cornell M, Wong HM, Hedeler C, Paton NW, Rattray M, Hubbard SJ, Oliver SG, Talbot NJ. 2008. Comparative genome analysis of filamentous fungi reveals gene family expansions associated with fungal pathogenesis. PLoS One. 4;3(6):e2300. https://doi.org/10.1371/journal.pone.0002300.

Solórzano, R., Ramírez Maguiña, H. A., Johnson, L., Ureta Sierra, C., and Cruz, J. 2025. Current Progress in Microbial Biocontrol of Banana Fusarium Wilt: A Systematic Review. Agronomy. 15(3), 619. https://doi.org/10.3390/agronomy15030619

Spadaro, D. 2017. The puzzle of bakanae disease through interactions between Fusarium fujikuroi and rice. Front Biosci 9:333–344. https://doi.org/10.2741/e806.

Spaepen, S., and Vanderleyden, J. 2011. Auxin and plant-microbe interactions. Cold Spring Harb. Perspect. Biol. 3:a001438. https://doi.org/10.1101/cshperspect.a001438

Ton, J., Flors, V., and MauchMani, B. 2009. The multifaceted role of ABA in disease resistance. Trends in Plant Science. 14(6): 310–317. https://doi.org/10.1016/j.tplants.2009.03.006

Trail, F. 2009. For Blighted Waves of Grain: *Fusarium graminearum* in the Postgenomics Era. Plant Physiology 149: 103–110. https://doi.org/10.1104/pp.108.129684.

Troncoso, C., González, X., Bömke, C., Tudzynski, B., Gong, F., Hedden, P., and Rojas, M. C. 2010. Gibberellin biosynthesis and gibberellin oxidase activities in *Fusarium sacchari*, *Fusarium konzum* and *Fusarium subglutinans* strains. Phytochemistry. 71(11–12): 1322–1331. https://doi.org/10.1016/j.phytochem.2010.05.006

Tsavkelova, E., Oeser, B., Oren-Young, L., Israeli, M., Sasson, Y., Tudzynski, B., and Sharon, A. 2012. Identification and functional characterization of indole-3-acetamide-mediated IAA biosynthesis in plant-associated *Fusarium* species. Fungal Genetics and Biology 49: 48–57. https://doi.org/10.1016/j.fgb.2011.10.005.

Tudzynski, B. 2005. Gibberellin biosynthesis in fungi: genes, enzymes, evolution, and impact on biotechnology. Appl. Microbiol. Biotechnol. 66:597–611. https://doi.org/10.1007/s00253-004-1805-1

Udomkun, P., Wiredu, A. 2017. Mycotoxins in sub-Saharan Africa: Present situation, socio-economic impact, awareness, and outlook. Food Control, 72, 110–122. https://doi.org/10.1016/j.foodcont.2016.07.039

Van Der Lee, T., Zhang, H., Van Diepeningen, A., and Waalwijk, C. 2015. Biogeography of *Fusarium graminearum* species complex and chemotypes: a review. Food Additives and Contaminants: Part A 32:453–460. https://doi.org/10.1080/19440049.2014.984244.

Vitale, J., Boyer, T., Uaiene, R., and Sanders, J. H. 2007. The economic impacts of introducing Bt technology in smallholder cotton production systems of West Africa: A case study from Mali. AgBioForum, 10(2), 71–84.

Vrabka, J., Niehaus, E.-M., Münsterkötter, M., Proctor, R. H., Brown, D. W., Novák, O., Pěnčik, A., Tarkowská, D., Hromadová, K., Hradilová, M., Oklešťková, J., Oren-Young, L., Idan, Y., Sharon, A., Maymon, M., Elazar, M., Freeman, S., Güldener, U., Tudzynski, B., Galuszka, P., and Bergougnoux, V. 2018. Production and role of hormones during interaction of *Fusarium* species with maize (Zea mays L.) seedlings. Front. Plant Sci. 9:1936. https://doi.org/10.3389/fpls.2018.01936.

Wiemann, P., Sieber, C. M. K., von Bargen, K. W., Studt, L., Niehaus, E.-M., Espino, J. J., Huß, K., Michielse, C. B., Albermann, S., Wagner, D., Bergner, S. V., Connolly, L. R., Fischer, A., Reuter, G., Kleigrewe, K., Bald, T., Wingfield, B. D., Ophir, R., Freeman, S., Hippler, M., Smith, K. M., Brown, D. W., Proctor, R. H., Münsterkötter, M., Freitag, M., Humpf, H.-U., Güldener, U., and Tudzynski, B. 2013. Deciphering the cryptic genome: genome-wide analyses of the rice pathogen *Fusarium fujikuroi* reveal complex regulation of secondary metabolism and novel metabolites. PLoS Pathog. 9:e1003475. https://doi.org/10.1371/journal.ppat.1003475.

Wilson, W., Dahl, B., and Nganje, W. 2018. Economic costs of *Fusarium* Head Blight, scab and deoxynivalenol. World Mycotoxin J. 11:291–302. https://doi.org/10.3920/wmj2017.2204.

