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Benzoxazinoids influence rhizosphere establishment and root colonization by PGPB

Jeroen Baatsen

Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

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Master of Science in Biochemistry and Biotechnology

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“Messieurs, c'est les microbes qui auront le dernier mot." (Gentlemen, it is the microbes who will have the last word.)”

— Louis Pasteur

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CONTENTS

RESUMO

Os benzoxazinóides influenciam o estabelecimento na rizosfera e a colonização radicular pelo BPCP

Benzoxazinoides (BXs) formam um grupo de metabólitos secundários produzidos por muitas plantas da família das gramíneas (Poaceae). A liberação e ativação dos BXs durante o ataque de patógenos suprimem fortemente doenças de espécies de pragas e a forragem de insetos herbívoros em partes aéreas da planta. Ao mesmo tempo, os BXs são produzidos constitutivamente e liberados na rizosfera predominantemente durante o desenvolvimento inicial da planta, onde eles afetam a diversidade microbiana. Os derivados de ácido hidroxâmico dos BXs, como 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) e 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA), em geral, são mais reativos, mas têm meia-vida mais curta do que os derivados de lactama 2-benzoxazolinone (BOA) e 6-methoxy-2-benzoxazolinone (MBOA). Independentemente, o MBOA é mais eficiente na supressão de vários patógenos fúngicos e influência a interação da rizosfera com microrganismos ao longo das gerações de plantas. A chave para entender a simbiose planta-microrganismo é o conhecimento sobre os meios de comunicação química entre os simbiontes e as mudanças fisiológicas que essas moléculas sinalizadoras provocam em cada simbionte. Portanto, nosso objetivo foi estudar os mecanismos pelos quais uma troca interespecífica de informações precede o estabelecimento da simbiose. Para obter mais informações sobre esses processos, investigamos como o MBOA medeia a colonização das raízes pelas bactérias promotoras do crescimento das plantas (BPCP) *Azospirillum brasilense* Ab-V5, *Bacillus thuringiensis* RZ2MS9, *Pantoea agglomerans* 33.1 e *Pseudomonas protegens* Pf-5, e o efeito adverso em várias espécies fúngicas do gênero *Fusarium*. A resposta bacteriana ao MBOA exógeno é específica para cada BPCP e dependente da dose. Curiosamente, linhagens de *Fusarium* isoladas de hospedeiros que não produzem BX foram suscetíveis ao MBOA em baixas doses, enquanto *Fusarium* isolado do milho (hospedeiro produtor de BX) é tolerante. Padrões de colonização radicular por Ab-V5 e Pf-5 foram estudados mais detalhadamente, revelando uma preferência por fendas e pelos pelos radiculares como locais primários de colonização. MBOA não influenciou a formação de biofilme por Pf-5 nas raízes de Arabidopsis, mas o biofilme de Ab-V5 foi aprimorado. Por fim, os resultados de experimentos in vitro foram validados cruzadamente por ensaios transcriptômicos em Ab-V5, nos quais uma proteína reguladora de quimiotaxia mostrou uma upregulação relativa no tratamento com 0,05 mM de MBOA, e pudemos correlacionar a quantidade de genes diferencialmente expressos relacionados à produção de biofilme com a concentração de MBOA. O transcriptoma de Pf-5, no entanto, foi pouco afetado, o que foi consistente com resultados previamente obtidos. Concluímos que MBOA em concentrações intermediárias estimula a forma móvel de Ab-V5, enquanto concentrações elevadas de MBOA provocam uma mudança metabólica em preparação para a colonização radicular.

Palavras-chave: Benzoxazinoides, BPCP, Colonização radicular, *Fusarium*, Quimoitaxia, Biofilme, Transciptoma

ABSTRACT

Benzoxazinoids influence rhizosphere establishment and root colonization by PGPB

Benzoxazinoids (BXs) form a group of secondary metabolites produced by many plants of the grass family (*Poaceae*). Release and activation of BXs upon pathogen attack strongly suppresses disease of pest species and foraging of herbivorous insects in areal parts of the plant. At the same time, BXs are constitutively produced and set free in the rhizosphere predominantly during early plant development, where they affect microbial interaction. Hydroxamic acid BX derivatives such as 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA) in general are more reactive but have a shorter half-life than the lactam derivatives 2-benzoxazolinone (BOA) and 6-methoxy-2-benzoxazolinone (MBOA). Regardless, MBOA is more efficient at suppressing several fungal pathogens and influences microbial rhizospheric interactions over generations of plants. Key to understanding plant-microbe symbiosis is knowledge about the means of chemical communication between symbionts, and the physiological changes those signaling molecules evoke on each symbiont. Therefore, we aimed to study the mechanisms by which an interspecies exchange of information precedes the initiation of symbiosis establishment. In order to gain more insight into these processes, we investigated how MBOA mediate root colonization by the plant growth promoting bacteria (PGPB) *Azospirillum brasilense* Ab-V5, *Bacillus thuringiensis* RZ2MS9, *Pantoea agglomerans* 33.1 and *Pseudomonas protegens* Pf-5, and the adverse effect on several fungal species of the pathogenic *Fusarium*. We found that bacterial response to exogenic MBOA was specific for each PGPB and dose dependent. Curiously, *Fusarium* strains isolated from non-BX-producing hosts were susceptible to MBOA at low doses, while maize isolated *Fusarium* (from a BX-producing host) was tolerant. Root colonization patterns by Ab-V5 and Pf-5 were studied in more detail, showing preference for crevices and root hairs as primary colonization sites. MBOA did not influence Pf-5 biofilm formation on *Arabidopsis* roots but Ab-V5 biofilm was improved. Finally, results from *in vitro* experiments were cross validated by transcriptomic assays on Ab-V5 where a chemotaxis regulatory protein showed a relative upregulation in 0.05 mM MBOA treatment and we could correlated the amount of differential expressed genes related to biofilm production with MBOA concentration. The Pf-5 transcriptome however, was little affected, which was consistent with previously obtained results. We conclude that MBOA in intermediate concentrations stimulates the motile form Ab-V5, while high concentrations of MBOA evokes a metabolic switch in preparation of root colonization.

Keywords: Benzoxazinoids, PGPB, Root colonization, *Fusarium*, Chemotaxis, Biofilm, Transciptome

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LIST OF ABBREVIATIONS

AAMPO	2-acetylamino-7-methoxy-phenoxazin-3-one
AAPO	2-acetylamino-phenoxazin-3-one
ACC	1-amino cyclopropane-1-carboxylic acid
AHL	*N*-acetylhomoserine lactone
AI	auto inducer
AMPO	5-methoxy-2-aminophenoxazin-3-one
APO	2-amino-3H-phenoxazin-3-one
BOA	2-benzoxazolinone
BX	Benzoxazinoids
CAGE	cap analysis gene expression
cDNA	complementary / copy DNA
CFU	colony forming units
CLSM	confocal laser scanning microscopy
DAPG	2.4-diacetylphoroglucinol
DEG	differentialy expressed Gene
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
EIL3	ETHYLENE INSENSITIVE3 (EIN3)-LIKE3
EPS	extracellular polymeric substances
EST	expressed seqience tag
f.sp.	formae speciales
HCT	horizontal chromosome transfer
HDMBOA	2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one
HGT	horizontal gene transfer
hpi	hours post inoculation
IAA	indole-3-acetic acid
ISR	induced systemic resistance
JA	jasmonic acid
KMB	Kings medium B
LB	Luria-Bertani
logFC	log fold change
LPS	lipopolysaccharides
MAMP	microbial-associated molecular pattern
MBOA	6-methoxy-2-benzoxazolinone
mRNA	messenger RNA
ncRNA	non-coding RNA
NGS	next generation sequencing
NPR1	Nonexpressor of PR1
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGPR	Plant Growth Promoting Bacteria
QQ	quorum quenching
QS	quorum sensing
RNA-seq	RNA sequencing
ROS	reactive oxygen species
rpm	rounds per minute
rRNA	ribosomal RNA
SA	salicylic acid
SAGE	serial analysis of gene expression
SAM	S-adenosyl-methionine
SEM	Scanning Electron Microscopy
TAD	tight adhesion
VOC	volatile organic compound

1. INTRODUCTION

One of the most difficult challenges society is facing nowadays is coping with world hunger. The increase of atmospheric carbon dioxide and global average temperatures each year account for more frequent extreme weather events that lead to decreasing crop yield and productivity (Mirón et al. 2023). With a 5 % decrease of arable land yearly (Borrelli et al. 2020), increasing land use and crop efficiency pose viable, if not the only solutions for keeping up with the current and future food demands.

In this context, it appears that the root microbiome can play an important role in improving crop efficiency. A compelling amount of evidence demonstrate how the plant microbiome in its whole forms a safety network repelling pathogens (Glenn et al. 2001, 2003a, Oikawa et al. 2004, Copaja et al. 2006, Søltoft et al. 2008, Glenn and Bacon 2009, Niemeyer 2009, Ahmad et al. 2011, Meihls et al. 2013, Śmist et al. 2016); improves abiotic stress tolerance and water and nutrient absorption (Gond et al. 2015, Vurukonda et al. 2016, Schütz et al. 2018, Kumar et al. 2019, Dehghani and Mostajeran 2020, Liu et al. 2020, Marques et al. 2020, Subiramani et al. 2020, Aloo et al. 2022), eventually leading to the overall biomass increase of the host plant (Hungria et al. 2010, 2015, 2018, Mishra et al. 2011, Quecine et al. 2012a, Oliveira et al. 2017). Most importantly, microbial inoculation of crops grants a buffer capacity to withstand various adverse abiotic and biotic stress, abolishing the need for energy demanding fertilizers and agrochemicals (Daramola and Hatzell 2023). On the long term, depletion of soil nutrients by over application of fertilizers can be evaded by intelligent and accurate inoculation of plant growth promoting bacteria (PGPB) aiming at sustainable agriculture in hand with environmental stability (Mohanty et al. 2021, Shah et al. 2021). Hence, manipulating the plant microbiome can provide significant increases in yield gain especially in more challenging and heterogeneous terrain (Teste et al. 2017, Schütz et al. 2018).

To optimize benefits gained from a healthy rhizobiome, plants naturally manipulate the composition of symbionts in the soil by secreting a plethora of primary and secondary metabolites (Bais et al. 2006, Bever et al. 2013, Bulgarelli et al. 2013). In various plants of the grass family, benzoxazinoids (BXs) in the soil fulfill an important role in shaping the root microbiome (Chen et al. 2010, Hu et al. 2018b, Cotton et al. 2019, Cadot et al. 2021). Specifically 6-methoxy-2-benzoxazolinone (MBOA) is efficient in suppression of fungal pathogens (Oikawa et al. 2004) and promotes herbivore tolerance by inducing systemic

defense through rhizobiome structuring (Hu et al. 2018b). Given the moderate 5.4 days half-life of MBOA (Etzerodt et al. 2008) and the lasting biosynthesis and presence in the soil (Cambier et al. 2000, Hu et al. 2018b), the influence of MBOA is sustained throughout the next generation of plant progeny (Hu et al. 2018b).

The efficacy of microbial inoculants however, depends on many factors such as cultivar, environment (Pacheco da Silva et al. 2022) and inoculation level (Renoud et al. 2022). Prominent causes of unsuccessful introduction of microbial inoculants are the incompetence to compete with the native soil microbiome (Herschkovitz et al. 2005) or the inability to adapt to the local environmental factors, characteristic for the soil type and cultivar (Martinez-Viveros et al. 2010). Therefore, conditioning of the soil with MBOA might improve environmental conditions and increase the success rate of microbial inoculation in crop cultivation.

For testing this hypothesis, we addressed the following research questions: 1. How does exogenous MBOA affect bacterial mechanisms of PGPB pertaining to plant root colonization, and how do *Fusarium* strains tolerate MBOA; 2. Does MBOA inflict observable changes on root colonization *in planta*; and 3. How does MBOA affect bacterial gene expression?

To complement the lack of knowledge concerning MBOA and its effect on bacterial rhizosphere competence and root colonization, we tested four different PGPB: *Azospirillum brasilense* Ab-V5, *Bacillus thuringiensis* RZ2MS9, *Pantoea agglomerans* 33.1 and *Pseudomonas protegens* Pf-5. We assessed specific and common behavioral responses to MBOA treatment, when testing for tolerance, biofilm formation, and chemotaxis. At the same time, we analyzed whether strains of the phytopathogenic and necrotrophic *Fusarium* were tolerant to MBOA and if there was any correlation with adaptation to the host plant they were isolated from. Those experiments are discussed in Chapter 3, where we demonstrate how each PGPB shows a specific and dose dependent response in biofilm formation to MBOA treatment, and attraction of *A. brasilense* Ab-V5 to MBOA in a modified capillary assay, in both high and moderate dosage. Furthermore, we could infer that *Fusarium* isolated from BX producing hosts were more tolerant to MBOA than BX free hosts.

Based on the results of the biofilm and chemotaxis assays used for research question 1, we chose *A. brasilense* Ab-V5 and *P. protegens* Pf-5 for more detailed examination by microscopy in Chapter 4, to respond to research question 2. Because in Ab-V5 elements of the Che1 chemotaxis pathway plays a role in adhesion indirectly (Bible et al. 2008, 2012, Siuti et al. 2011), we evaluated adherence of Ab-V5 to *Arabidopsis thaliana* roots and peroxidase

activity under influence of ambient MBOA. In Chapter 4, we showed how root colonization by Ab-V5 and Pf-5 resulted in a higher peroxidase activity independent of the MBOA treatment, thus demonstrating how colonization may evoke a systemic immune response. The number of adhering cells however, was not notably different. As expected, we could draw the same conclusions from biofilm observed with microscopy on live plant roots with *in vitro* measurements of biofilm from Ab-V5 and Pf-5 at the same time point after inoculation. Ab-V5 showed enhanced biofilm after 120 hours of inoculation and treatment with MBOA, while biofilm of Pf-5 was not significantly different form control treatments.

We proceeded by analyzing Ab-V5 and Pf-5, both PGPB responding very differently on MBOA treatment. From static *in vitro* cultures, we analyzed the transcriptome of both strains in Chapter 5, answering research question 3. Findings from bioinformatics analysis are in line with conclusions we drew from the two preceding chapters. As in all assays, Pf-5 is more tolerant to MBOA which translates to very limited influence on gene expression profiles. However, in Ab-V5 lots of genes in energy metabolism were relatively upregulated while symbiosis related genes were relatively dowregulated. This includes genes for biofilm formation, nitrogen metabolism and tight adhesion (TAD) pili. Chemotaxis, on the other hand, was stimulated by relative upregulation of a regulatory gene in comparison with the control treatment.

With this research project we hope to have contributed to the current state of knowledge by studying the complex influence of MBOA on fundamental mechanisms of plant root colonization, such as bacterial biofilm and chemotaxis. Despite the well-studied bacterial feature biofilm as a mechanism for attachment and protection from the environment (Costerson et al. 1995, Bloemberg and Lugtenberg 2004, Ramey et al. 2004, Larsen et al. 2008, Pizzirani-kleiner 2011, Arruebarrena Di Palma et al. 2013, Ueda and Saneoka 2015, Shumilova et al. 2016, Flemming et al. 2016, Pagnussat et al. 2016, Yin et al. 2019, Mina et al. 2019, Shelud'ko et al. 2019, Viruega-Góngora et al. 2020), the number of studies reporting on the effect of BXs on bacterial biofilms is very limited (Guo et al. 2016) not including any studies conducted on PGPB. Similarly, the scientific reports on chemotactic response to BX is limited to only one study elaborating on chemotactic behavior of the PGPB *Pseudomonas putida* towards DIMBOA via capillary assays, microarray and fluorescent microscopy (Neal et al. 2012).

Similarly, there has not been any report on a genome-wide transcriptomic analysis on PGPB so far when treated with MBOA. It has been found though, that treatment of *P. putida* with 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) caused the induction of

chemotaxis related genes (Neal et al. 2012). In the other way around, colonization of maize plants with *A. brasilense* genotype specifically modified the secondary metabolite profile of host plants especially within the group of BXs (Walker et al. 2011), a similar feedback on the BX metabolism was observed when maize plants were inoculated with *P. fluorescens* MZ05 (Zhou et al. 2020). When *A. thaliana* was inoculated with *A. brasilense*, genes related to defense, hormones and the cell wall were induced in the host plant which partly depended on auxin production of the bacteria (Spaepen et al. 2014). Interestingly, when wheat roots, from a BX producing grass plant, were infected by *A. brasilense*, bacterial chemotaxis, biofilm and nitrogen fixation related genes were upregulated (Camilios-Neto et al. 2014).

We further elaborate on the current state of knowledge concerning all aspects that relate to the research field within the context of this doctoral thesis in the next chapter. Chapter 2, 'Literature' can be used at all times as a holdfast for explaining certain concepts discussed in the chapters to follow.

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2. REVIEW OF LITERATURE

In 2021, more than one billion people suffered either acute or chronic hunger globally, as many as 13 percent of the world population (World Food Programme (WFP) 2022). The global pandemic caused by COVID-19 complicated matters even more, by hindering accessibility of territories; the transport of goods and by soaring fuel prices. Other drivers of hunger are conflict and climate crisis. Whether it will be feasible to keep rising global temperatures in check will depend on current actions undertaken. Global heating, climate extremes like floods, droughts, storms and disruption of seasonal changes, affect food accessibility and strikes where is needed the most (Mirón et al. 2023). Climate variability calls upon actions undertaken on a global scale including improvement of the efficiency within the agroindustry sector. Important aspects for crop development to meet those demands are sustainability of land use and versatility of crops to withstand adverse conditions.

Traditionally, farmers apply significant amounts of agrochemicals and fertilizers to overcome hurdles in yield gain. Yet, excessive use of conventional fertilizers cause acidification of the soil and makes nutrients unavailable for uptake by crops (Gupta et al. 2015), rendering the opposite effect on the long term. Unavoidably, for satisfying a long lasting intensification of the agroindustry, alternatives to such traditional methods must be drawn on. Both sustainability and flexibility can be attained by employing microbial inoculations, to omit drought stress (Gond et al. 2015, Vurukonda et al. 2016, Dehghani and Mostajeran 2020, Marques et al. 2020), for biocontrol (Pieterse et al. 1996, Whipps 2001, Quecine et al. 2014, 2016, Saraf et al. 2014, Loper et al. 2016) and increase crop yield (Hungria et al. 2010, 2015, 2018, Mishra et al. 2011, Quecine et al. 2012a, Oliveira et al. 2017). Microbial inoculants are typically found in the artificial group of plant growth promoting bacteria (PGPB): soil-dwelling bacteria that have positive effects on plant health.

In what follows we will discuss: 1. the use and features of PGPB as microbial inoculants; 2. the diversity; biosynthesis; mode of action and role of benzoxazinoids (BX) in root colonization; 3. Biology, occurrence and diseases caused by *Fusarium* and 4. History, sequencing platforms and data analysis of RNA sequencing data.

2.1. Plant growth promoting bacteria

2.1.1. Commercial inoculants

The term “rhizosphere” was introduced for the first time in 1904 by Lorenz Hiltner (Hartmann et al. 2008), stating that the rhizosphere or “soil influenced by roots” creates the

capacity for bacteria to fixate nitrogen. Furthermore, it was noted that exudates of legume plants attract different bacteria to the rhizosphere than mustard or oats, in respect to their specific nutritional needs. Preceding elaborate studies on symbioses of nodule forming bacteria in legume plants, development of the first microbial inoculant was launched in 1891 (Nobbe et al. 1891, Nobbe and Hiltner 1893). Those studies elaborated on the specificity of the host plant to enable symbiosis with the microbial inoculant and specific handling and preparation methods for keeping the inoculant viable.

Microbial inoculants can be applied to crops in the form of a liquid solution coating the seeds, or in presence of a carrier material such as cork, perlite or bargasse (Albareda et al. 2008). Inoculating crops with PGPB proved challenging, due to unsuitable environmental conditions or the inability to compete with the native microbiome (Catroux et al. 2001). More recently, encapsulated bacteria are being used for inoculation, granting protection from the environment and allowing slow release in the soil and adaptation of the bacteria to the new environment (John et al. 2011). A well-studied polymer for encapsulating PGPB is alginate, which polymerizes in the presence of calcium and has the capability to contain substantial amounts of bacteria (Zohar-Perez et al. 2002). In alignment with the environment, gel forming bio-polymers serve well by the fact that they are non-toxic and provide an energy source (Bahsan et al. 2014). Typically combinations of polymers being used for encapsulation are pectin, starch, dextrin and vegetable proteins (Khan et al. 2013, Nesterenko et al. 2013).

2.1.2. Bacterial features pertaining to root colonization

Considering the relative large amount of plant metabolites released in the soil, plant hosts are main drivers for soil community structuring. Up to 11 % of the host net fixed carbon plus 10 – 16 % of fixed nitrogen metabolites are set free by rhizodeposition (Jones et al. 2009, Pausch and Kuzyakov 2018, Sasse et al. 2018). Apart from metabolites, in maize plants, root cap border cells are detached that remain active for at least a week in the soil (Vermeer and McCully 1982). After a substrate-driven accumulation of a candidate population for rhizosphere colonization, follows a host genotype dependent selection of epiphytes and endophytes competent for colonizing the rhizoplane and interior of the host plant (Bulgarelli et al. 2013). This stringent filtering determines the difference between the bacterial density in the rhizosphere of $10^7 – 10^9$ colony forming units (CFU) g^{-1} (Benizri et al. 2001) and $10^5 – 10^7$ CFU g^{-1} (Benizri et al. 2001, Bais et al. 2006) in the rhizoplane. This genotype specific selection is based on the innate immune system of plants which blocks pathogen invasion upon recognition of microbial-associated molecular patterns (MAMPs) by pattern recognition

receptors at the cell surfaces (Jones and Dangl 2006). To engage in a symbiotic relation, bacteria need to be able to evade or suppress the host immune response, like pathogenic microbes are capable of (Boller and He 2009). Production of auxin by PGPB can inhibit salicylic acid (SA) signaling, and suppress innate immune response (Kunkel and Harper 2018). In *B. velezensis* auxin production is indispensable for suppressing plant immune response and reactive oxygen species (ROS) production, which was induced by root colonization at first (Tzipilevich et al. 2021). Yet a lot remains to be uncovered regarding the exact mechanisms of evading the plant immune response by PGPB.

Important to many PGPB for exerting the abilities that improve plant performance as described below in section 2.1.3. PGPB functions, is proper plant or rhizosphere colonization (Compant et al. 2010). Rhizosphere competent bacteria profit from root colonization by occupying a protective ecological niche that provides stable environmental conditions and nutrients (Senthilkumar et al. 2011). In order to establish an intimate relation with the host plant, PGPB communicate for simultaneous undertaken actions within the bacterial community; need to be recruited by chemotaxis from the bulk soil and anchor themselves on the root surface by production of biofilm.

2.1.2.1. Quorum sensing

A wide range of bacteria produce auto inducer (AI) molecules that are perceived by other individual cells and stimulate their own biosynthesis. In this way bacteria are able to monitor their population density and act accordingly, through quorum sensing (QS) (Fuqua and Winans 1994). Many processes are regulated that way, controlling bacterial behavior in various environments. The threshold to be reached for AI to regulate a certain process, depends on the QS system as well as the regulators involved. *N*-acylhomoserine lactones (AHLs) are synthesized from S-adenosyl-methionine (SAM) by the LuxI synthase (Schaefer et al. 1996) in α-, β- and γ-proteobacteria (A and Chen 2011) with varying acyl chain lengths donated by acyl chain carrying proteins. Sensing of AHLs happens via interaction with the N-terminal receptor domain of LuxR proteins, while its C-terminal domain has DNA-binding properties (Koch et al. 2005). Some Gram-positive bacteria and Gram-negative bacteria make use of the AI-2 system which is a mixture of compounds resulting from cyclization of 4,5-dihydroxy-2,3-pentanedione mediated by LuxS synthase, also found in the pathogens *Salmonella typhimurium* and *Vibrio cholerae* (Surette et al. 1999). Several other QS molecules are derived from fatty acids (He and Zang 2008); from amino acids and peptides (Holden et al. 1999, Monnet et al. 2014).

QS regulates a wide variety of processes falling into four classes: cell behavior; cell maintenance; horizontal gene transfer and microbe-host interactions (Fuqua and Winans 1994, Whitehead et al. 2001, Jimenez et al. 2012, Monnet et al. 2014). In this section we will limit ourselves to processes regulated by QS that pertain to root colonization and microbe-host interactions.

To make it even more complex, it is possible for bacteria to contain several QS signaling pathways at the same time, for instance the biocontrol strain *P. fluorescens* 2P24 has both the LuxR/LuxI and PcoR/PcoI systems. 2.4-diacetylphoroglucinol (DAPG) production by *P. fluorescens* is regulated by a density dependent manner, though not under control of any QS mechanism (Delany et al. 2000, Schnider-Keel et al. 2000). Consequently, when the *PcoI* gene has been knocked-out, DAPG is produced as normal, though biofilm formation and colonization of wheat roots is strongly impaired (Wei and Zhang 2006). Similarly, in *Serratia plymuthica* AHL signaling is indispensable for colonization of bean roots; for biocontrol of the phytopathogen *P. aphanedermatum* and for activation of induced systemic resistance (ISR) (Pang et al. 2009). In rhizobia-legume symbiosis, QS has a significant impact on the intimate host-symbiont relation in several cases, but is not always indispensable. For example, in *Rizobium elite* CNPAF512 mutants defect in the LuxI-type AHL synthesis gene *cinI* or LuxR-type AHL regulator gene *cinR* caused decreased N fixation and aberrant bacteroid development in nodules (Daniels et al. 2002). In contrast, in *R. leguminosarum* bv. *viciae,* mutants of the *cinI* or *cinR* genes did not impair symbiosis and even increase the number of nodules (Rosemeyer et al. 1998, Wisniewski-Dyé et al. 2002).

Several important abilities and processes of bacteria are controlled by QS, as exemplified above. For decent root colonization, chemotaxis and biofilm formation are of paramount importance. Both are often regulated by QS (Solano et al. 2014, Jani et al. 2017, Fukami et al. 2018a, Zhang et al. 2020), via highly intertwined regulatory mechanisms (Bahlawane et al. 2008, Jani et al. 2017, Berne and Brun 2019). In *Sinorhizobium meliloti*, the AHL regulator protein ExpR inhibits visNR expression by binding in its operon region, which is a regulator of the flagellation gene set (Bahlawane et al. 2008). Although flagellar independent movement of bacteria is promoted by ExpR by production of extracellular polymeric substances (EPS) reducing friction with the contact surface and allows bacteria to spread by passive movement through sliding (Nogales et al. 2012).

Depending on the bacteria and the environment it is adapted to, AI influence biofilm formation differently. In *V. cholerae* and *Staphylococcus aureas,* accumulation of AI represses biofilm formation, while in *P. aruginosa* biofilm production is stimulated in the

presence of high AI concentrations (de Kievit and Iglewski 2000, Bronesky 2016). When exposed to fluid flow, the produced AI in the environment are carried away, consequently, biofilm production in *V. cholerae* and *S. aureus* is stimulated (Kim et al. 2016). After establishment of biofilm, cells that are shielded form fluid flow by neighboring cells experience a buildup in AI which represses biofilm formation. Thus, bacteria within a biofilm adapt different roles based on AI levels and hence QS exerted genetic control, defined by their spatially distribution (Kim et al. 2016).

Even though QS is a mechanism widespread in prokaryotes and archaea, AI also affects eukaryotes. The diverse functions bacterial AI have on their plant host correlate with the length of the acyl chain, AHL containing short acyl chains have plant growth stimulating properties, while AHL with long acyl chains stimulate ISR and pathogen defense (Schikora et al. 2011, 2016, Zarkani et al. 2013, Schenk et al. 2014, Calatrava-Morales et al. 2018). In the other way around, plants can produce compounds that perturb QS (Rasmussen et al. 2005, Koh et al. 2013), sometimes resulting in pathogen tolerance. Inside the human gut, epithelial cells can produce AI-2 mimics as a response to interaction with bacteria. Those mimics interact with the AI-2 receptor LuxP/LsrB in *Salmonella typhimurium* and control QS dependent gene regulation (Ismail et al. 2016). Fungal derived QS molecules play important roles in fungal morphogenesis, biofilm formation and pathogenicity (reviewed in WONGSUK; PUMEESAT; LUPLERTLOP, 2016). Moreover, some mycotoxic compounds also suppress quorum sensing such as fusaric acid from *Fusarium* species, which on top of that inhibits antibiotic production of biocontrol bacteria (Manefield et al. 1999, Van Rij et al. 2005, Quecine et al. 2016).

2.1.2.2. Chemotaxis

To support a rich microbiome in the soil, plants release large amounts of fixed carbon and nitrogen in the form of primary and secondary metabolites, mucilage and proteins (Bais et al. 2006). The majority of root exudates are primary metabolites such as carbohydrates, amino acids and organic acids while secondary metabolites like flavonols, lignins, coumarins, and indole compounds make up a smaller moiety (Bardi and Vivanco 2009). Low molecular weight compounds are released by passive transport over the plasma membrane via concentration driven diffusion, vesicle transport and through ion channels (Bardi and Vivanco 2009, Dreyer et al. 2012). Alternatively, metabolites are translocated via transporter proteins in the plasma membrane by an active transport mechanism (Baetz and Martinoia 2014). Two families of membrane bound transporter proteins exist: ATP-Binding Cassette (ABC) and

Multidrug and toxic compound extrusion (MATE) transporters. ABC transporters are called primary transporters that harness biochemical energy from ATP hydrolysis for transport of various substrates (Orelle et al. 2018), while MATE are secondary transporters which make use of the electrochemical differential to facilitate transport over the plasma membrane (Weston et al. 2012).

One percent of exudated secondary metabolites are small organic compounds with a lipophilic character and a low boiling point, grouped in volatile organic compounds (VOCs) (Schmidt et al. 2015, Venturi and Keel 2016). Their physiochemical characteristics allow them to spread easily and have a wide area of influence in the surrounding soil, making them suitable chemo-attractants (Ali et al. 2010, Van Dam et al. 2016, Schulz-bohm et al. 2018). Other secondary metabolites within the group benzoxazinoids (BXs), are highly toxic and has therefore a strong influence on the microbial composition of the soil microbiome (Hu et al. 2018b, Cotton et al. 2019, Cadot et al. 2021) and pathogen defense (Niemeyer 2009, Ahmad et al. 2011, Neal and Ton 2013). Apart from these properties further discussed in section 2.2. Benzoxazinoids, DIMBOA causes chemo attraction of *P. putida* towards the rhizosphere of maize roots (Neal et al. 2012).

The presence of carbon and nitrogen rich component is the soil determines the preference of bacteria to move towards nutrient rich environments by sensing chemical gradients in the circumference. This directed movement is referred to as chemotaxis, a well-studied bacterial behavior (Colin and Sourjik 2017, Bi and Sourjik 2018). By rotating bundle forming flagella in the same direction, *E. coli* travels short distances until it rotates by tumbling. Chemotaxis manipulates the duration of straight runs along chemical gradients, resulting over time in a net directed diffusion of the bacteria (Larsen et al. 1974).

Sensory protein complexes are mainly located at the poles of *E. coli* (Yang and Briegel 2020), existing of the receptor proteins CheW and CheA. CheW contains a scaffolding domain and modulates the activity of CheA upon ligand binding, and CheA is a histidine kinase (Parkinson et al. 2015). The mobile regulatory protein CheY is phosphorylated by CheA and induces rotation of the flagellar motor. Dephosphorylation of CheY is carried out by CheZ, CheC or CheX, to maintain the function of the histidine kinase CheA in steering flagellar rotation (Silversmith 2010).

Another regulatory mechanism is constituted by methylation and demethylation of receptor proteins by CheR and CheB, altering its affinity for ligands by conformational changes (Kehry and Dahlquist 1982). The CheR and CheB system is much slower than the CheW, CheA and CheY mediated mechanism, which allows the modulation of bacterial

movement according to temporal changes in environments (Yi et al. 2000, Kalinin et al. 2009).

Amino acids are principally perceived by Tsr and Tar receptors that show overlap in their downstream signaling pathway, via CheA mediated CheY phosphorylation (Berg 2003). Those receptors are coupled by signalization pathway, but also physically influencing each other by a neighbor assisted mechanism showing similar methylation sites in receptor clusters (Li and Hazelbauer 2005). Consequently, the combination of the stimulation of both receptors result in different responses than when stimulated separately. More specifically, for instance rising aspartate concentrations does not influence the time interval of tumbling by *E. coli*, also referred to as 'perfect adaptation' (Alon et al. 1999). While ambient serine causes *E. coli* to switch course slower by tumbling according to the concentration increase, the serine response is therefore not perfectly adapted (Berg and Brown 1972). Now, when the serine concentration in kept constant, increase in aspartate makes *E. coli* tumble slower and hence the response to aspartate is no longer perfectly adapted (Wong-Ng et al. 2016). Perfect adaptation is of ecological relevance for bacteria when encountering swiftly altering peak concentrations of compounds during swimming in aqueous environments (Celani and Vergassola 2010).