Windels, C. E. (2000). Economic and social impacts of *Fusarium* head blight: Changing farms and rural communities in the Northern Great Plains. Phytopathology, 90(1), 17–21. https://doi.org/10.1094/PHYTO.2000.90.1.17

Wu, H.-S., Raza, W., Fan, J.-Q., Sun, Y.-G., Bao, W., Liu, D.-Y., Huang, Q.-W., Mao, Z., Shen, Q.-R., and Miao, W.-G. (2008). Antibiotic effect of exogenously applied salicylic acid on in vitro soilborne pathogen, *Fusarium oxysporum* f.sp. *niveum*. Chemosphere 74: 45–50. https://doi.org/10.1016/j.chemosphere.2008.09.027.

Xu, F., Song, Y. L., Yang, G. Q., Wang, J. M., Liu, L. L., and Li, Y. H. 2015. First Report of *Fusarium pseudograminearum* from Wheat Heads with *Fusarium* Head Blight in North China Plain. Plant Disease 99: 156–156. https://doi.org/10.1094/PDIS-05-14-0543-PDN.

Yabuta, T. 1935. Biochemistry of the ‘bakanae’ fungus of rice. Agric Hortic, 10, 17-22.

Yang, J., Duan, G., Li, C., Liu, L., Han, G., Zhang, Y., and Wang, C. 2019. The Crosstalks Between Jasmonic Acid and Other Plant Hormone Signalling Highlight the Involvement of Jasmonic Acid as a Core Component in Plant Response to Biotic and Abiotic Stresses. Front. Plant Sci. 10: 1349. https://doi.org/10.3389/fpls.2019.01349.

Zhan, L., Chen, L., Hou, Y., Zeng, Y., and Ji, Z. 2024. Bakanae Disease Resistance in Rice: Current Status and Future Considerations. Agronomy 14: 1507. https://doi.org/10.3390/agronomy14071507.

Zhang, J., Zheng, Q., Guo, W., and Zong, X. 2020. Salicylic acid biosynthesis and metabolism: a divergent pathway for plants and lacking in filamentous fungi. Front. Plant Sci. 11: 705. https://doi.org/10.3390/biom11050705

Zheng, Z., Hou, Y., Cai, Y., Zhang, Y., Li, Y., Zhou, M., Wang, H., Liu, X., and Xu, J. R. 2015. Whole-genome sequencing reveals that mutations in myosin-5 confer resistance to the fungicide phenamacril in *Fusarium graminearum*. Scientific Reports. 5 : 8248. https://doi.org/10.1038/srep08248

Zhou, Y., Zhou, X. E., Gong, Y., Zhu, Y., Cao, X., Brunzelle, J. S., Li, J., Xu, H. 2020. Structural basis of *Fusarium* myosin I inhibition by phenamacril. PLoS Pathogens. 16(3): e1008323. https://doi.org/10.1371/journal.ppat.1008323

CHAPTER 7

7.1. PREFACE

Title: General Conclusions: *Methylobacterium* as candidates for the restoration and fortification of the phytobiome

Author: Daniel Palberg

CHAPTER 7

7.2 CONCLUSIONS: *Methylobacterium* IN BIOLOGICAL CONTROL

Critique of biological control (BC) is often underpinned by the ease with which synthetic agents can be integrated into existing crop protection regimen, and the rate at which these products diminish signs of disease. These attributes, combined with the abundance of marketed agrochemical formulations, impart the impression that biocontrol methods are slow – requiring years to establish a new ecological balance which deters pathogen proliferation – and ineffective. This has resulted in the 'first-line' fallacy – that engaging with phytopathogens directly using synthetic agents is the most convenient and effective option – which prescribes large and abrupt changes to the phytopathogen population as necessary for adequate disease control. Often brief, the period of eradication is afterwards fraught with years of fluctuating pathogen density and smaller secondary outbreaks in a cyclical sequence (i.e. the 'grand cycle'). While this approach has pulled producers from the brink of collapse, it has resulted in selection pressures which favour fungicide resistance.

With the exclusion of parasitism, pathogenicity is often the result of a disturbance of biological balance which imparts physiological stress in the host (salinity, pH, drought, injury) or upsets the phytobiome (Berendsen et al. 2012; Mendes et al. 2013; van der Heijden and Hartmann, 2016). Pathogenicity is anomalous to plant-microbe relationships – the number of disease-causing interactions is exceedingly small relative to the total scale of plant interactions. Given that microbial biodiversity is a key factor influencing the severity of disease, perhaps the inadvertent disruption of protective endophytes – especially those which have shared a long history with the host

– by modern farming practices contributes to the depreciation of a natural phytopathogen-supressing biological balance (Ghorbani et al. 2008).

Here, we present evidence characterizing the endogenous phytohormone profile of *Methylobacterium* (Chapter 2), highlighting both the uniquely high levels of active cytokinin free bases (CK-FB) secreted by several members in addition to the broader portfolio of phytohormones produced by this genus, and their potential contributions within the wider phytobiome.