2.1.2.3. Biofilm and root attachment

In order to adhere to various surfaces and substrates, bacteria commonly produce biofilm which is a protective coat composed of a polymeric matrix of EPS as main component (Costerson et al. 1995). Biofilms can contain diverse communities and grants protection against drought, ultraviolet radiation, extreme pH, pressure, antibiotics and more (Yin et al. 2019). Encapsulated in biofilm, PGPB are provided an advantage over free-living bacteria by being anchored in a nutrient rich and protective environment. *Rhizobium, Pseudomonas, Azospirillum, Agrobacterium* and *Salmonella* share a similar two-step root attaching mechanism to the root surface (Wheatly and Poole 2018). The first step is characterized by reversible, loose binding of bacteria to the surface, where after bacteria tightly adhere and form aggregates in the second step of attachment. In the following we will summarize the attachment mechanisms by those two phases in *Azospirillum brasilense, Pseudomonas putida* and *P. fluorescens.*

Because both bacterial cells and root epidermal cells carry a net negative charge, bacteria need to overcome electrostatic repulsion in order to physically reach the root surface (Berne et al. 2015). Therefore, during the first phase, *A. brasilense* propels its self towards the

root and makes contact with the surface using its polar flagellum, a non-specific and reversible interaction (Croes et al. 1993, Mora et al. 2008). *A. brasilense* Cd defect in the flagellin modification genes *flmA* and *flmB* resulted in non-motile cells; were incompetent in maize root adsorption and EPS and lipopolysaccharides (LPS) production, as a result of impaired polar flagellum assembly (Rossi et al. 2016). Similarly, in *A. brasilense* Sp245, bacteria with defect polar flagellum and lateral flagella accumulated less biofilm biomass (Shelud'ko et al. 2019). In both the first and second phase of attachment, the polar flagella and outer membrane proteins on the cell surface of *Azospirillum* are involved in absorption onto to root and in aggregation with other bacteria (Burdman et al. 2001). Bacteria that successfully attached to the root surface in the first phase are stimulated to proceed to the second phase which is marked by production of polysaccharide fibrils and aggregation of bacteria (Jofré et al. 2004). Factors that involve adherence in the irreversible second step in *Azospirillum* are polysaccharides rich in arabinose, LPS, outer membrane proteins and lectines (Michiels et al. 1991, De Troch and Vanderleyden 1996). *A. brasilense* Sp245 mutated in the *mmsB1* and *fabG1* genes, were impaired in LPS production and showed reduced hydrophobicity, cell aggregation and mature biofilm biomass (Shumilova et al. 2016). In the first two days, aggregated bacteria start forming biofilm producing micro colonies. It takes 3 to 5 days to form a mature biofilm, with an average thickness of 28 – 39 µm on the surface of a glass coverslip, depending on the strain (Viruega-Góngora et al. 2020). Root colonization patterns differ among *A. brasilense* strains: while *A. brasilense* Sp245 is able to penetrate the root epidermis and internally colonize root hairs and vasculature, colonization by *A. brasilense* Sp7 is limited to the root surface (Schloter and Hartmann 1998, Vande Broek et al. 1998a).

In *Pseudomonas putida* and *P. fluorescens* pili have a role in motility and primary attachment, and outer membrane porin F partakes in both steps of root attachment (Vesper 1987, De Mot and Vanderleyden 1991, Crespo and Vervalde 2009). *P. putida* and *P. fluorescens* possess two large adhesion proteins LapA and LapF that irreversibly attach the bacteria to the root and mark the onset of colony formation (Fuqua 2010). Furthermore, biosynthesis of cellulose fibrils ensures surface colonization, strengthening the biofilm by interaction of the LPSs and the cellulose matrix, a characteristic widely spread among *Pseudomonas* species (Spiers et al. 2003, Ude et al. 2006). Despite the similarities in root attachment mechanisms, *P. putida* and *P. fluorescens* differ in the way they occupy the surface of the root. *P. putida* produces a thick continuous biofilm spreading over the entire root, while biofilm from *P. fluorescens* is thinner and localized around fissures (Bloemberg et

al. 2000, Bloemberg and Lugtenberg 2004). The environmental conditions that stimulate biofilm formation in *Pseudomonas* is strain dependent: where *P. protegens* produces biofilm in nutrient rich environments, *P. fluoresens* and *P. putida* are stimulated to form biofilm in nutrient poor conditions (Ueda and Saneoka 2015).

Colonizing host tissue, PGPB undergo a transition from a motile lifestyle in bulk soil, to a sessile one when adhering to the root surface. Even being a complex process clearly marked by elaborate regulatory mechanisms, there does not seem to be a genetic reprogramming behind vegetative growth in biofilms (Whiteley et al. 2001, Sauer et al. 2002). Because the production of biofilm is dependent on nutrient availability (Arruebarrena Di Palma et al. 2013, Ueda and Saneoka 2015, Wang et al. 2017, Shelud'ko et al. 2020), the bacteria rather seem to adapt cellular motility and adhesion in function of environmental parameters.

2.1.3. PGPB functions

The soil microbiome is a highly dynamic and complex environment harboring millions of interacting communities composed of bacteria, archaea, fungi and viruses (Jansson and Hofmockel 2020). The microbiome of the soil has a huge impact on plant health (Bender et al. 2016), therefore it does not surprise that plants deposit around 10 % of carbon products in the rhizosphere for fueling the microbiome (Jones et al. 2009, Pausch and Kuzyakov 2018, Sasse et al. 2018). Hence, plants strongly influence the microbial composition of the soil by release of carbon in the form of primary and secondary metabolites (Hartmann et al. 2009, Cesco et al. 2010). To exploit nutritious secretes, bacteria are attracted to the rhizosphere and compete for the metabolites form the host plant (Neal et al. 2012, Pausch and Kuzyakov 2018, Sasse et al. 2018). The influence on composition of the soil microbiome, exerted by plants can last and be maintained for successive generations (Hu et al. 2018b, Wei et al. 2019). In return, PGPB lend their advantageous properties, relieving the host plant from various biotic (Neal et al. 2012, Hu et al. 2018b, Mendes et al. 2018, Liu et al. 2020, Gu et al. 2022), abiotic stresses (Vurukonda et al. 2016, Fukami et al. 2018b), and facilitating nutrient uptake (Schütz et al. 2018, Aloo et al. 2022). In general, healthy plants profit from a root microbiome with high diversity, in the case of *Arabidopsis thaliana*, with high abundance of *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* phyla, while containing less bacteria from the *Firmicutes* phylum (Lundberg et al. 2012, Bulgarelli et al. 2013).

2.1.3.1. Biostimulation

The stimulation of plant growth by PGPB is accomplished by means of three general mechanisms in any combination thereof: phytostimulation, biofertilization and biocontrol. Through production of phytohormones, many PGPB possess the ability to directly induce growth of the host plant by altering its hormone balance, which is referred to as phytostimulation (Bloemberg and Lugtenberg 2001).

In this context, the influence of ethylene and auxin on plant growth are the best studied cases. By production of 1-amino cyclopropane-1-carboxylic acid (ACC) deaminase by PGPB, the precursor ACC for ethylene, ACC is catabolized lowering the ethylene content in the root. A diminished ethylene level stimulates DNA synthesis, cell division and root and shoot growth (Burg 1973). Some bacterial endophytes that reportedly release ACC deaminase are *Achromobacter*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Rhizobium* and *Rhodococcus* (Belimov 2001, Ghosh et al. 2003, Szdiderics et al. 2007, Govindasamy et al. 2008, Duan et al. 2009). Synthesis of indole-3-acetic acid (IAA), a naturally occurring auxin class plant hormone, is principally produced from exuded tryptophan (Kravchenko et al. 2004). IAA biosynthesis by several PGPB stimulates root proliferation of the host plant (Dobbelaere et al. 1999, Khalid et al. 2004); can function as a signaling molecule (Spaepen et al. 2007); is indispensable for *Arabidopsis* root colonization with *Bacillus velezensis* FZB42 (Tzipilevich et al. 2021) and highly impacting for colonization with *Azospirillum brasilense* Sp245 (Méndez-Gómez et al. 2021) and *Bacillus thuringiensis* RZ2MS9 of tomato (Batista et al. 2021).

Several PGPB emit a species specific blend of VOCs to elicit plant growth responses. Recently, in four PGPB species, 121 VOCs were identified able to affect metabolism of the host plant (Mhlongo et al. 2022). Next to promoting plant growth (Ryu et al. 2003), VOCs can enhance drought stress response by elevated antioxidant enzymes when maize plants are expose to VOCs from *Pseudomonas pseudoalgcaligenes* (Yasmin et al. 2021) and when *A. thaliana* plants are exposed to *Burkholderia pyrrocinia* VOCs (Luo et al. 2022).

2.1.3.2. Biofertilization

Inoculation of PGPB may in addition to phytostimulators serve as biofertilizers. Depending on their properties and roles in the soil, there are several types of biofertilizing PGPB. Biofertilizers can consist of a consortium of PGPB or solely as a single strain exhibiting multiple PGPB features. A meta-analysis on a global scale, showed that biofertilization is the most efficient in dry climates rendering a yield increase of 20 %,

lowering progressively in wetter environments resulting on average in a 16.2 % yield increase over all regions (Schütz et al. 2018). The efficiency in phosphate and nitrogen use depends on the availability in the soil, ranging for phosphate from 15-25 kg/ha while nitrogen efficiency use was optimal with at least 45 kg/ha available in the soil (Schütz et al. 2018).

The most commonly used biofertilizers are nitrogen fixing PGPB, bacteria that convert atmospheric nitrogen (N_2) into ammonia (NH_3) requiring the Nif protein complex (Streicher et al. 1972). The N_2-fixing bacteria *Rhizobium* and *Bradyrhizobium* form nodules limiting oxygen exposure in the roots of various leguminous crops (Murray 2011), and are commercially available as biofertilizers (Adeleke et al. 2019). Other free-living N_2-fixers not limited to leguminous crops are *Azospirillum* (Boddy et al. 1986, Garcia de Salamone et al. 1996), *Azoarcus* (Stein et al. 1997), *Azotobacter* (Adeleke et al. 2019), and *Pantoea agglomerans* (Verma et al. 2001). However, often inoculation of these biofertilizers results in an increase in root development explaining the yield gain rather than the fixation of nitrogen *in se* (Okon et al. 1998).

Potassium and phosphate content of the soil is often limited and largely inaccessible for take up by plants, since they are mainly found in insoluble forms. However, K and P solubilizing PGPB increase availability and absorption of K and P (Ahmad et al. 2019, El-Deen et al. 2020, Imran et al. 2020, Laxita and Shruti 2020) by acidification of the environment (Mantelin and Touraine 2004, Richardson et al. 2009) and by exudation of chelating agents or enzymes (Hameeda et al. 2008). Similarly, by a general zinc deficiency in arable soils, plants benefit from symbiosis with Zn solubilizing biofertilizers. In wheat *Azospirillum, Azotobacter, Pseudomonas* and *Rhizobium* species improve Zn solubility and uptake (Naz et al. 2016), while *Azobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* harbor phosphate solubilizing bioferilizers (Sturz and Nowak 2000, Sudhakar et al. 2000, Mehnaz and Lazarovits 2006).

Iron (Fe) uptake from the soil is hampered in most neutral and alkaline soil types by its low solubility (Kobayashi et al. 2010). To facilitate Fe absorption by plants certain PGPB produce siderophores or low-molecular-weight compounds that chelate Fe^{3+} enabling uptake (Ahmed and Holstrom 2014). Distinct classes of siderophores can be distinguished specific for the bacterial genus: *Bacillus* produces bacillibactin (Wilson et al. 2006), *Eschericia* produces enterobactin (Saharan and Nehra 2011) and *Pseudomonas* species produce pyoverdine (Cézard et al. 2015). Interestingly, an interplay exists between iron assimilation and auxin regulation, since exogenous auxin treatment increased iron uptake (Housh et al.

2021). Auxin levels, in turn, control ACC oxidase determining ethylene production and which is a Fe^{2+} family of oxygenases (Zhang et al. 1997). Thus this interplay may be regulated through the relation between auxin and ethylene which are highly intertwined (Madhaiyan et al. 2007).

2.1.3.3. Biocontrol

Besides providing benefits for plant growth by modulating hormone balances and nutrient uptake, PGPB may extend the plant immune response granting an extra layer of protection against pathogens. In general, there are two mechanisms that accomplish disease suppression, which is either by direct competition of PGPB and the microbial pathogen in the rhizosphere, or indirectly by stimulating the plant immune system (Teixeira et al. 2019).

Strong competitors in the rhizosphere are bacteria that disperse fast and colonize aggressively. Those antagonistic bacteria produce typically a range of allelochemicals to suppress growth of other microbes. Allelopathy is defined as “chemical elicited interactions between plants or pathogens” (Siegler 1996), which is mediated by exudation of antibiotics, volatiles and enzymes. Strong antagonistic strains are found in the genera *Bacillus* and *Pseudomonas* (Jayaprakashvel and Mathivanan 2011). *Pseudomonas protegens* Pf-5 for instance is a well-studied example of a biocontrol PGPB for its production of antibiotics (Henkels et al. 2014, Loper et al. 2016). From the secondary metabolites produced by *P. protegens* Pf-5, DAPG, rhizoxin, pyrrolnitrin, were the most effective against *Fasurium oxysporum* (Quecine et al. 2016). Interestingly, while *P. protegens* Pf-5 inhibits growth of *F. oxysporum*, the toxin fusaric acid produced by *Fusarium* diminishes antibiotics production by *P. protegens* Pf-5 at the same time (Quecine et al. 2016). Likewise, *Burkholderia ambifaria* and *Mucor rouxii* could both detoxify fusaric acid by hydroxylation of the butyl sidechain (Crutcher et al. 2017) and inhibit fungal growth (Simonetti et al. 2018).

En plus, release of cell wall hydrolyzing enzymes is part of many biocontrol PGPB strategies for suppressing pathogens (Santoyo et al. 2021). β-1,3-glucanase was crucial for *Pseudomonas cepacia* to be able to degrade cell walls of *Rhizoctonia solani*, *Pythium ultimum*, and *Sclerotium rolfsii* (Fridlender et al. 1993), while chitanase and β-1.4-glucase from *Bacillus* sp. BPR7 enabled suppression of *F. oxysporum*, *F. solani* and *R. solani in vitro* (Kumar et al. 2012). Apart from directly acting on a pathogen by means of antibiotics, biocontrol PGPB can simply outcompete pathogens by depleting nutrients such as glucose and asparagine (Elad and Chet 1987, Mohammed and Caunter 1995), or by depriving the environment from iron by production of siderophores (Pal et al. 2001, Tarpet et al. 2016).

Many strains produce a plethora of antibiotics and suppressing metabolites, though some may be depending on the environmental conditions for their production (Duffy and Défago 1999). Therefore, the use of multiple biocontrol strains in the microbial inoculant can offer a more complete and flexible protection especially in highly variable climates and heterogeneous environments.

Furthermore, PGPB can enhance disease tolerance by stimulating the host plants' own immune system through eliciting ISR, effective against a broad range of pathogens (van Loon et al. 1998). ISR is activated through a SA independent pathway via an ethylene and jasmonate dependent defense mechanism, triggered by recognition of MAMPs of beneficial microorganisms (Pieterse et al. 1998, 2014, Thomma et al. 1998). When triggered, the R2R3-MYB-like transcription factor MYB72 is activated and interacts with ETHYLENE INSENSITIVE3 (EIN3)-LIKE3 transcription factor EIL3, which act together via an intermediate in the ethylene signaling pathway causing ISR in systemic tissue. In this signal transduction pathway, Nonexpressor of PR1 (NPR1) as a key component by inducing a set of jasmonate and ethylene responsive genes (Pozo et al. 2008, Van der Ent et al. 2008).

At the site of pathogen challenging, callose disposition in the form of papillae is induced for reinforcing cell walls and PR-proteins, hydrolytic enzymes, phytoalexins and phenolic compounds accumulate (Ramamoorthy et al. 2001, Pokhare et al. 2012). ISR activation is not related with major transcriptional changes, nor accumulation of phytohormones but renders the host plant in a state of elevated awareness enabling the plant to activate inducible resistance mechanisms faster upon subsequent pathogen challenging (Choudhary et al. 2007, Conrath 2011). *P. simiae* WCS417 for instance, stimulates MYB72 which is besides the ISR pathway strongly induced during Fe deficiency for iron mobilization (Palmer et al. 2013). MYB72 and BGLU42 both are required for production and excretion of iron mobilizing coumarins which in turn specifically suppress the pathogens *F. oxysporum* and *Verticillium dahliae* (Stringlis et al. 2018). Other well studied examples of ISR eliciting bacteria are *Pantoea agglomerans* (Liu et al. 1995, Jeun et al. 2002) and *A. brasilense* Ab-V5 (Fukami et al. 2018b), the latter also popular for its nitrogen fixing properties and IAA production (Steenhoudt and Vanderleyden 2000, Ona et al. 2005).

Several bacterial components can elicit ISR in plants such as flagella, components of the envelope, or various metabolites and antibiotic (Meziane et al. 2005). Quorum sensing (QS) molecules, which regulate various physiological process in bacteria (Grandclément et al. 2016, Mukherjee and Bassler 2019) depending on the bacterial community density, are able to elicit ISR as well and constitute an important mechanism for root colonization in several

bacteria (Schuhegger et al. 2006, Wei and Zhang 2006, Pang et al. 2009, Calatrava-Morales et al. 2018). QS molecules such as AHLs differ in their functionality depending their acyl chain: while in general QS molecules with shorter acyl chains promote plant growth, longer acyl chains induce ISR and confer pathogen resistance (Schikora et al. 2011, 2016, Zarkani et al. 2013, Schenk et al. 2014). Priming of plant defense via AHLs is accompanied with increased levels of SA and oxylipids, which regulate stomata closure when challenged with *P. syringae* and induce SA and ethylene dependent defense genes (Schuhegger et al. 2006, Schenk et al. 2014). Therefore, induced resistance by AHLs differs from both ISR and systemic acquired resistance, by its independence of jasmonate, and by different gene expression of typically SA regulated genes (Schenk et al. 2014).

Having such an impacting effect on bacterial behavior, certain microbes disturb QS signals for competing in the soil, for instance by degrading QS molecules with enzymes, which is referred to as quorum quenching (QQ). Several strains within the genera *Bacillus* (Shaheer et al. 2021), *Actinobacteria* (Devaraj et al. 2017), *Pseudomonas* (Jayanna and Umesha 2017), *Comamonas* (Uroz et al. 2007), *Arthrobacter* (Park et al. 2003) and *Streptomyces* (Park et al. 2005) are able to breakdown AHLs. For instance, *B. thuringiensis* produces lactonases that are able to cut the lactone ring of AHL (Dong et al. 2002), while acylases from *Rolstonia* and *Pseudomonas aeruginosa* can hydrolyze the amide moiety of AHLs (Lin et al. 2003, Jayanna and Umesha 2017).

Interestingly, plants when challenged with a certain stress, actively reach out for symbionts to cope with that particular limitation or issue. For example, *Fusarium culmorum* infected *Carex arenia* roots stimulate the emission of VOC that attract bacteria producing fungicidal components over significant distances, while uninfected plants do not recruit bacteria equally efficient (Schulz-bohm et al. 2018). Sugar beets that were challenged with *Rhizoctonia solani* attract *Flavobacterium* and *Chitinophaga,* to inhibit fungal propagation (Carrión 2019). Maize plants exposed to *Fusarium graminearum,* recruit *Bacillus amyloliquefaciens* OR2-30 more efficiently compared to non-challenged plants (Xie et al. 2022). Similarly, in nitrogen limiting conditions, flavonoids are set free in higher quantities to attract nitrogen fixing PGPB to the roots of leguminous plants (Hassan and Mathesius 2012).

2.2. Benzoxazinoids

Many plants have the ability to condition the soil by modifying local environmental parameters that in turn influence the plants' performance. Primary and secondary metabolites in plant root exudates contribute in establishing a plant soil-feedback which determine plant

diversity and succession (Teste et al. 2017) particularly by influencing the root microbiome (Bever et al. 2013, Kudjordjie et al. 2019). A substantial amount of secondary metabolites deposited by maize roots exists of Benzoxazinoids (BX), a highly toxic group of secondary metabolites in Poacea. Root associated bacterial and fungal communities are strongly affected by BXs, aiding in plant growth and defense for successive generations (Hu et al. 2018b) and can attract certain PGPB to the root surface (Neal et al. 2012). Meanwhile, the main function of BXs in plant defense is limiting the growth of microbial and herbivorous pest species (Niemeyer 2009, Ahmad et al. 2011, Neal and Ton 2013).

2.2.1. Discovery and occurrence

BX is a group of secondary metabolites widely spread in grass species including maize, wheat and rye, (Niemeyer 1988) and found in some eudicotyledonous species (Baumeler et al. 2000, Schullehner et al. 2008), that possess the 2-hydroxy-2H-1,4-benzoxazin-3,4-one base structure. The earliest studies on BXs date back from 1955, in which BXs were isolated form rye seedlings (Virtanen and Hietala 1955a, 1955b). Virtanen and Hietala demonstrated soon after how cleavage of the glucoside moiety of 2,4-dihydroxy-1,4-benzoxazin-3-one-glucoside (DIBOA-glc) only happened in unheated disrupted plant tissue, while from boiled plant material and hence containing denatured β-glucosidases, only the glucoside form was obtained (Virtanen and Hietala 1960). In contrast to phytoalexins, which are secondary metabolites that are *de novo* synthesized, BXs are phytoanticipins, constitutively produced and sequestered in an inactive, glycosylated form in the vacuole (VanEtten et al. 1994). Upon herbivore attack hydroxylation allows activation of the bioactive aglycone benzoxazinones by vacuole bound β-glucosidases, which spontaneously split in benzoxazolinones and formic acid (Niemeyer 1988) (both benzoxazinones and benzoxazolinones are referred to as BXs). Despite being constitutively produced, BX production is boosted upon herbivory attack by insects in maize seedlings (Köhler et al. 2015) and in mature leaves (Maag et al. 2016).

BX content varies in its composition of derivatives and their concentrations according to plant organs or tissue (Cambier et al. 1999, Villagrasa et al. 2006), age (Cambier et al. 1999, Köhler et al. 2015) and plant species (Eljarrat and Barceló 2001, Copaja et al. 2006, Schulz et al. 2013). For instance in wheat and maize DIMBOA is the most abundant BX (Villagrasa et al. 2006, Köhler et al. 2015) while DIBOA is the most prevalent in rye (Oikawa et al. 2004, Rakoczy-Trojanowska et al. 2017). In maize, early during plant development BX levels are the highest while they decline and stabilize over the first months (Ebisui et al. 1998,

Hu et al. 2018b). 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one-glucoside (DIMBOA-Glc) is predominantly found in aerial parts and in the roots shortly after germination but diminishes fast during the first and second week after germination respectively (Cambier et al. 2000). After that period, 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one-glucoside (HDMBOA-Glc) and its more stable breakdown product 6-methoxy-benzoxazolin-2-one (MBOA), become more dominant in the roots (Cambier et al. 2000, Hu et al. 2018b).

DIMBOA is enriched in maize crown roots (Robert et al. 2012) and is able to chelate iron, facilitating iron uptake (Bigler et al. 1996). As opposed to generalist herbivores, the crown rootworm can sequester and is resistant to DIMBOA (Robert et al. 2012). Moreover, its larvae exploit DIMBOA-Fe complexes for foraging on nutrient rich crown roots (Hu et al. 2018a). In Fe poor soils, growth of the fall armyworm is suppressed by DIMBOA, while in rich soils harboring free Fe the average biomass of the fall armyworm is increased (Hu et al. 2021). This effect may be caused by the elevated Fe content in the plants, the army fall armyworm feeds on. Apart from these examples, BXs were proven effective against a number of other nematodes, fungus, aphids and other herbivorous insects (Cambier et al. 2000, Niemeyer 2009, Ahmad et al. 2011, Hu et al. 2018b).

2.2.2. Biosynthesis

The first step in BX biosynthesis is mediated by the BX1 gene, converting indole-3-glycerolphosphate into indole (**Figure 1**). This first step forms a shared branch point with tryptophan and auxin synthesis via the shikimate pathway, the conversion in this case by tryptophan synthase is performed in conjunction with the tryptophan synthase beta-subunit, whereas BX1 acts as a monomer (Frey et al. 2000). Free indole can also be formed by indole-3-glycerol phosphate lyase (IGL) induced by herbivory attack, and set free for defense priming (Erb et al. 2015, Hu et al. 2018a), besides serving as an metabolic intermediate. The conversion of indole to DIBOA is carried out by the cytochrome P450 dependent monooxygenases BX2 - BX5 by successively adding four oxygen atoms, which are all substrate specific (Frey et al. 1995) (**Figure 1**). Glucosylation of BXs catalyzed by the two UDP-glucosyltransferases BX8 and BX9 (Von Rad et al. 2001), prevents ring opening and self-toxicity, since hydroxylation and *O*-methylation by BX6 and BX7 respectively, takes place in the cytoplasm (Frey et al. 2003, Jonczyk et al. 2008) (**Figure 1**). BX6 and BX7 convert DIBOA-Glc to DIMBOA-Glc, and are both stored in the vacuole. Possibly, synthesis of DHBOA-Glc, HDMBOA-Glc and HM_2BOA-Glc is mediated by the same enzymes for

DIBOA production, however, metabolic pathways of lactam forms remain to be uncovered. Upon pathogen and herbivory insect invasion, glucosylated BX species are hydrolyzed by β-glucosidases GLU1 and GLU2, converting their substrates into highly reactive forms (Czjzek et al. 2001) (**Figure 1**).

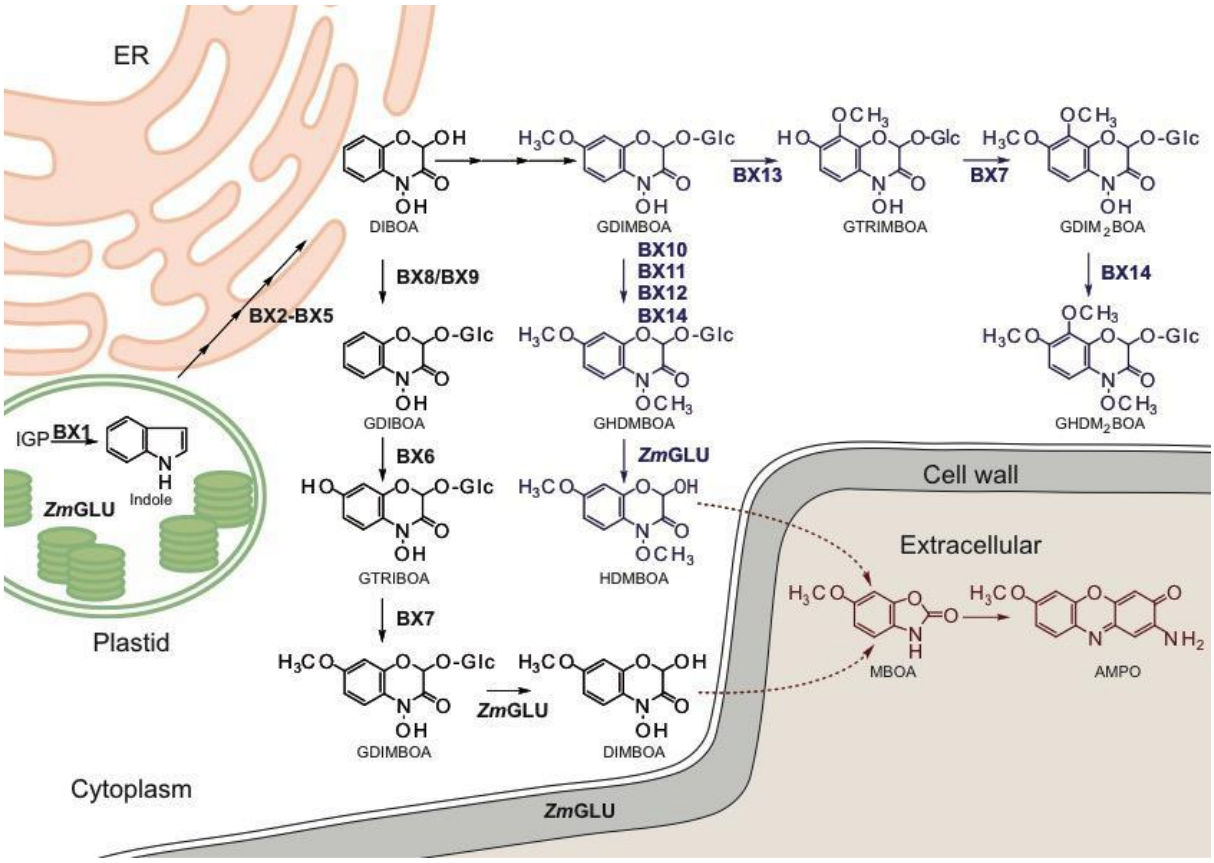

Figure 1: Benzoxazinoid (BX) biosynthesis pathways in maize. Constitutive BX compounds and related enzymes are in black and induced modifications and their related compounds in blue. 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) and 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA) are rapidly converted into 6-methoxy-2-benzoxazolinone in aqueous milieu, indicated in red. Reproduced with permission (Niculaes et al. 2018)

The exact amount of BX produced by plants is often compromised by sample handling due to the activity of β-glucosidases in the samples and the short half-life of the resulting benzoxazinone aglucons (Grambow et al. 1986, Cambier et al. 1999). Furthermore, variation of BX content relate to plant organs or tissue (Cambier et al. 1999, Villagrasa et al. 2006), and age (Cambier et al. 1999, Köhler et al. 2015). Between species the difference in BX production is substantial: in the grains of wheat and rye respectively around 4,8 and 95 µg g^{-1} dry weight is found (Tanwir et al. 2013); in the shoots of rye 1900 µg g^{-1} dry weight (Schulz et al. 2013) and maize can accumulate a few mg g^{-1} dry weight (Meihls et al. 2013, Köhler et al. 2015).

2.2.3. Reactivity

The aglucon benzoxazinones are cyclic hydroxamic acids that contain a highly reactive α-oxo-aldehyde group upon ring opening (Atkinson et al. 1992). The instability of the metabolic intermediate makes benzoxazinones react with thiols (Atkison et al. 1991) and amines (Pérez and Niemeyer 1989) in amino acid residues of proteins; catalytic centers of enzymes which interrupts their functionality (Cuevas et al. 1990) and disrupts metabolic processes such as electron transport (Massardo et al. 1994). In general, hydroxamic acids are more phytotoxic than lactams, DIBOA being the strongest allelopathic natural occurring BX (Macías et al. 2005, 2006). Apart from plants, BXs are toxic for various insects and microorganisms (Copaja et al. 2006, Søltoft et al. 2008, Niemeyer 2009, Ahmad et al. 2011, Meihls et al. 2013). Again, hydroxamic acids being more toxic than lactams and increasingly more potent in relation with the level of methylation (Søltoft et al. 2008). On the other hand, the conversion of DIMBOA-Glc to HDMBOA-Glc was thought of as a mechanism for increasing MBOA content by spontaneous degradation, the latter being better at suppressing conidia germination and germ tube growth of *Bipolaris maydis*, *Curvularia lunata* and *Alternaria alternate* (Oikawa et al. 2004).

As a result of the strong fungistatic effect of BX metabolites on *Fusarium, Phythophtora, Rhizoctonia, Phoma, Alternaria, Blumeria,* and *Botrytis* (Glenn et al. 2001, 2003a, Oikawa et al. 2004, Glenn and Bacon 2009, Śmist et al. 2016), the necrotrophic *F. verticillioides* possesses the gene clusters *FDB1* and *FDB2* required for the detoxification of the benzoxazolinons MBOA and 2-benzoxazolinone (BOA) (Glenn et al. 2001, Glenn and Bacon 2009), acquired by horizontal gene transfer events from *Colletotrichum* and *Aspergillus* (Glenn et al. 2016). The detoxification of BX metabolites by fungal endophytes was exemplified by the survival of BX sensitive endophytes after colonization by BX tolerant *Fusarium* (Saunders and Kohn 2008). An arylamine N-acetyltransferase *NAT1* within the *FDB2* cluster converts the BOA breakdown product 2-aminopheno (2-AP) into the non-toxic (2-hydroxyphenyl) malonamic acid (HPMA) (Glenn et al. 2003a, Glenn and Bacon 2009). Interestingly, the biocontrol PGPB *Bacillus mojavensis*, is able to neutralize the ability of *F. verticillioides* to process BX into non-toxic metabolites, allowing accumulation of 2-amino-3H-phenoxazin-3-one (APO) in the soil which is toxic for *F. verticillioides* (BACON et al., 2007).

Abiotic factors like pH (Niemeyer et al. 1982) and chemical solvents (Bravo and Niemeyer 1985) can facilitate the closure of the open ring benzoxazinone intermediate, lowering its reactivty. Stability of benzoxazinones highly depends on the functional group

bound to the N atom: lactam forms (N-H) do not convert into benzoxazolinone forms, while hydroxamic acids (N-OH) are rapidly converted and *N*-*O*-methyl derivatives even faster. For example the hydroxamic acid DIBOA has a half-life of 25 h, while the half-life of the *N*-*O*-methyl derivative HDMBOA is only 1.8 h in pH 5.5 (Maresh et al. 2006).

Once DIMBOA and DIBOA are degraded into the benzoxazolinones MBOA and BOA, they undergo microbial transformation into 5-methoxy-2-aminophenoxazin-3-one (AMPO) (Kumar et al. 1993) and APO (Gagliardo and Chilton 1992), and decompose further into 2-acetylamino-7-methoxy-phenoxazin-3-one (AAMPO) (Etzerodt et al. 2006) and 2-acetylamino-phenoxazin-3-one (AAPO) (Understrup et al. 2005) respectively. In degradation experiments with start concentrations of 2400 nmol g^{-1} and pH 6.8, the half-life of MBOA was 5.4 days (Etzerodt et al. 2008). At the same time, degradation experiments with a start concentration of 3000 nmol g^{-1} and pH 6.8, a half-life of 3.1 days was determined for BOA (Understrup et al. 2005).

2.2.4. BXs in plant-soil feedback

Continuous cultivation of a single crop causes depletion of nutrients and accumulation of species specific phytopathogens in the soil, which has led to the practice of crop rotation (Dias et al. 2015). Crop rotation profits from plant-soil feedback improving soil microbiota diversity, which in turn enhances nutrient availability, pest control and plant growth (Dias et al. 2015). Hence, plant-soil feedback is of paramount importance to plant health (Teste et al. 2017) and can be a great resource for improving agricultural efficiency and productivity (Mariotte et al. 2017).

The unique composition of the microbial community of the rhizosphere is explained for 5 % by genetic factors of the host plant, while physio-chemical factors are conclusive determinants for the structure of the root associated microbiome (Bulgarelli et al. 2013, Hacquard et al. 2015). Therefore, plants manipulate soil characteristics in favor of PGPB by secretion of root exudates, the influence thereof can differ according to genetic variation among genotypes (Sasse et al. 2018). Release of BX accounts for the same amount of variation as the genetic aspect on soil microbial community composition and exerts a strong effect on soil fungi (Cadot et al. 2021). Furthermore, BX production and conditioning of soil with MBOA results in a generation of more tolerant plants against the *Spodoptera frugiperda* caterpillar via restructuring of the soil microbiome (Hu et al. 2018b). Biosynthesis or the lack of BX synthesis imposes major changes in root metabolic readout, with emphasis on flavonoid anabolism and strongly regulates root associated microbes (Cotton et al. 2019).