For the first time, definitive evidence is also provided (Chapter 3) demonstrating the sensitivity of *Methylobacterium* – including endophytic strains – to commercial herbicide formulations containing glyphosate. Importantly however, our findings show that glyphosate cytotoxicity is strongly linked to cell permeability and conclusively establish that access to the intracellular matrix is essential for replicating the bactericidal effects observed in experiments involving commercial glyphosate formulations.

In Chapter 4, we demonstrate broad compatibility between over 30 members of *Methylobacterium* and commonly used fungicide formulations. This compatibility suggests safe integration possibilities with products containing active ingredients that operate via distinct mechanisms of action – particularly those not targeting components of conserved metabolic pathways (Shikimate) shared between bacteria and plants.

In Chapter 5, we show evidence of necrotrophic behaviour of two strains of *F. graminearum* towards soybean (*Glycine max*) seedlings in both soil and artificial growth medium. Crucially, our work has demonstrated that the presence of an

endophyte, namely *M. organophilum*, even at relatively low inoculum density (10^6 CFU/mL) improves germination and seedling development. Importantly, the presence of *M. organophilum* appears to preserve the health of the seed coat by slowing its digestion by *F. graminearum*, restoring the normal coat shedding process, and preventing secondary injury to the embryonic tissue (Figure 5.8. and Figure 5.9.). Seedlings co-inoculated *with M. organophilum* and *F. graminearum* had lower disease severity scores (DSS), longer taproots and taller stems (43% and 31%, respectively), thinner mycelial networks, less fruiting bodies, and an absence of soft rot in the maturing cotyledons (Figure 5.8c and 5.8d. and Figure 5.9c. and 5.9d.). These results suggest that *Methylobacterium* may have an important role to play in slowing the progression of serious disease by soilborne pathogens including *Fusarium*.

Across North America, corn-soybean or corn-soybean-wheat rotations are often paired with reduced-tillage or no-tillage systems and while these practices may help preserve overall soil health, coupled with early sowing they create conditions which delay seedling emergence (cooler, wetter) and increase susceptibility to preemergence damping off (Broders et al., 2007). Plant residues from overwintering cover crops also provide sustenance for necrotrophic *Fusarium* and increase soil inoculum for subsequent seasons. Despite advancements in treatment, pre- and post-emergence diseases of soybean continue to be a challenge, especially as the host-range of phytopathogens like *Fusarium* is broad enough to include all members of a corn-soybean-wheat rotation.

We suggest that microbiome enrichment with antagonists like *Methylobacterium* could stave off infection by maintaining unfavourable conditions for

the pathogen until abiotic conditions improve. Evidence of the direct beneficial effects of *Methylobacterium* on plants are multitudinous and span from improving tolerance to salinity (Lee et al. 2015) and drought (Jorge et al. 2019), to enhancing defense responses (Madhaiyan et al. 2006) and growth (Abadi et al. 2020; Abanda-Nkpwatt et al. 2006; Kuklinsky-Sobral et al. 2004; Maneewan and Khonsarn, 2017; Meena et al. 2012; Senthilkumar and Krishnamoorthy, 2017). Incorporation of bacterial endophytes into existing crop protection strategies may offset the number of synthetic fungicides required for adequate and stable phytopathogen control, especially with newfound understanding of compatibility (Chapter 3 and Chapter 4) between these organisms and synthetic agents (Palberg and Emery 2025).

That phytopathogens can establish and even flourish in entirely new ecosystems is proof that otherwise foreign species can invade an established ecological community, so long as the biological balance is tipped in favour of pathogen proliferation: a suitable host, favourable abiotic conditions, and a weakened or disrupted phytobiome. This lends support to the notion that the same introduction and flourishing of beneficial symbionts in nonnative environments is possible, and potentially an important contributor in tipping the balance to favour the host.

Beyond their well-documented roles in plants (e.g. tissue growth and differentiation, seed development, nutrient balance, stress responses, senescence), phytohormones are increasingly found at the interface between phytopathogenic fungi and their hosts. For example, the biosynthesis and secretion of cytokinins (CKs) by *Claviceps purpurea*, a biotrophic cereal pathogen with cosmopolitan distribution, formed an important component of the infectious process as isolates with impaired CK

synthesis were observably less virulent in rye (Hinsch et al. 2015). CKs have been similarly implicated in other pathogen-host combinations including infection of maize by *Ustilago maydis* (Morrison et al. 2017) and *Colletotrichum graminicola* (Behr et al. 2012), rice blast caused by *Magnaporthe oryzae* (Chanclud et al. 2016), and stem canker of rape by *Leptosphaeria maculans* (Trdá et al. 2017). Hormones, including the CKs, are similarly intertwined in diseases caused by fusaria – by either disruption of host production and trafficking broadly, or by inciting fluctuations locally at the site of ingress – including seedling blight of rice (bakanae disease) by *F. proliferatum* and *F. fujikuroi* (Quazi et al. 2015), mango malformation by *F. mangiferae* (Nicholson and Van Staden, 1988; Van Staden and Nicholson, 1989), and crown rot of wheat by *F. pseudograminearum* (Sørensen et al. 2018). Increasingly, phytohormone biosynthesis is found across various clades of soil-dwelling organisms, and seem to be intrinsically linked to ecological stability, even being proposed as indicators of soil health and productive capacity, complimentary to traditional soil health parameters (Perera et al. 2025). Work undertaken previously by our group revealed the wide diversity of phytohormone profiles in the secretome of *Methylobacterium* isolates, with the freebase (FB) isoform, *trans*-zeatin (tZ), generally dominating over less bioactive CK types (Palberg et al. 2022).

Collectively, the data presented here illuminates a nuanced and ecologically integrated view of BC and underscores the potential for leveraging bacterial endophytes, such as *Methylobacterium* spp., as proactive agents to maintain an ecological balance unfavorable for pathogen proliferation. Nevertheless, numerous questions remain regarding the precise molecular and ecological mechanisms by which *Methylobacterium* achieves biocontrol efficacy, particularly in the context of hormone-

mediated interactions. The evident complexity of phytohormone dynamics at the pathogen-host-antagonist interface – demonstrated by diverse cytokinin profiles and their roles in pathogen virulence across different plant-pathogen systems – warrants further comprehensive and targeted studies to validate the practical utility and scalability of *Methylobacterium*-based biocontrol strategies.

Ultimately, integrating beneficial microbial symbionts into contemporary agricultural practices necessitates a fundamental shift in perception – from interpreting BC as simply a slower and indirect method, to an appreciation for its capacity for sustained and stable disease suppression, once ecological rebalancing has been achieved. As we continue to deepen our understanding of microbial interactions, and the hormone crosstalk that underpins them, BC may in time, emerge as an integral pillar of sustainable agriculture.

7.3 THE GOLDILOCKS PARADOX

Phytohormones are increasingly recognized as pivotal mediators in interkingdom signalling, significantly influencing plant developmental processes, immune responses, and host-microbe interactions (Berger et al. 2020; Nakano et al. 2022). Nevertheless, their roles in BC and pathogenesis remain strangely paradoxical. Hormones that confer pathogen resistance in one context may, conversely, enhance susceptibility or disease severity in another (Chanclud and Morel, 2016; Pieterse et al. 2014). These apparent contradictions underscore the fundamentally nonlinear and context-dependent nature of hormone perception, which underpins what I proffer as the "*Goldilocks Paradox*" – a conceptual framework which posits that while the success or failure of phytopathogens certainly involves hormone signals, is not dictated solely by them. Instead, success or failure is likely determined by a precise and contextually appropriate equilibrium of hormone profile and concentrations, timing, developmental stage of the host, receptivity of the pathogen, and a wide breadth of other environmental factors.

Unlike traditional antimicrobial agents whose effects are typically dose-dependent, phytohormones function within delicately balanced signalling networks characterized by conditional, reversible, and hormetic responses. Hormones such as CKs, auxins, salicylic acid (SA), jasmonic acid (JA), and gibberellins (GAs) regulate interconnected signalling pathways that modulate transcriptional activity, morphological development, stress responses, and communication in both plants and microbes (Grich et al. 2024; Chanclud and Morel, 2016; Qi et al. 2019). The pathogenic success of fungi including *Fusarium*, likely hinges upon the accurate perception and

integration of host-derived hormonal cues – particularly during early infection phases – to coordinate developmental transitions essential for host colonization (Chapter 5).

Hormonal sensitivity then, potentially provides a strategic entry point for BC through targeted disruption of pathogen signalling. A model I posit involves signal hijacking, wherein antagonistic microbes, such as *Methylobacterium*, interfere with fungal development by synthesizing hormone analogs or intermediates that mimic signals of the host that are perceived by pathogenic fungi like *Fusarium* as aberrant or premature developmental cues. If, under natural conditions, pathogenic fungi rely on a specific set of these host-derived signals to trigger critical processes (enzymatic secretion, or vesicle trafficking) required for colonization, bacterial mimicry could result in inappropriate fungal responses. Consequently, the pathogen might initiate energetically costly processes prematurely in unfavorable environments, leading to resource depletion, inappropriate morphological fitness, and ultimately impaired viability. This model parallels known incidences of signal disruption documented in bacterial quorum sensing, where deceptive signals interrupt coordinated virulence expression (Tzipilevich et al. 2021).

A variation on the model would alternatively involve signal masking, wherein bacterial antagonists degrade, sequester, or competitively inhibit host-derived hormonal signals essential for fungal perception and response. Enzymatic pathways such as cytokinin oxidases (CKX) or salicylate hydroxylases (SAX) in antagonistic bacteria could reduce the abundance of hormones important to overall pathogenic competency and prevent or delay effective infection even in compatible hosts (Di Pietro et al. 2001; Rispail and Di Pietro, 2010).

Importantly though, hormone effects are not isolated or linear: phytohormones exist in interactive suites and dynamically modulate receptor sensitivity and signalling pathways (Liu et al. 2017; Vanstraelen, M., and Benková, 2012). For example, elevated JA can antagonistically repress SA-mediated defenses in dicotyledonous plants, while CK-to-IAA ratios regulate cell division and differentiation in fungi and plants (Yang et al. 2019; Grich et al. 2024). Small perturbations within these interconnected networks may therefore generate nonlinear, context-specific outcomes.