Colonization of maize by *Azospirillum* renders a strain specific impact on secondary metabolism, altering relative content of BX derivatives according to the *Azospirillum* strain of the inoculum (Walker et al. 2011). Similarly, inoculation of maize with *P. fluorescence* MZ05 augments DIMBOA content in the leaves by stimulating BX biosynthesis genes, which significantly impacts disease tolerance against the foliar pathogen *Setosphaeria turcica* (Zhou et al. 2020). In conclusion, plants can manipulate the soil microbiome with secondary metabolites such as BX, while individual PGPB in turn influence metabolism of host synthesized metabolites. Given the vast diversity of the soil microbiome, a rigid positive feedback loop can be established to maintain and expand the root associated microbes by improving the local biotic and abiotic soil properties.

2.3. *Fusarium*

2.3.1. Taxonomy and phylogeny

Benzoxazinoids are not exclusively able to recruit PGPB, on the other hand, they have a toxic effect on a spectrum of pathogenic fungi. A dominant soil-dwelling filamentous fungus, *Fusarium*, contains many toxin producing, pathogenic species of economic importance. Infection can cause blights, wilts and rots making crops unsuitable for consumption. Inferred from phylogeny analysis it was found that *Fusarium* emerged around 91 Mya, overlapping with the evolution of flowering plants (Smith et al. 2010, Geiser et al. 2013), which explains why ancient *Fusarium* strains are principally associated with woody angiosperm species that emerged early in evolution (Soltis et al. 2008). The *Fusarium* genus consists of 20 highly diverse monophyletic groups and nine out groups (O'Donnell et al. 2013). *F. verticillioides* and *F. oxysporum* are closely related species, while *F. solani* is more distant and had even been considered to be split from the *Fusarium* family as a taxa within the family of *Neocosmospora*. However, recently the monophyly of *Fusarium* was proven with the inclusion of *F. solani* (Geiser et al. 2021).

2.3.1. Life cycle

The genus *Fusarium* has been given eleven different heterotypic synonyms based on their sexual form, e.g. *Gibberella*, *Albonectria* and *Cyanonectria* (Geiser et al. 2013). Their diverse host specificity and host range determine distinct life cycles among *Fusarium* species. For instance, less than 20 % of *Fusarium* species invest in meiotic spores for sexual reproduction and propagate strictly by clonal reproduction via production of mitotic spores (Ma et al. 2013). In *Fusarium*, the sexual cycle involves the production of recombinant

ascospores through cyclical mating, occurring upon fertilization with a different mating type, or the generation of clonal ascospores resulting from 'selfing' in homothallic species. Following spore germination, a haploid mycelium develops, initiating the asexual cycle (**Figure 2**). Within *Fusarium* species, three types of conidia exist: microconidia, macroconidia, and chlamydospores. Both sexual and asexual cycle-derived spores disperse via air and infect new tissues.

Infection of maize plants by *F. verticillioides* is characterized by a necrotrophic defense response, for instance by accumulation of the PR protein PRm (Murillo et al. 1999). PR proteins and peroxidases were produced in higher quantities in fusarium resistant cultivars compared to susceptible ones, though the induction of expression of the respectable genes was reduced (Maschietto et al. 2016). At the site of infection, ROS accumulate that can cause lipid peroxidation of membranes, proteins, enzymes and nucleic acids (Arora et al. 2002, Asada 2006, Sharma et al. 2012). In order to limit damage to cell organelles caused by high levels of ROS, the plant maintains a robust enzymatic antioxidant system consistent of ascorbate peroxidase, catalase, peroxidase and superoxide dismutase (Arora et al. 2002, Mittler 2002, Asada 2006).

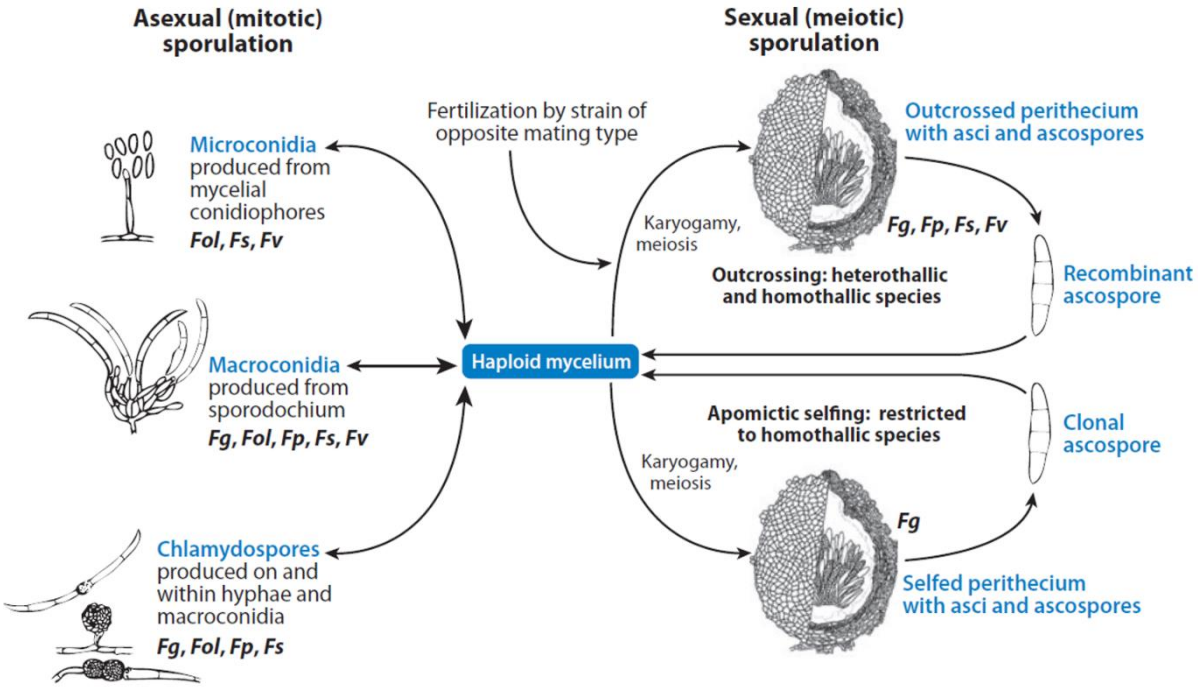

Figure 2: Scheme of diverse reproductional mechanisms of *Fusarium* species. *Fg, F. graminearum; Fol, F. oxysporum* f. sp. *lycopersici; Fp, F. pseudograminearum; Fs, F. solani* f. sp. *pisi; Fv, F. verticillioides.* Reproduced with permission (Ma et al. 2013).

Despite the fungistatic character of BXs, some *Fusarium* strains are able to detoxify those toxic secondary metabolites. For instance, *F. verticillioides* is more tolerant to BXs than other species of *Fusarium* (Richardson and Bacon 1995, Saunders and Kohn 2008). *F. verticillioides* has a narrower host range, infecting predominantly BX-producing cereals whereas *F. oxysporum* has a broader range of plant hosts (Armstrong and Armstrong 1981). *F. verticillioides* can convert benzoxazilinones such as BOA and MBOA in non-toxic breakdown products (Glenn et al. 2003b, Bacon et al. 2007, Glenn and Bacon 2009) granting *F. verticillioides* to exist frequently as symptomless symbionts in several maize species (Kommedahl and Windels 1981, Bacon and Hinton 1996, Merritt et al. 2005).

2.3.3. Characteristics

Interestingly, the differences in genome size among the *Fusarium* species *F. graminearum, F. oxysporum* f. sp. *lycopersici, F. verticillioides,* and *F. solani* is principally determined by the constitution of repetitive sequences (Ma et al. 2013). The amount of repetitive sequences, which is low in both *F. verticillioides* and *F. graminearum,* and high in *F. oxysporum* and *F. solani,* reflects the host range of the respective strains. Important mechanisms for acquiring or expending pathogenicity are either by mutation of effector proteins or by horizontal gene / chromosome transfer (HGT/HCT) (Mehrabi et al. 2011). The four afore mentioned *Fusarium* species have high synteny between their core chromosomes, while *F. oxysporum* and *F. solani* each have their species specific chromosomes enriched in repetitive sequences and pathogenicity related genes. One such chromosome attributed to pathogenicity in tomato infecting *F. oxysporum*, chromosome 14, can be transferred during co-cultivation of pathogenic and non-pathogenic *F. oxysporum* strains via HCT, and thus can lead to the generation of new pathogenic strains (Ma et al. 2010).

Our lab found out that biocontrol of *F. verticillioides* and *F. oxysporum* was established by antibiosis from co-inoculation with *P. protegens* Pf-5 (Quecine et al. 2016). The three antifungal metabolites rhizoxin, pyrrolnitrin and DAPG produced by *P. protegens* Pf-5 were most impacting on antibiosis of *Fusairum*. Interestingly, when fusaric acid, which is biosynthesized by many *Fusarium* species including *F. verticillioides*, *F. oxysporum* and *F. solani* (Munkvold 2017), was added to the culture medium, production of DAPG by *P. protegens* Pf-5 was diminished and altered the transcription of its biosynthetic genes. Regardless, when infection has well been established, *F. verticillioides* causes ear rots, stalk rots and seedling blight (Nelson 1992, Munkvold 2003, The CIMMYT maize program 2004). In addition to pre-harvest crop losses due to *Fusarium* related diseases, exudation of

mycotoxins by *Fusarium* species causes post-harvest losses that poses risks to human health by its carcinogenic effect (IARC 2002).

2.4. Transcriptomics

Transcriptomics is the field of research that studies the complete set of all RNA transcripts produced by an organism at a certain time point and in a specific tissue. Messenger RNA (mRNA) is an intermediate molecule in the exchange of information stored in DNA with the expression of proteins (CRICK 1958, 1970), while non-coding RNAs (ncRNA) perform an array of regulatory functions (Li and Liu 2019). Analysis of the transcriptome provides a valuable source of the way genes are regulated and the function of genes that were not annotated, at a certain time point and in certain environmental conditions. By studying gene expression in its entirety, one is able to reveal orchestrated trends in gene regulation. Therefore, the amount of information gained is unparalleled by more targeted analyses. Ever since the early 90s, technological advances and innovations have known various turning points and revolutionized current knowledge about Biology. In Chapter 5, transciptomics is used in the form of RNA sequencing for analyzing expression profiles of *A. brasilense* Ab-V5 in MBOA treatments. Therefore, we will provide a brief introduction in transcriptomic techniques and analysis with an emphasis on RNA sequencing, which is most relevant for understanding the analysis performed in Chapter 5.

2.4.1 History

First research on transcriptome sequencing was conducted in the early 80s, using Sanger sequencing of random transcripts or expressed sequence tags (EST) (Sim et al. 1979, Sutcliffe et al. 1982). EST are short fragments of complementary DNA (cDNA) that are used for identifying and quantifying transcripts. Gene expression analysis using EST subsided with the advent of high throughput techniques such as microarrays and RNA sequencing (RNA-seq), even though the large EST GenBank library of over 485 million entries (08/2023) (Re3data.org - Registry of Research Data Repositories 2021) that was built over time, can still be used for the design of probes in microarrays experiments (Close et al. 2004).

In 1995, microarrays was developed, which allowed the analysis of thousands of transcripts simultaneously, based on hybridization with a predefined set of probes (Heller 2002). Over the decades to come, the technique was fine-tuned by improving the sensitivity of fluorescence detection, the specificity of probes and the amount of probes that could fit an array (Pozhitkov et al. 2007). The use of microarrays only allows the analysis of known genes

for which probes has been designed, shows cross-hybridization artifacts, has a limited dynamic range, low specificity and a high cost (Jaksik et al. 2015). To improve precision and implement high throughput, other tag based techniques such as serial analysis of gene expression (SAGE) and cap analysis gene expression (CAGE) were developed that could directly related transcript numbers with expression levels (Velculescu et al. 1995, Shiraki et al. 2003). However, cloning of tags is highly labor intense, depends on expensive automated Sanger sequencing which requires big quantities of RNA and is not suitable for analyzing expression profiles of isoforms.

These limitations gave rise to the development of RNA-seq using Next-Generation Sequencing (NGS) methods for revealing transcript abundance. This requires the construction of cDNA libraries from RNA molecules by reverse transcription using automated sequencing-by-synthesis approach. The first RNA-seq project was first published in 2006 using 454 pyrosequencing, generating 10^5 transcripts originating from prostate cancer cells (Bainbridge et al. 2006). Soon after in 2008, Illumina technologies (San Diego, CA) transformed NGS by developing sequencing technology with the ability to reproduce up to 52 billion reads per run, depending on the specific kit used (Mortazavi et al. 2008, Illumina 2022).

2.4.2. Sequencing platforms

Before performing RNA-seq, attention should be given to sequencing strategies in developing an experimental design. Depending on the research goals, details to be considered in an experimental set up for RNA-seq are the amount of biological and technical repeats; the sequencing depth and coverage; the sequencing platform and the length of the reads they generate. When the priority of RNA-seq is gene expression quantification rather than gene discovery for instance, it is interesting to consider a higher number of biological repeats instead of increasing the sequencing depth when facing monetary constrains for the project (Tarazona et al. 2011, Haas et al. 2012). On the other hand, when assembling a *de novo* transcriptome, sequencing depth and coverage become paramount parameters. For repetitive sequences and discovery of splice variants, long reads (> 1000 bp) are the better choice, while short reads (< 100 bp) provide more depth and statistical power (Kovaka et al. 2019, Yasir et al. 2022, De La Cerda et al. 2023, Nip et al. 2023).

After RNA is isolated from cells grown in the desired conditions, a library is prepared from a subset of RNA species. Therefore, RNA is filtered from the samples based on size, for small RNA; on C-terminal polyadenyl for eukaryotic mRNA or by ribosomal depletion for separating prokaryotic mRNA from ribosomal RNA which makes up more than 80 % of the

RNA pool. Subsequently, RNA is reverse transcribed into copy DNA (cDNA) which allows for amplification through polymerase chain reaction (PCR) and ligation of sequencing adaptors.

The majority of NGS platforms handle sequencing-by-synthesis methods by either an ensemble or single-molecule approach, which includes sequencing DNA copies in parallel or sequencing a single DNA molecule respectively (Bentley et al. 2008, Eid et al. 2009). In the ensemble approach e.g. during Illumina sequencing, DNA molecules are fixed on the surface of a flowcell and clone-wise amplified by PCR with fluorescently labeled nucleotides, relating the received fluorescent signal with expression levels of genes. This technique has the advantage of a very low error-rate and the requirement of a low amount of RNA. Illumina sequencing is best known for its short-read sequencing, providing a substantial sequencing depth which ideal for differential expression analysis (Tarazona et al. 2011, Haas et al. 2012).

In contrast, single-molecule-based approaches as for instance used in Single Molecule Real-Time sequencing by Pacific Biosciences (PacBio) and in nanopore sequencing by Oxfort Nanopore Technologies (ONT), is the best option for reference-free transcriptome assembly, identification of repetitive elements, and analysis of splice variants due to the production of long reads (Kovaka et al. 2019, Yasir et al. 2022, De La Cerda et al. 2023, Nip et al. 2023). Single-molecule-based approaches synthesize DNA strands in a continuous, template guided polymerization of fluorescent nucleotides by DNA polymerase. Though coping with a higher error rate than Illumina sequencing, being PCR independent, this technique allows a uniform coverage unaffected by amplification bias. Generating long reads facilitates the reproduction of reads into transcripts and the identification of splice forms. Therefore, the choice of the sequencing platform depends on the research objective.

2.4.3. Data analysis

After the sequencing output is obtained in FASTQ file format, a bioinformatics data analysis pipeline follows. Throughout the great availability of bioinformatics tools, of which many are free of cost, pipelines vary but boil down to the same data processing procedure for differential expression analysis. In general, a typical pipeline for organisms with a draft genome sequence available, consists of three parts. First, reads are mapped to an annotated reference genome or transcriptome; in the second part, transcripts are counted and finally, differential expression of transcripts between treatments is calculated. When no genomic data is available, the genome sequence can be obtained via *de novo* genome assembly. The informatics work flow of genome assembly is slightly different from that of differential

expression analysis aided by the availability of a reference genome. First, the raw reads are preprocessed to remove adaptor sequences, barcodes or low quality sequences. The trimmed reads are then forged into contigs, which are a consensus sequences of overlapping reads. Subsequently, contigs are combined into a genome assembly by genome finishing tools that fill up gaps between contigs. The resulting genome assemblies go through rounds of evaluation and re-evaluation until the desired quality has been reached. Finally, genes can be identified by annotation programs to be used as a tool for drug design, identification of disease related genes or for taxonomic analysis, to sum up some applications. Considering the relevance to this research project, in what follows we will focus on differential expression analysis.