Complicating our understanding of the intricate interplay of signals across the pathogen-antagonist-host axis, however, is the broader microbial community in the phytobiome. The introduction or enrichment of hormone-modulating bacteria like *Methylobacterium* may in fact induce broader metabolic shifts within the phytobiome, influencing other residents through probiosis. Such community-level metabolic adjustments could amplify or dampen hormone – and other metabolite – signals, drastically altering the environment surrounding the pathogen. Such manipulations, however, are not without risk: enriching the phytobiome with a specific group could unintentionally displace native microbial residents and consequently reduce biodiversity. A less diverse microbiome could then become skewed in a way that favors a different taxon of opportunistic pathogens.

While phytohormones are players in pathogen–host–antagonist interactions, their role likely represents only one component within a much broader framework. This is especially the case with antagonistic bacteria which likely confront pathogenic fungi with an array of orthogonal pressures including substrate occupation and nutrient

competition (Bonaterra et al. 2022). Bacterial symbionts probably also secrete a variety of enzymes that disrupt pathogen integrity, and their rapid replication could enable the formation of inhibitory biofilms. Yet, even with these interlocking mechanisms at play, a long history of research efforts involving BC prove that antagonism is far from consistent or guaranteed (Bonaterra et al. 2022). The success of BC, then, hinges on a confluence of factors, and in this context, hormone signalling represents one finely tuned layer; too much modulation may disrupt host balance or favor the pathogen, while too little may fail to produce effective defenses.

The conceptual framework of the *Golidlocks Paradox* overall, aligns closely with existing ecological models which emphasize context dependency in microbial interactions (antagonism, mutualism, or commensalism), where outcomes are not determined by intrinsic microbial properties alone, but heavily influenced by external variables as well (Hassani et al. 2018). Ultimately, the *Goldilocks Paradox* as presented here, highlights a fundamental principle of both developmental biology, complex systems, and ecological interactions: it is not simply the presence or absence of signals that determines an outcome, but the precision, balance, and timing of those signals within the phytobiome. Effective microbial antagonism, therefore, almost certainly depends on deepening our understanding of these signals to navigate the inherently multifactorial nature of BC and guide the design of plant protection strategies.

7.4 CHALLENGES & FUTURE DIRECTIONS

Despite the formal establishment of BC as a distinct subdiscipline, widespread implementation of BC strategies remains limited. Multiple barriers impede the translation of promising laboratory results to consistent field-level outcomes. Primary among these is the inherent complexity and variability of natural ecosystems and agricultural practices. Interactions among pathogens, hosts, and antagonists occur within ecological networks influenced significantly by environmental factors such as soil type, climate, microbial community composition, and cultivation practices (Massart et al. 2015). Such complexity routinely frustrates efforts to replicate promising *in vitro* and greenhouse results in natural field conditions. Further exacerbating these challenges, intimidating regulatory hurdles and stringent approval criteria steepen the long path toward marketability, while unclear guidelines for use and incompatibility with existing equipment or techniques exacerbate producer hesitancy towards adoption (Leahy et al. 2014).

To address these barriers, strategic adjustment to regulation specifically tailored for BC agent testing could foster an environment more conducive to rigorous field trials. Targeted investments in infrastructure designed for large-scale field research would also enable the evaluation of BC agents in realistic agricultural settings – including comprehensive assessment of microbiome enrichment on the native microbiota and crop yields – which are notoriously difficult to capture reliably in greenhouse studies (Bardin et al. 2015).

Importantly though, BC should not be viewed through the lens of classical approaches to crop protection which emphasize complete pathogen eradication, as BC

ultimately operates through ecological rebalancing: establishing conditions unfavorable to pathogen proliferation. This fundamental conceptual shift will require reshaping expectations around efficacy and performance where BC is deployed, and success instead measured in terms of overall ecological stability, biodiversity, pathogen suppression, crop health, and sustainability (Köhl et al. 2019).

The relationship between agricultural practices and microbial dynamics further underscores the importance of ecological rebalancing. For example, studies have documented disruptions to microbial communities following glyphosate-based herbicide (GBH) applications, which correlate with increased diseases prevalence across various cropping systems (Zobiole et al. 2011). Intriguingly, work carried out herein (Chapter 3) also show evidence of disruption to *Methylobacterium* by GBH's, including many species which are dominant endophytic symbionts. Therefore, leveraging native microbial populations to enrich or refortify the phytobiome post-treatment with agrichemicals might also represent an ecologically sustainable approach to pathogen control.

Future research to advance BC effectiveness should undoubtedly include extensive field-level evaluations to enhance the validity of ecological fitness and scalability. Investigating the impacts of pathogen-antagonist interactions among resident microbial populations is crucial to understand broader community-level effects. Further *in-planta* studies should assess hormonal dynamics across the host-pathogen-antagonist axis and use of isotopically labeled nutrients would assist with the precise tracking of phytohormone synthesis and movement between interacting species. Advanced microscopy techniques (transmission electron microscopy (TEM) and

scanning electron microscopy (SEM)) could also be deployed to visualize interactions at the antagonist-pathogen interface, potentially revealing critical behaviors like biofilm formation that contribute to BC effects.

The design of novel experimental setups which enable spatial separation in co-culture systems, perhaps using semipermeable methylcellulose membranes would significantly advance understanding of diffusible factors and their roles in pathogen inhibition. Despite difficulties in maintaining membrane integrity experienced in this work, refining these approaches would allow greater precision in defining molecular interactions.

Given the dependency of the agriculture sector on monoculture and synthetic inputs, transitioning to biologically-centered management practices will require shifts in agronomic planning, regulatory frameworks, grower incentives and perceptions, scientific investment in systems-based approaches, and even consumer expectations. Encouragingly, emerging evidence suggests that the integration of beneficial microbial communities into crop management does in fact bolster resilience against pathogens, reduce reliance on synthetic inputs, and mitigate environmental impacts (Massart et al. 2015; Köhl et al. 2019). Ultimately, embracing BC as a mainstream crop protection strategy requires acknowledgment and acceptance of its nuances and inherent variability – recognizing that its success, often depends on complex interactions, context-specific conditions, and long-term systems-level thinking rather than immediate, uniform outcomes.

7.5 REFERENCES

Abadi, V.A.J.M., Sepehri, M., Rahmani, H.A., Zarei, M., Ronaghi, A., Taghavi, S.M., and Shamshiripour, M. 2020. Role of dominant phyllosphere bacteria with plant growth–promoting characteristics on growth and nutrition of maize (*Zea mays* L.). J. Soil Sci. Plant Nutr. 20(4): 2348–2363. https://doi.org/10.1007/s42729-020-00302-1.

Abanda-Nkpwatt, D., Müsch, M., Tschiersch, J., Boettner, M., and Schwab, W. 2006. Molecular interaction between *Methylobacterium extorquens* and seedlings: Growth promotion, methanol consumption, and localization of the methanol emission site. Journal of Experimental Botany 57:4025–4032. https://doi.org/10.1093/jxb/erl173.

Bardin, M., Ajouz, S., Comby, M., Lopez-Ferber, M., Graillot, B., Siegwart, M., and Nicot, P. C. 2015. Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? Frontiers in Plant Science. 6:566. https://doi.org/10.3389/fpls.2015.00566.

Behr, M., Motyka, V., Weihmann, F., Malbeck, J., Deising, H. B., and Wirsel, S. G. R. 2012. Remodeling of Cytokinin Metabolism at Infection Sites of *Colletotrichum graminicola* on Maize Leaves. MPMI 25:1073–1082. https://doi.org/10.1094/MPMI-01-12-0012-R.

Berendsen, R. L., Pieterse, C. M., Bakker, P. A. 2012. The rhizosphere microbiome and plant health. Trends Plant Sci. 17(8):478-86. https://doi.org/10.1016/j.tplants.2012.04.001.

Berger, S., Van Wees, S.C.M., Nybroe, O., and Großkinsky, D.K. 2020. Editorial: Cross-Frontier Communication: Phytohormone Functions at the Plant–Microbe Interface and Beyond. *Frontiers in Plant Science*, 11:386. https://doi.org/10.3389/fpls.2020.00386

Bonaterra, A., Badosa, E., Daranas, N., Francés, J., Roselló, G., and Montesinos, E. 2022. Bacteria as Biological Control Agents of Plant Diseases. Microorganisms. 10(9): 1759. https://doi.org/10.3390/microorganisms10091759

Broders, K. D., Lipps, P. E., Paul, P. A., and Dorrance, A. E. 2007. Evaluation of *Fusarium graminearum* associated with corn and soybean seed and seedling disease in Ohio. Plant Disease. 91: 1155–1160. https://doi.org/10.1094/PDIS-91-9-1155.

Chanclud, E., and Morel, J.-B. 2016. Plant hormones: a fungal point of view. Molecular Plant Pathology 17:1289–1297. https://doi.org/10.1111/mpp.12393.

Di Pietro, A., García-Maceira, F. I., Méglecz, E., and Roncero, M. I. G. 2001. A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. Molecular Microbiology. 39:1140–1152. https://doi.org/10.1111/j.1365-2958.2001.02307.x.

Grich, N., Huynh, T., Kisiala, A., Palberg, D., Emery, R. J. N. 2025. The biosynthesis and impacts of cytokinins on growth of the oyster mushroom, *Pleurotus ostreatus*. Mycologia. 117(1): 76-94. https://doi.org/10.1080/00275514.2024.2401320.

Hassani, M. A., Durán, P., and Hacquard, S. 2018. Microbial interactions within the plant holobiont. Microbiome 6:58. https://doi.org/10.1186/s40168-018-0445-0.