From the cultivation of cells to the sequencing of RNA and alignment of reads, there are many steps where samples and data can get biased in a certain form. To prevent introducing bias in downstream procedures of data analysis in the bioinformatics pipeline, it is of paramount importance to incorporate sufficient quality assessments. At the level of RNA isolation, in the advent of sequencing a bioanalyzer (Agilent) is a commonly used instrument for determining accurately the quality of isolated RNA, which judges the sample quality inferred form gel electrophoresis and photo spectrometry readouts. After sequencing, FastQC software (Andrews 2010) is frequently included in the analysis to score the overall sequence quality of the obtained reads per sample, structured in separated parameters that display acceptable or problematic aspects. Information such as k-mer representation, GC percentage and per base quality can lead to the identification of adaptor sequences or terminal low quality sequences, which are best trimmed before proceeding to the next step of data processing. Low quality of read ends can arise from erroneous priming during random amplification in the library preparation procedure (Lin et al. 2012). At the stage of read alignment, errors can occur by ambiguous mapping of reads primarily at splice junctions. Misalignment is more prominent when lacking annotation of isoforms in the reference genome (Kleinman and Majewski 2012, Pickrell et al. 2012).

Once the actual sequencing by an NGS platform has been done and trimming and filtering of the raw data output has finished, reads are mapped to an annotated reference genome for identifying transcripts. In eukaryotes, read alignment of RNA-seq data is hampered by the occurrence of splice junctions between exons. Transcripts frequently cover various splice junctions making alignment a challenging task. Hence, the occurrence of splice junctions is an important feature to consider when selecting an alignment tool, although most modern read alignment tools take into account splice junctions while mapping reads. The

most commonly used tools for mapping are: STAR (Dobin et al. 2013) and TopHat (Trapnell et al. 2009).

When read alignment has been accomplished, mapped reads proceed to assembly into full length transcripts and are counted. This can either be done by reconstruction of transcripts from neighboring reads or from read alignment to a reference genome (Grabherr et al. 2011, Li et al. 2011, Schulz et al. 2012, Mezlini et al. 2013). Currently, best known bioinformatics tools for transcript quantification are Cufflinks (Trapnell et al. 2010) and HTSeq (Anders et al. 2015). Read counts must be corrected for systemic variation caused by gene length in the reference genome per total amount of aligned reads, expressed as reads per kilobase of transcripts per million mapped reads (RPKM) and paired fragments per kilobase of transcripts per million mapped reads (FPKM) for paired-end data. By incorporating a likelihood function of reads mapping to splice variants, the problem of quantifying reads that align to highly similar splice variants can be abolished by using tools as Cufflinks (Trapnell et al. 2012) and MISO (Katz et al. 2010) for analysis of eukaryotic data.

Often, the goal of RNA-seq experiments is to find differential expressed genes (DEGs) among treatments. Initially, the same statistical test for microarray data were applied on RNA-seq data assuming a normal distribution (Smyth 2004, Grant et al. 2005). Later on, a Poisson distribution was claimed to be a better fit for RNA-seq data (Marioni et al. 2008). In practice though, adapting Poisson lead to many false positives, making the negative binominal distribution the most suitable distribution to cope with overdispersion and large sampling error (Anders and Huber 2010, Robinson and Oshlack 2010). Additionally, samples vary in sequencing depth, which makes certain genes accumulate more counts irrespective of the treatment. To account for sequencing depth variation over samples, instead of counts, FPKM or RPKM metrics can be used. However, after normalization highly expressed genes can relatively diminish the counts of lowly expressed genes, calling for more complex statistical strategies (Robinson and Oshlack 2010, Srivastava and Chen 2010). The most common tools for calculating differential expression are DESeq (Anders and Huber 2010) and edgeR (Robinson et al. 2010). Using different programs for differential expression analysis does not exclusively lead to the same results and consequently, scientific conclusions (Liu et al. 2021). Since they employ different steps along the analysis process, the preference of a certain tool depends on the data.

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3. 6-METHOXY-2-BENZOXAZOLINONE (MBOA) HAS A SPECIES-SPECIFIC EFFECT ON PGPB BIOFILM, CHEMOTAXIS AND A HOST-SPECIFIC INFLUENCE ON *Fusarium* CONIDIA GERMINATION

Abstract

Benzoxazinoids (BXs) are a group of secondary metabolites produced by Poaceae, with significant effects on soil microbial inhabitants. This study investigates the impact of BXs, particularly the lactam derivative 6-methoxy-2-benzoxazolinone (MBOA), on plant growth-promoting bacteria (PGPB) and pathogenic fungi. Four different PGPB: *Azospirillum brasilense* Ab-V5, *Bacillus thuringiensis* RZ2MS9, *Pantoea agglomerans* 33.1 and *Pseudomonas protegens* Pf-5 plus five isolates of necrotrophic *Fusarium* species were evaluated for their tolerance, chemotactic response, and biofilm formation in the presence of MBOA. From the PGPB we tested, Ab-V5 was the most sensitive to MBOA and at the same time the most responsive. Ab-V5, exhibited a time dependent biofilm formation pattern, from which we concluded that biofilm production was slowed down. Results from other PGPB were less pronounced but generated a species-specific read out. We conducted a chemotaxis experiment based on the capillary assay, and found a positive chemotactic response of Ab-V5 towards MBOA. Additionally, MBOA tolerance varied among *Fusarium* species, with the isolate from BX-producing maize displaying higher resistance than those from non-BX-producing hosts. The results suggest that MBOA positively influences establishment of certain PGPB in the rhizosphere depending on the concentration, and exhibits a negative effect on pathogenic fungi. This study provides valuable insights into the complex interactions between BXs, soil microbes, and plant health, shedding light on the potential use of BXs in agroindustry and crop breeding.

3.1. Introduction

Benzoxazinoids (BXs) form a highly toxic group of secondary metabolites produced by several cereal crops with a strong impact on microbial diversity in the soil (Hu et al. 2018b, Cotton et al. 2019, Kudjordjie et al. 2019, Cadot et al. 2021). The discovery of BXs date back from almost 70 years ago (Virtanen and Hietala 1955b, 1955a) gaining increasingly more interest in the decades after for its potential use in the agroindustry (Schulz et al. 2013) and attained some success in breeding high BX content crop varieties, tolerant to insect feeding (Klun et al. 1970, Grombacher et al. 1989, Barry and Darrah 1991, Gianoli et al. 1996). BXs are indole-3-glycerolphosphate derived phytoalexins, constitutively expressed and stored as their inactive glucosylated form in vacuoles (Frey et al. 2000). Glucosylated BXs are hydrolyzed rapidly upon herbivory and pathogen attack by GLU1 and GLU2, rendering highly reactive aglucon BXs derivatives (Czjzek et al. 2001).

Ample evidence shows that BX have a strong influence on individual soil microbes or on the soil microbiome as a whole (Hu et al. 2018b, Cotton et al. 2019, Kudjordjie et al. 2019, Cadot et al. 2021). For instance, BX related compounds have a strong fungistatic effect on *Fusarium, Phythophtora, Rhizoctonia, Phoma, Alternaria, Blumeria,* and *Botrytis* (Glenn et

al. 2001, 2003a, Oikawa et al. 2004, Glenn and Bacon 2009, Śmist et al. 2016), while at the same time, BXs from maize root exudates attract the PGPB *P. putida* (Neal et al. 2012). Concurrently, maize root colonization by *Azospirillum brasilense, Pseudomonas putida* and *Pseudomonas fluorescens* three well studied plant growth promoting bacteria (PGPB), causes a strain specific positive feedback on BX metabolism, establishing complex interactions between host and symbionts (Walker et al. 2011, Planchamp et al. 2015, Zhou et al. 2020).

One BX derivate, the lactam 6-methoxy-2-benzoxazolinone (MBOA) is a spontaneous break down product of the hydroxamic acids 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA) and 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), which has a half-life of 5.4 days in the soil (Etzerodt et al. 2008). Soil deposition of MBOA has a persistent effect on microbiome structuring lasting for the next progeny of maize plants and confers improved pest tolerance through jasmonate signaling and activation of plant defense mechanisms (Hu et al. 2018b). Next to herbivore tolerance, MBOA is among BX metabolites the most efficient in suppressing conidia germination and germ tube growth of the fungi *B. maydis, C. lunata* and *A. alternate* (Oikawa et al. 2004).

Apart from studies carried out on *P. putida* KT2440 (Neal et al. 2012, Neal and Ton 2013), the effect of BX on PGPB remains to be uncovered. To shed light on the mechanism by which MBOA manipulates soil structuring, we evaluated four different PGPB: *Azospirillum brasilense* Ab-V5, *Pseudomonas protegens* Pf-5, *Pantoea agglomerans* 33.1 and *Bacillus thuringiensis* RZ2MS9, as well as different isolates of the nonobligate plant pathogen *Fusarium: F. verticillioides* T4 from maize; *F. verticillioides* from sugarcane; *F. oxysporum* from banana; *F. oxysporum* R5 from pea and *F. solani* from soja. The BX breakdown products MBOA and BOA are stable in native soil conditions and considering its effectivity in fungal suppression, are promising allelochemicals (Fomsgaard et al. 2004). Therefore, we hypothesized that MBOA might inflict a positive effect on rhizosphere establishment of PGPB and a negative effect on pathogenic fungi.

First we monitored biomass of PGPB and *Fusarium* by either measuring the optical density at 600 nm (OD_{600}) in liquid medium at predetermined time points or by determining its radial growth on solid growth medium, in the presence of MBOA. We analyzed biofilm formation of the PGPB as an estimate for their competence for colonizing root surfaces. For many plant-associated bacteria, the production of a complex extracellular polymeric substances (EPS) containing matrix or biofilm, is an intrinsic component in the plant-bacterial interaction by ensuring an intimate contact between symbionts (for reviews on biofilm in plant-associated bacteria: (Ramey et al. 2004, Danhorn and Fuqua 2007)). Secondly,

chemotactic behavior was determined by a modified capillary assay. Allowing directed movement of bacteria, chemotaxis is an indispensable feature of bacteria to localize nutrient rich root exudates and guide root colonization (Colin et al. 2021). Despite elaborate research on BX for pest control, little is known about bacterial physiological responses to BX which are inherently associated with plants and pivotal for plant health. Hence, the objective of this study was to analyze the impact of MBOA on commercial and potent PGPB, and harmful pathogens, examining the potential use of MBOA as an agricultural tool for improving soil conditions for PGPB establishment.

3.2. Methods

3.2.1. Microbial strains and media

The bacterial strain *Bacillus thuringiensis* RZ2MS9 previously isolated from the rhizosphere of guarana plants (*Paullinia cupana*) (Batista et al. 2018) and *Pantoea agglomerans* 33.1 isolated from Eucalyptus (*Eucalyptus grandis*) (Quecine et al. 2012b) were routinely cultivated on Luria-Bertani (LB) medium (Sambrook et al. 1989), *Pseudomonas protegens* Pf-5 from cotton rhizosphere (*Gossypium hirsutum*) (Howell and Stipanovic 1978) was grown on Kings medium B (KMB) (King, Eldora et al. 1954). *Azospirillum brasilense* Ab-V5 previously isolated form maize (*Zea mays*) (Hungria et al. 2010) was grown at 28°C on DYGS medium (Rodriguez et al. 2004), Nfb-malic medium and MSM medium (Dobereiner and Day 1976). All bacterial strains were stored at -80 °C in 15 % glycerol in the Molecular Genetics Lab (Piracicaba, Brazil) and freshly prepared at the onset of each experiment on their respective cultivation mediums.

Fusarium is a filamentous fungus that has a substantial impact on various economically important crops, causing wilts, blights and rots, and further affecting crop yield by post-harvest contamination caused by production of mycotoxins (Woloshuk and Shim 2013). *F. verticillioides* T4 isolated from maize; *F. verticillioides* from sugarcane; *F. oxysporum* from banana; *F. oxysporum* R5 from pea and *F. solani* from soja were routinely grown on Potato Dextrose (PD) medium of pH 5.6 at 28 °C. Conidial spores were stored in 15 % glycerol at – 80 °C and freshly prepared prior to inoculation form 7-day old fungal cultures.

3.2.2. Effect of MBOA on microbial biomass

We assessed to what concentration of MBOA the PGPB subject to this study were tolerant, by obtaining growth curves when bacteria where grown supplemented with

increasing concentrations of MBOA. Therefore, pre-cultures were freshly prepared on the onset of the experiment from bacterial stock and grown until early logarithmic phase in the PGPBs' respective growth medium, and diluted in 100 mL Erlenmeyer flasks containing 20 mL liquid growth medium amended with 0.00 mM, 0.05 mM, 0.50 mM or 1.00 mM MBOA (product no. 543551, Sigma-Aldrich) from stock solutions prepared in acetone. The 0.00 mM MBOA treatment contained 0.5 % acetone which equals the amount of MBOA solution in the other treatments. Over the time course of 12 hours, every three hours 1 mL per culture was analyzed by spectrophotometry to determine the OD_{600} with a dual beam Genesys 50 UV-Vis spectrophotometer (Thermo Scientific, Massachusetts), while flasks remained shaking at 120 rpm and 28 °C. The experiment contained four biological repeats per treatment and was carried out twice. Considering the overall negative influence of 0.50 mM MBOA, we included next to 0,50 mM MBAO a 0.05 mM MBOA treatment to study a more gentle effect on bacterial physiology in contrast to the 0.50 mM treatment.

The effect of MBOA on the before mentioned *Fusarium* isolates was evaluated by measuring the two parameters: germination of conidia and fungal biomass. *Fusarium* was grown in liquid PDB culture medium amended with 0.00 mM, 0.05 mM, and 0.50 mM MBOA at 28 °C at 150 rpm for 12 hours, starting from an initial 10^6 conidia ml^{-1} concentration, to determine the percentage of germinated conidia counted in an improved Neubauer chamber (Beoco, Hamburg, Germany). Control treatments were given the same amount of acetone as a substitute for MBOA solution in a final concentration of 0.5 % acetone. In addition, we grew *Fusarium* on solid PDA plates to score the biomass on solid plates after 7 days of incubation, by measuring the size of the culture radiating from the center, where a mycelial agar plug (8,0 mm) of cultured *Fusarium* was placed at the advent of inoculation. All *Fusarium* assays were carried out using four biological repeats and at least three technical repeats.

3.2.3. Effect of MBOA on bacterial biofilm

To determine the influence of MBOA on the production of biofilm by the PGPBs Ab-V5, RZ2MS9, 33,1 and Pf-5, a microtiter plate biofilm assay was carried out, using sterile polystyrene 96-well plates as described in Merritt *et al*. 2005 (Merritt et al. 2005). Briefly, overnight bacterial cultures were diluted until OD_{600} of 0.05 (approximately 10^8 bacteria) in MSM, DYGS or LB liquid growth medium, and supplemented with either 0.00 mM; 0.05 mM or 0.50 mM MBOA. Microtiter plates were filled with 100 µL of bacterial culture using at least eight repetitions of each treatment including non-inoculated controls for 72, 96, 120 and

144 hours of stationary incubation at 28 °C. The microtiter plates were then rinsed to remove planktonic bacteria. Biofilm was stained with 125 µL 0.01 % (w/v) crystal violet per well for 20 minutes. After removal of the unbound crystal violet, wells were filled with 150 µL 100 % ethanol for 15 minutes which was then transferred in an optically clear microtiter plate and analyzed with a Multiskan FC Microplate Photometer (Thermo Scientific, Massachusetts) at OD_{590}.

3.2.4. Effect of MBOA on bacterial chemotaxis

Chemotaxis responses of the same PGPB mentioned above, were assessed by a modified capillary assay (Adler 1972). Briefly, sterile syringes of 0.5 mL with needles of 0.25 µm aperture were filled with MBOA or acetone equivalent in phosphate buffered saline (PBS, 8 g/L NCL, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) of pH 7.4. The syringes were inserted into 15 mL Falcon tubes containing 5 mL of washed bacteria in PBS with an OD_{600} of 0.05. After incubation at room temperature for 15 minutes, syringes were ejected and every 100 µL was directly plated on 15 % agar DYGS or LB plates, rendering five plates per syringe. Colony forming units (CFU) were counted digitally using ImageJ software (Scion Corporation, Maryland). For every treatment at least four replicates were used and the experiment was carried out in triplicate.

3.2.5. Statistical analysis

All quantitative data was analyzed using R software (Bunn and Korpela 2018) and tested for normality via the Shapiro-Wilk normality test. Normal distributed data was subjected to a one-way ANOVA and a subsequent Tukey multiple comparisons of means or a Welch Two Sample t-test for testing two groups. Not normal distributed data was analyzed with a Kruskal-Wallis rank sum test or Wilcoxon rank sum test with continuity correction (a.k.a. Mann–Whitney U test).

3.3. Results

3.3.1. Ab-V5 is the most susceptible PGPB to MBOA.

From the four PGPB that we evaluated for MBOA tolerance by measuring bacterial growth in liquid culture during the time span of 12 hours, *A. brasilense* Ab-V5 showed the most sensitivity, followed by *P. agglomerans* 33.1, and *P. protegens* Pf-5, in that specific order. The three mentioned PGPB exhibited decreased growth rates from 6 hours post inoculation (hpi) onwards when supplemented with at least 0.50 mM while *B. thuringiensis*

RZ2MS9 did not grow significantly less (p = 0.05) in MBOA amended medium (**Figure 3**). The same results were obtained from repeating the experiment and allowed for optimizing the MBOA concentrations used in the subsequent experiments.