Hinsch, J., Vrabka, J., Oeser, B., Novák, O., Galuszka, P., and Tudzynski, P. 2015. *De novo* biosynthesis of cytokinins in the biotrophic fungus *Claviceps purpurea*. Environmental Microbiology 17:2935–2951. https://doi.org/10.1111/1462-2920.12838.

Jorge, G. L., Kisiala A, Morrison E., Aoki, M., Nogueira, A. P. and Emery, R. J. N. 2019. Endosymbiotic *Methylobacterium oryzae* mitigates the impact of limited water availability in lentil (*Lens culinaris* Medik.) by increasing plant cytokinin levels. Environ Exp Bot. 162: 525–40. https://doi.org/10.1016/j.envexpbot.2019.03.028

Köhl, J., Kolnaar, R., and Ravensberg, W. J. 2019. Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Frontiers in Plant Science 10:845. https://doi.org/10.3389/fpls.2019.00845.

Kuklinsky-Sobral, J., Araújo, W. L., Mendes, R., Geraldi, I. O., Pizzirani-Kleiner, A. A., and Azevedo, J. L. 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environmental Microbiology 6: 1244–1251. https://doi.org/10.1111/j.1462-2920.2004.00658.x.

Leahy, J., Mendelsohn, M., Kough, J., Jones, R., and Berckes, N. 2014. Biopesticide oversight and registration at the U.S. Environmental Protection Agency. In Biopesticides: State of the Art and Future Opportunities, ed. A. D. Gross, J. R. Coats, S. O. Duke, and J. N. Seiber, pp. 3–18. American Chemical Society, Washington, D.C.

Lee, Y., Krishnamoorthy, R., Selvakumar, G., Kim, K., and Sa, T. 2015. Alleviation of salt stress in maize plant by co-inoculation of arbuscular mycorrhizal fungi and *Methylobacterium oryzae* CBMB20. Journal of the Korean Society for Applied Biological Chemistry 58:533–540. https://doi.org/10.1007/s13765-015-0072-4.

Liu, J., Moore, S., Chen, C. and Lindsey, K. 2017. Crosstalk Complexities between Auxin, Cytokinin, and Ethylene in Arabidopsis Root Development: From Experiments to Systems Modeling, and Back Again. Mol Plant. 10(12): 1480-1496. https://doi.org/10.1016/j.molp.2017.11.002.

Madhaiyan, M., Poonguzhali, S., Ryu, J. and Sa, T. 2006. Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. Planta, 224(2), 268–278. https://doi.org/10.1007/s00425-005-0211-y

Maneewan, K. and Khonsarn, N. 2017. Selection of bioinoculants for tomato growth enhancement and pathogen resistance. Asia-Pacific J Sci Technol. 22(3):1-9. https://doi.org/10.14456/apst.2017.37

Massart, S., Martinez-Medina, M., and Jijakli, M. H. 2015. Biological control in the microbiome era: challenges and opportunities. Biological Control 89:98–108. https://doi.org/10.1016/j.biocontrol.2015.06.003.

Meena, K. K., Kumar, M., Kalyuzhnaya, M. G., Yandigeri, M. S., Singh, D. P., Saxena, A. K., Arora, D. K. 2012. Epiphytic pink-pigmented methylotrophic bacteria enhance germination and seedling growth of wheat (*Triticum aestivum*) by producing phytohormone. Antonie Van Leeuwenhoek. 101(4):777–86. https://doi.org/10.1007/s10482-011-9692-9

Mendes R, Garbeva P, Raaijmakers JM. 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol Rev. 37(5): 634-63. https://doi.org/10.1111/1574-6976.12028.

Morrison, E. N., Emery, R. J. N. and Saville, B. J. 2017. Fungal derived cytokinins are necessary for normal *Ustilago maydis* infection of maize. Plant Pathol, 66: 726-742. https://doi.org/10.1111/ppa.12629

Nakano, M., Omae, N., and Tsuda, K. 2022. Inter-organismal phytohormone networks in plant–microbe interactions. Current Opinion in Plant Biology. 68:102258. https://doi.org/10.1016/j.pbi.2022.102258

Nicholson, R. I. D., and Van Staden, J. 1988. Cytokinins and Mango Flower Malformation. I. Tentative Identification of the Complement in Healthy and Malformed Inflorescences. Journal of Plant Physiology 132:720–724. https://doi.org/10.1016/S0176-1617(88)80235-9.

Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C., and Bakker, P. A. 2014. Induced systemic resistance by beneficial microbes. Annual Review of Phytopathology 52: 347–375. https://doi.org/10.1146/annurev-phyto-082712-102340.

Palberg, D., Kisiała, A., Jorge, G.L., and Emery, R.J.N. 2022. A survey of *Methylobacterium* species and strains reveals widespread production and varying profiles of cytokinin phytohormones. BMC Microbiol. 22(1): 1–17. https://doi.org/10.1186/s12866-022-02454-9.

Perera, I., Kisiala, A., Thompson, K. A., and Emery, R. J. N. 2025. Soil health improvements under cover crops are associated with enhanced soil content of cytokinins. Plant Biol J 27:265–278. https://doi.org/10.1111/plb.13743.