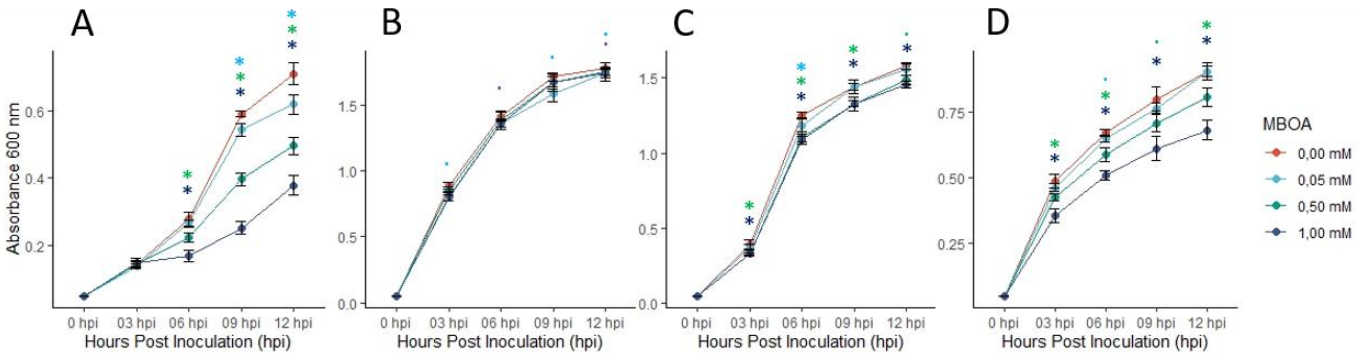

Figure 3: Growth curves of PGPB strains (A) *A. brasilense* Ab-V5, (B) *B. thuringiensis* RZ2MS9, (C) *P. protegens* Pf-5 and (C) *P. agglomerans* 33.1 in a series of MBOA concentrations. Pre-cultures were diluted to a start OD of 0.05, and bacterial inoculums were supplemented with 20 fold concentrated MBOA suspension or an equal amount of acetone in control treatments. At an interval of three hours, the OD was measured at 600 nm by spectrophotometry, over a time span of 12 hours. Error bars in the graphs show standard deviation. Significant codes: '***': 0,001, '**': 0,01, '*': 0,05, '.': 0,1, ' ': 1.

3.3.2. Ab-V5 is attracted to MBOA

We tested the four PGPB Ab-V5, RZ2MS9, 33.1 and Pf-5 for chemotactic behavior in a modified capillary assay, using the MBOA concentration of 0.50 mM which had a moderate effect on optical density in the growth curves recorded over 12 hours. Ab-V5 was the only bacteria that exhibited a chemotactic response by accumulating a significant higher number of CFU collected in the assay compared to the control treatment containing acetone without MBOA (**Figure 4B**). Next we analyzed chemotaxis to intermediate (0.05 mM) and high (0.50 mM) levels of MBOA using the same experimental set up, only testing Ab-V5. Again, we observed a significant chemotactic response of Ab-V5, independent of the MBOA concentration that was used in the assay (**Figure 4A**).

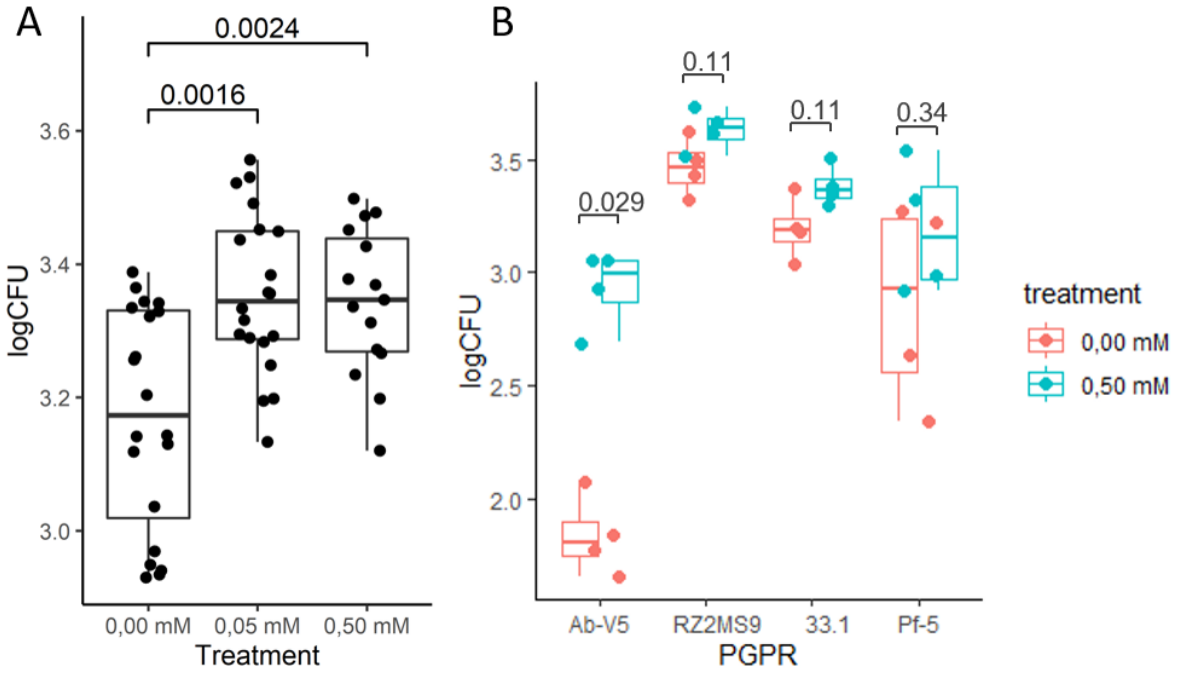

Figure 4: Ab-V5 exhibits chemotaxis towards MBOA, independent of the concentration. Ab-V5 pre-cultures were washed and diluted in PBS to a final OD600 of 0.05 and used in a modified capillary assay. After 15 minutes of incubation at room temperature, the collected bacteria in 0.5 mL syringes were plated out and counted. A: Chemotaxis assay on *A. brasilense* Ab-V5 with 0.05 mM and 0.50 mM MBOA. B: Chemotaxis assay on *A. brasilense* Ab-V5, *B. thuringiensis* RZ2MS9, *P. agglomerans* 33.1 and *P. protegens* Pf-5 with 0.50 mM MBOA. P-values from ANOVA tests were calculated to analyze significant differences between MBOA and control treatments, and are depicted in the graphs.

3.3.3. MBOA influences bacterial biofilm production *in vitro* in a species specific manner

Different growth media were tested before hand to optimize biofilm production in statical conditions. We therefore scored biofilm production of Ab-V5 in DYGS, LG, MSM and NFB, LB, of Pf-5 in KMB and LB, of RZ2MS9 and 33.1 in LB liquid growth medium in microtiter plates (**Supplementary Figure 1C**). To determine the time point at which biofilm production was optimal, preliminary assays included measurements at 72, 96, 120 and 144 hpi (**Supplementary Figure 1A, 1B**). Measurements of biofilm on predetermined time points resulted in characteristic readouts for each PGPB, none of which showed linear relationship with the applied MBOA concentration (**Figure 5B**). Pf-5, which produced in general the highest levels of biofilm showed a very strong decline in biofilm accumulation when treated with 0.50 mM, while its growth rate was not affected as much as in the growth curve. Both Ab-V5 and RZ2MS9 showed improved biofilm production 0.05 mM MBOA. While 33.1 produced less biofilm at 0.05 mM, the 0.50 mM treatment did not seem to significantly impact biofilm (**Figure 5B**).

The maximum amount of biofilm produced by Ab-V5 did not coincide among treatments, as it did with Pf-5 and 33.1 at 120 hpi and 144 hpi respectively (**Supplementary**

Figure 1A, 1B). The maximum amount of biofilm production by Ab-V5 was shifted after 72 hpi from the control treatment to the 0.05 mM treatment after 120 hpi (**Figure 5A**). The results regarding the timing of biofilm production are consistent with how MBOA influences the optical density in liquid cultures under agitation (**Figure 3**), where MBOA lowered the growth rate of Ab-V5 more than Pf-5 and 33.1.

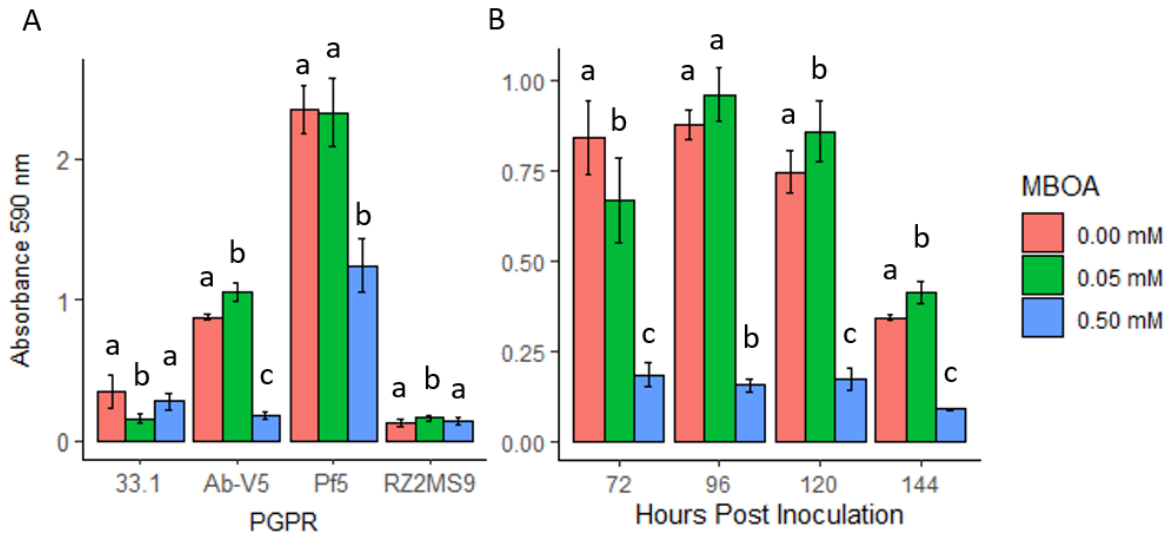

Figure 5: Biofilm formation by PGPB in microtiter plates. A. Biofilm production of *A. brasilense* Ab-V5 after 120 hpi, *B thuringiensis* RZ2MS9 after 48 hpi, *P. agglomerans* 33.1 after 144 hpi and *P. protengens* Pf-5 after 72 hpi. B. *A. brasilense* Ab-V5 biofilm between 72 and 144 hpi. Biofilm was determined in a 96-well microtiter plate by spectrometry, reading the absorbance at 590 nm after staining with 0.1 % Crystal Violet. Error bars in the charts represent standard deviation, different characters indicate significance at the level of 0.05.

3.3.4. MBOA affects conidia germination and biomass of *Fusarium* spp. related to their plant host

We tested the tolerance of different *Fusarium* species and isolates to MBOA, to assess the variance by genetic background, in between species, and the effect of the host plant. From growing *Fusarium* spp. in liquid and solid medium, we found that *F. oxysporum* isolated from pea was the most sensitive isolate we tested. Its conidia germinated significantly less in medium containing 0.05 mM MBOA (**Figure 6C**), while conidia from *F. oxysporum* from banana (**Figure 6A**) were only sensitive to 0.50 mM, similar to *F. solani* from soja (**Figure 6E**) and *F. verticillioides* from sugar cane (**Figure 6G**). Only germination of *F. veriticillioides* from maize conidia (**Figure 6I**) was not affected by MBOA in concentrations up to 0.50 mM. A similar response was observed in the biomass assay, *F. oxysporum* from pea (**Figure 6D**) again being the most sensitive, growing to a lesser extent on plates containing at least 0.05 mM MBOA. *F. oxysporum* from banana (**Figure 6B**) only showed a

significant decrease in hyphal growth on 0.50 mM MBOA plates, while *F. solani* from soja (**Figure 6F**); *F. verticillioides* from sugar cane (**Figure 6H**) and *F. verticillioides* from maize (**Figure 6J**) remained unaffected (**Supplementary Figure 2**).

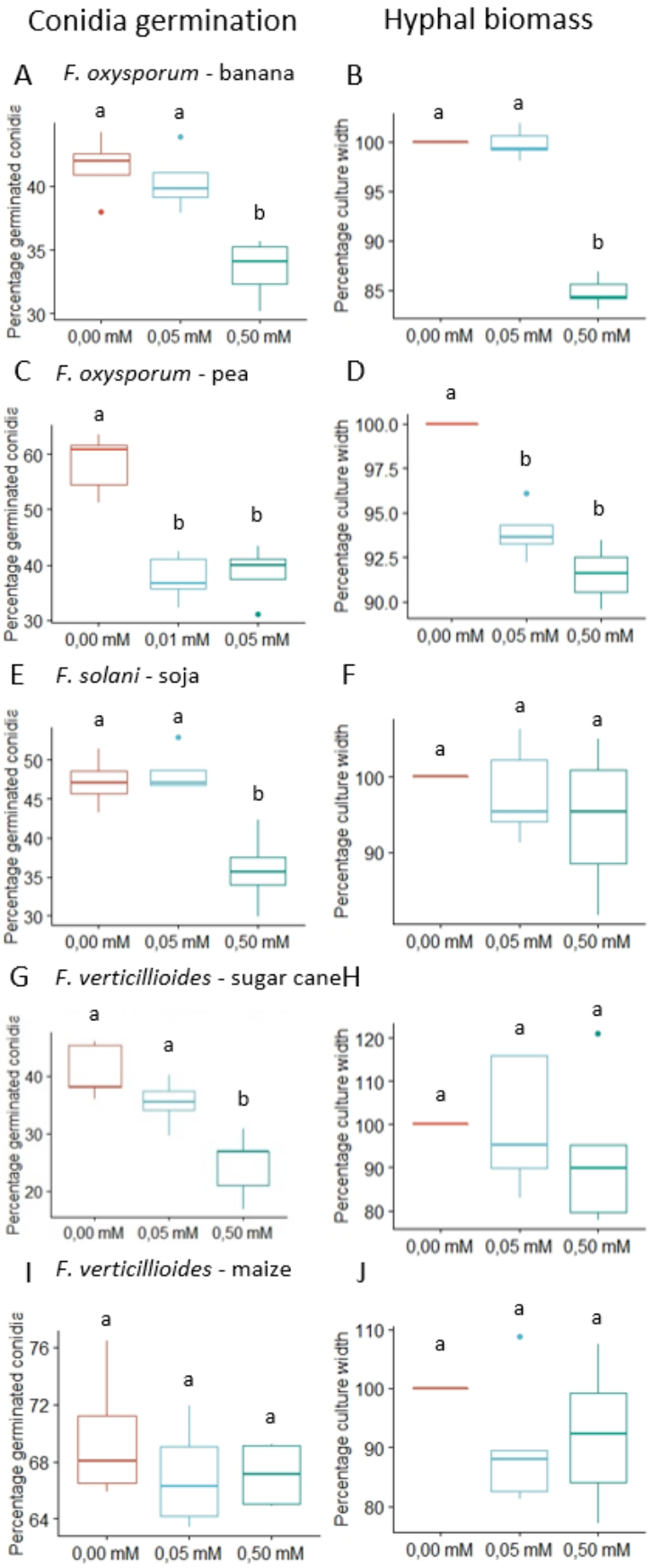

Figure 6: Tolerance assays of *Fusarium* isolates towards ambient MBOA. We assessed to tolerance of *Fusarium* isolates by germinating conidia in liquid culture, and by growing *Fusarium* on MBOA amended plates. *F. oxysporum* (A – D) was the most sensitive species, while *F. solani* from soja (E, F) and *F. verticillioides* from sugar cane (G, H) were more tolerant. *F. verticillioides* from maize (I, J) was completely tolerant to MBOA. Data was analyzed by a Wilcoxon rank sum exact test. Error bars in the charts represent standard deviation, different letters indicate significance at the level of 0.05.

3.4. Discussion

For gaining insights in the general mode of action of MBOA on microorganisms associated with plants, we studied four distinct PGPB and pathogenic *Fusarium* spp. isolated from different host plants. Evaluating the PGPB for MBOA tolerance, Ab-V5 displayed the most sensitivity to MBOA, which interestingly is frequently associated with BX producing grass plant species (Michiels et al. 1991, Croes et al. 1993, Vande Broek et al. 1998b, Housh et al. 2021). Furthermore, we observed that MBOA stimulated chemotaxis of Ab-V5, both towards 0.05 mM and 0.50 mM MBOA, while other PGPB were not attracted. Chemotaxis, being indispensable for root colonization (Vande Broek et al. 1998b, O'Neal et al. 2020), and in extension for exerting the growth promoting properties of many PGPB (Parke 1991, Bloemberg and Lugtenberg 2001), was also observed with *Pseudomonas putidi* KT2440 towards DIMBOA in an *in vitro* capillary assay using 0.237 mM DIMBOA by Neal *et al.* 2012 (Neal et al. 2012).