Qi, P.-F., Zhang, Y.-Z., Liu, C.-H., Chen, Q., Guo, Z.-R., Wang, Y., Xu, B.-J., Jiang, Y.-F., Zheng, T., Gong, X., Luo, C.-H., Wu, W., Kong, L., Deng, M., Ma, J., Lan, X.-J., Jiang, Q.-T., Wei, Y.-M., Wang, J.-R., and Zheng, Y.-L. 2019. Functional analysis of FgNahG clarifies the contribution of salicylic acid to

wheat (*Triticum aestivum*) resistance against *Fusarium* head blight. Toxins (Basel) 11:59. https://doi.org/10.3390/toxins11020059.

Quazi, S. A. J., Meon, S., Jaafar, H., and Ahmad, Z. A. B. M. 2015. The role of phytohormones in relation to bakanae disease development and symptoms expression. Physiological and Molecular Plant Pathology 90:27–38. https://doi.org/10.1016/j.pmpp.2015.02.001.

Rispail, N., and Di Pietro, A. 2010. The two-component histidine kinase Fhk1 controls stress adaptation and virulence of *Fusarium oxysporum*. Molecular Plant Pathology 11:395–407. https://doi.org/10.1111/j.1364-3703.2010.00613.x.

Senthilkumar, M. and Krishnamoorthy, R. 2017. Isolation and characterization of tomato leaf phyllosphere *Methylobacterium* and their effect on plant growth. Int J Curr Microbiol Appl Sci. 6(11): 2121–36. https://doi.org/10.20546/ijcmas.2017.611.250

Sørensen, J. L., Benfield, A. H., Wollenberg, R. D., Westphal, K., Wimmer, R., Nielsen, M. R., Nielsen, K. F., Carere, J., Covarelli, L., Beccari, G., Powell, J., Yamashino, T., Kogler, H., Sondergaard, T. E., and Gardiner, D. M. 2018. The cereal pathogen *Fusarium pseudograminearum* produces a new class of active cytokinins during infection. Molecular Plant Pathology 19: 1140–1154. https://doi.org/10.1111/mpp.12593.

Spaepen, S., and Vanderleyden, J. 2011. Auxin and plant-microbe interactions. Cold Spring Harbor Perspectives in Biology 3:a001438. https://doi.org/10.1101/cshperspect.a001438.

Trdá, L., Barešová, M., Šašek, V., Nováková, M., Zahajská, L., Dobrev, P. I., Motyka, V., and Burketová, L. 2017. Cytokinin Metabolism of Pathogenic Fungus *Leptosphaeria maculans* Involves Isopentenyltransferase, Adenosine Kinase and Cytokinin Oxidase/Dehydrogenase. Front. Microbiol. 8:1374. https://doi.org/10.3389/fmicb.2017.01374.

Tudzynski, B. 2005. Gibberellin biosynthesis in fungi: genes, enzymes, evolution, and impact on biotechnology. Applied Microbiology and Biotechnology 66:597–611. https://doi.org/10.1007/s00253-004-1805-1.

Tzipilevich, E., Russ, D., Dangl, J. L., and Benfey, P. N. 2021. Plant immune system activation is necessary for efficient root colonization by auxin-secreting beneficial bacteria. Cell Host and Microbe 29:1507–1520. https://doi.org/10.1016/j.chom.2021.08.005.

Vanstraelen, M., and Benková, E. 2012. Hormonal interactions in the regulation of plant development. Annual Review of Cell and Developmental Biology 28, 463–487. https://doi.org/10.1146/annurev-cellbio-101011-155741

van der Heijden MG and Hartmann M. 2016. Networking in the Plant Microbiome. PLoS Biol. 14(2):e1002378. https://doi.org/10.1371/journal.pbio.1002378.

Van Staden, J. and Nicholson, R. I. D. 1989. Cytokinins and mango flower malformation II. The cytokinin complement produced by *Fusarium moniliforme* and the ability of the fungus to incorporate [8-14C]adenine into cytokinins. Physiological and Molecular Plant Pathology 35:423–431. https://doi.org/10.1016/0885-5765(89)90061-1.

Yang, Y.-X., Ahammed, G. J., Wu, C., Fan, S.-Y., and Zhou, Y.-H. 2019. Cross-talk among jasmonate, salicylate, and ethylene signalling pathways in plant disease and immune responses. Journal of Integrative Plant Biology 61:1112–1127. https://doi.org/10.1111/jipb.12750.

Zobiole, L. H. S., Kremer, R. J., Oliveira, R. S., and Constantin, J. 2011. Glyphosate affects chlorophyll, nodulation, and nutrient accumulation of "second generation" glyphosate-resistant soybean (*Glycine max* L.). Pesticide Biochemistry and Physiology 99:53–60. https://doi.org/10.1016/j.pestbp.2010.10.005.

Zhang, J., Zheng, Q., Guo, W., and Zong, X. 2020. Salicylic acid biosynthesis and metabolism: a divergent pathway for plants and lacking in filamentous fungi. Frontiers in Plant Science 11:705.