Curiously, biofilm production was enhanced at intermediate concentration of MBOA in case of Ab-V5 and RZ2MS9; did not affect biofilm accumulation of Pf-5 and diminished biofilm of 33.1. The coinciding time point of maximal biofilm production by Ab-V5 and Pf-5 is supported by the results of Pagnussat *et al.* 2016, which reported enhanced static biofilm formation when bacteria were co-inoculated (Pagnussat et al. 2016). In case of Ab-V5, the treatment rendering the most biofilm was depending on the inoculation time, while in other PGPB the time point of maximal biofilm accumulation remained the same over treatments. Ab-V5 showed a shift in maximal biofilm accumulation from 72 hpi in control treatment to between 96 and 120 hpi in 0.05 mM MBOA treated Ab-V5, which might be the result of a delay in biofilm establishment. Since biofilm production is under regulatory control of quorum sensing mechanisms depending on cell density (Ding et al. 2011), the delay in biofilm establishment in microtiter assays on Ab-V5 may be related to the decreased growth rate we observed in growth curves, which was not that pronounced in Pf-5 and 33.1 that neither showed a shift in time point of maximal biofilm production. The fact that none of the PGPB showed a linear relation between biofilm formation and MBOA concentration suggests a sophisticated, more complex mechanism of biofilm regulation by the presence of MBOA, rather than a direct negative influence on biofilm by chemical interaction.

At the same time, MBOA suppressed germination of conidia in all *Fusarium* spp. with the exception of maize isolated *F. verticillioides* T4 and diminished biomass of *F. oxysporum* isolates. Interestingly, when comparing *F. verticillioides* species we observed a tolerance to MBOA of T4 form maize, while FV from sugarcane was susceptible. Comparable results

were obtained in a study of Richardson *et al.* 1995, where *F. verticillioides* isolated from maize converted up to 2.5 mM BOA and MBOA, while rice isolates did not catabolize any of the benzoxazolinones (Richardson and Bacon 1995). In contrast to sugarcane that does not produce BX, maize plants produce substantial amounts of BXs and hence, our results show how *Fusarium* spp. are able to adapt to a host plant according to its BX production. In general, *Fusarium* species associated with grasses exhibit higher tolerance to BXs, enabling *F. verticillioides* to live as a symptomless endophytes (Glenn et al. 2001, Bacon and Hinton 2011). *F. verticillioides* establishes tolerance by bioconversion of BOA and MBOA into N-(2-hydroxy-phenyl)malonamic acid and N-(2-hydroxy-4-methoxyphenyl)malonamic acid respectively (Richardson and Bacon 1995, Yue et al. 1998). BX content in the soil is therefore an interesting avenue for disease suppression of non-BX producing crops by crop rotation or combining different crops (Xu et al. 2015). Besides disease tolerance caused by fungal pathogens, BX enables the plant to withstand negative plant-soil feedback from competing plants, which acts via the soil associated microbiota (Gfeller et al. 2023).

In this pioneering study, the varying influence of MBOA on Ab-V5, RZ2MS9, 33.1 and Pf-5 biofilm and chemotaxis was demonstrated, which has nowhere been reported before. We conclude that the effect of MBOA on PGPB is not straightforward but depends on the concentration of MBOA and the PGPB species. Considering the inhibitory influence of MBOA on conidia germination of several *Fusarium* species, we deduce that the net effect of MBOA favors PGPB in the rhizosphere by chemo attraction and suppressing conidia germination of pathogenic fungus. Despite a diminishing MBOA production over time during plant development (Cambier et al. 2000, Hu et al. 2018b), pathogen control is reinforced once biocontrol PGPB such as Pf-5 and Pa 33.1 colonize the root surface (Bardin et al. 2003, Bonaterra et al. 2003, Plaza et al. 2004, Henkels et al. 2014, Majumder et al. 2014, Quecine et al. 2016). Finally, further investigation is required for testing MBOA at molecular level as an agent for soil establishment of commercial PGPB inoculums in agriculture applications.

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4. ROLE OF 6-METHOXY-2-BENZOXAZOLINONE (MBOA) IN ROOT COLONIZATION BY THE PLANT GROWTH PROMOTING BACTERIA (PGPB) *Azospirillum brasilense* AB-V5 AND *Pseudomonas protegens* PF-5

Abstract

Benzoxazinoids (BXs) form a group of secondary metabolites produced by many plants of the grass family (*Poaceae*). Release and activation of BXs upon pathogen attack strongly suppresses disease of pest species and foraging of herbivorous insects in areal parts of the plant. At the same time, BXs are constitutively produced and released in the rhizosphere predominantly during early plant development, where they affect the microbiome. Hydroxamic acid BX derivatives such as DIBOA, DIMBOA and HDMBOA in general are more reactive but have a shorter half-life than the lactam derivatives BOA and MBOA. Regardless, MBOA is more efficient at suppressing several fungal pathogens and influences microbial rhizosphere composition over generations of plants. Key to understanding plant-microbe symbiosis is knowledge about the means of chemical communication between symbionts, and the physiological changes those signaling molecules evoke on each symbiont. Therefore, we studied the mechanisms by which an interspecies exchange of information precedes the initiation of symbiosis establishment. In order to gain more insight into these processes, we investigated how MBOA mediate root colonization by the plant growth promoting bacteria (PGPB) *Azospirillum brasilense* Ab-V5 and *Pseudomonas protegens* Pf-5. In this work we studied root colonization patterns of the PGPB on roots of *Arabidopsis thaliana* and the impact of ectopically applied MBOA on biofilm formation. Both Ab-V5 and Pf-5 colonize root hairs and crevices on the root surface. Biofilm produced by Ab-V5 was more abundant and covered more surface of MBOA treated roots, while the amount of biofilm originating from Pf-5 was equal in both treatments. Peroxidase activity was unaffected by MBOA, yet was elevated in both Ab-V5 and Pf-5 inoculated *Arabidopsis* seedlings, with a more pronounced effect on Ab-V5 inoculation. We conclude that MBOA favors root colonization of Ab-V5 while colonization by Pf-5 is not improved.

4.1. Introduction

As plants germinate and develop, the region around the root system acquires a unique composition of microbial life with an abundance much higher than the circumference. This phenomena called ‘the rhizosphere effect’ (Whipps 2001), causes high competitive pressure among microbes in order to dominate the rhizosphere and colonize plant roots. The soil bacterial community structure strongly depends on the genotype of the host plant, which defines the chemical composition of root exudates (Haichar et al. 2008), and the soil type that harbors potential microbial symbionts (Berg and Smalla 2009). Consequently, many plants have the ability to condition the soil and modify the local environment, influencing the plants’ performance. Many cereals release 2-hydroxy-2,4-benzoxazin-3(4H)-one (HBOA) derived secondary metabolites or benzoxazinoids (BX) in the soil, such as 6-methoxy-2-benzoxazolinone (MBOA) that have a strong influence on the organization of the rhizosphere associated microbiome (Hu et al. 2018b, Cotton et al. 2019, Kudjordjie et al. 2019, Cadot et

al. 2021). Hence, by acquiring plant growth promoting bacteria (PGPB) host plants indirectly stimulate their own health which, in some cases, can be facilitated by exudation of BX (Hu et al. 2018b).

A variety of benefits can be obtained from symbiosis with PGPB. *Azospirillum brasilense* attributes its plant growth promoting effects mainly to its production of plant hormones such as indole-3-acetic acid, cytokines and gibberellins (Reynders and Vlassak 1979, Tien et al. 1979, Bottini et al. 1989), and to a lesser extent to fixation of atmospheric nitrogen through reduction of nitrate (NO_3) (Zimmer et al. 1984). Conversely, *P. protegens* is a biofilm forming PGPB (Ueda and Saneoka 2015) that attributes to plant health predominantly via secondary metabolite production, which makes it a strong antagonist of *Fusarium* species (Kidarsa et al. 2013, Henkels et al. 2014, Loper et al. 2016, Quecine et al. 2016) and suitable as a biocontrol agent.

Being a necrotrophic pathogen, *F. verticiollioides* highly profits from reactive oxygen species (ROS), which above a certain threshold can cause programmed cell death of the cell (Van Durme and Nowack 2016) and originates from the penetration peg and appressoria for weakening the host cell wall (Jennings et al. 1998, Segmüller et al. 2008) to invade the plant cell. As a countermeasure the plant cell aims at keeping ROS levels in check by means of several ROS scavenging mechanisms including peroxidases (Gill and Tuteja 2010, Heller and Tudzynski 2011). Furthermore, Shelud'ke *et al.* 2020 demonstrated how biofilm produced by *A. brasilense* harnesses peroxidase activity (Shelud'ko et al. 2020), providing a protective barrier from ROS.

To exert their properties on the host plant, successful colonization of the root surface is a requisite for many PGPB. A universal mechanism of attachment by bacteria in plant microbe-interactions is accomplished via formation of biofilms on the root surface of host plants, a multicellular community enclosed in a matrix composed of polysaccharides, proteins, lipids and extracellular DNA (Sutherland 2001, Ramey et al. 2004, Branda et al. 2005, Danhorn and Fuqua 2007, Wang et al. 2017). A resistant biofilm ensures an intimate and efficient symbiosis and improves protection against environmental stresses as well as resistance against antibiotics and favors nutrient absorption (Wang et al. 2017, Yannarell et al. 2019). The amount of studies reporting on the effect of BXs on bacterial biofilms is very limited (Guo et al. 2016) not including any studies conducted on PGPB.

In this pioneering work, we build on previous research demonstrating a positive effect of MBOA on Ab-V5 biofilm and chemotaxis, two important traits for root colonization (Bashan and Holguin 1993, 1994, Vande Broek et al. 1998b). We hypothesized that ambient

MBOA improves root colonization and tested therefore the two distinct PGPB *A. brasilense* Ab-V5 and *P. protegens* Pf-5. First, we analyzed biofilm on the root surface to assess the colonization process. In addition, we measured peroxidase activity in the roots as a second factor to score root colonization. We considered this feedback from the host plant in peroxidase activity as a measure for perceiving the PGPB. Peroxidases are typically expressed in higher levels upon microbial infection and in PGPB interactions (Lavania et al. 2006) limiting infection of microbial pathogens through production of ROS (Almagro et al. 2008).

4.2. Methods

4.2.1. Bacterial strain and media

The bacterial strain *A. brasilense* Ab-V5 previously isolated from maize (*Zea mays*) (Hungria et al. 2010) was grown at 28°C on DYGS medium (Rodriguez et al. 2004), *P. protegens* Pf-5 isolated form the cotton rhizosphere (*Gossypium hirsutum*) (Howell and Stipanovic 1978) was cultivated in bacterial growth medium Kings B (KMB) (King, Eldora et al. 1954) at 28°C. Bacterial stock was stored in 15 % glycerol at -80 °C in the Molecular Genetics Lab (Piracicaba, Brazil). On the onset of the experiment, bacterial cultures were freshly prepared from stock and grown in liquid medium until an optical density at 600 nm (OD_{600}) of 1.0, which was then diluted to an OD_{600} of 0.05 (approximately 10^8 bacteria) after washing the cells in phosphate buffered saline (PBS, 8 g/L NCL, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) of pH 7.4.

4.2.2. Plant growth conditions

Seeds of wildtype *Arabidopsis thaliana* Col-0 were surface sterilized by suspending the seeds in 70 % ethanol for two minutes, in 50 % hypochlorite for ten minutes and in 70 % ethanol followed by rinsing three times with sterile deionized water. Sterile seeds were placed on ½ MS plates containing 0.8 % agar and 1 % sucrose. After an incubation period in the dark for three days, plates were placed vertically in an incubation room at 22°C under a 16/8 hours light/dark regime, in order for the roots to grow on the surface of the ½ MS agar plates. After two weeks of incubation, seedlings were analyzed.

4.2.2. Brightfield epifluorescence microscopy, Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM)

Two weeks old *A. thaliana* seedlings were 96 hours before analysis inoculated with washed Ab-V5 or Pf-5 cultures of OD_{600} of 0.05 with or without the addition of 0.05 mM

MBOA (cat. no. 532-91-2, Sigma-Aldrich; Saint Louis, USA). Prior to bright field fluorescence microscopy, *Arabidopsis* seedlings were supplemented with 3 mL of 2 µg/mL NileRed solution (9-diethylamino-5H-benzo[a]phenoxazine-5-one) (cat.no. 7385-67-3, Sigma-Aldrich; Saint Louis, USA); incubated at room temperature for one hour at 120 rpm; rinsed with sterile Milli-Q (Merck, Darmstadt, Germany) purified water and carefully transferred on microscopic slides and sealed. Nile Red is a lipophilic stain that has an emission wave length around 540 nm when bound to neutral lipids and around 650 nm when bound to polar lipids (Greenspan and Fowler 1985, Diaz et al. 2008, Marin-Dett et al. 2022). Fluorescent microscopic analysis was carried out using an Axiophot II epifluorescence microscope (Zeiss, Oberkochen, Germany) with magnifications within the range of 100 – 400 times, with a 488 nm laser at 5% intensity and a 500 – 550 nm filter (fluorescein isothiocyanate (FITC), 510 nm) to capture green fluorescent signal, and a 590 nm long pass filter to capture the red fluorescent signal with the pinhole set at 150 µm. The microscope was mounted with a PCO CCD camera and the ISIS Metasystems software (Metasystems, Germany) was used to digitalize and to analyze the captured images.

Preparation of samples for scanning electron microscopy (SEM) included a primary fixation step with 2.5 % glutaradehyde in 0.2 M cacodylate; secondary fixation with 2 % osmium tetroxide overnight; dehydration in a series of ethanol solutions in increasing concentration (10%, 20%, 30%, 50% 70% ten minutes per step and three times in 100 % ethanol); drying with a Baltec EM CPD 300 (Baltec, Lichtenstein) critical point drying machine and gold coating with a Baltec SCD 050 (Baltec, Lichtenstein) gold coater. After mounting the samples on stubs, they were analyzed by a JEOL JSM-IT300LV (JEOL, Japan) located at the Phytopathology Department at ESALQ/USP (Piracicaba, Brazil) using an accelerating voltage of 20 kV and magnifications varying between 140 and 750 times, during SEM image analysis.

4.2.3. Adherence assay

Ab-V5 cultures treated with either 0.00 mM, 0.05 mM or 0.50 mM were grown until early log-phase and rinsed in PBS of pH 7.4 by centrifuging with a universal 320R benchtop centrifuge (Hettich, Westphalia, Germany) during 10 minutes at 4 °C and resuspending in PBS by pipetting. Two weeks old *A. thaliana* Col-0 seedlings were grown on sterile ½ MS 0.8 % phytoagar medium containing 1 % sucrose and inoculated with 5 mL of the rinsed *A. brasilense* Ab-V5 culture and diluted to an OD_{600} of 0.05. The seedlings were incubated for two hours shaking at 120 rpm and 28 °C. The areal parts of two Arabidopsis seedlings were

removed and the roots inserted in the Eppendorf micro centrifuge tubes with 1 mL PBS that were weighted beforehand to determine the mass of the roots. By shaking gently in 1 mL PBS three times roots were rinsed; ground with pestle and mortar in 0.5 mL PBS; 1000 times diluted and plated out on DYGS agar plates in triplicate to determine the attached bacteria by enumeration on plates.

4.2.4. Peroxidase assay

Peroxidase activity was evaluated by measuring oxidation of guaiacol by spectrophotometry. *A. thaliana* Col-0 seedlings grown on 0.8 % agar, 1 % saccharose, ½ MS medium were harvested two weeks after germination in samples of approximately 0.5 g. 72 hours before analysis, *A. thaliana* Col-0 seedlings were inoculated with washed Ab-V5 or Pf-5 bacteria treated with 0.00 mM, or 0.50 mM. The seedlings were homogenized in 0.5 mL 10 mM sodium acetate of pH 5, centrifuged for 25 minutes at 15 000 x g and 4° C where after the supernatant was used as protein extract for the peroxidase activity measurement. The measurements were started by mixing in a cuvette: 970 µL sodium acetate, 2.5 µL guaiacol 0.25 % (v/v), 6.0 µL hydrogen peroxide 30 % 100 V and 20 µL of protein extract. Absorbance of oxidized guaiacol was then measured every ten seconds along the timespan of one minute in a Genesys 30 spectrophotometer (Thermo Scientific, Waltham, USA) at 470 nm wavelength. From the data, normalized per gram of tissue, the coefficients of the regression lines were used to calculate the peroxidase activity expressed in absorbance per minute per gram.

4.2.5. Sequencing

To confirm the bacterial species that was observed by microscopy, DNA from inoculated *Arabidopsis* seedlings was isolated using a DNeasy Blood and Tissue kit (Qiagen, Venlo, The Netherlands). From total DNA, 16S PCR was performed according to Stets *et al.* 2015 (Stets et al. 2015) using *taq* DNA polymerase and 16S forward (TCGCTAGTAATCGCGGATCA) and reverse (TGTGACGGGCGGTGTGTA) primers. PCR products were purified from reactions with a Wizard SV gel and PCR clean-up system kit (Promega, Madison, USA) and send for sequencing to Laboratório de Malhoramento de Plantas in Centro de Energia Nuclear na Agricultura (Piracicaba, Brazil).

4.2.6. Statistical analysis

Data obtained from digital analysis of pictures from plates using the ImageJ software (Scion Corporation, Maryland) for counting colony forming units (CFU), and data from the peroxidase activity assay were statistically analyzed using the R software (Bunn and Korpela 2018). We tested for normality via the Shapiro-Wilk normality test. Normal distributed data was subjected to a one-way ANOVA and a subsequent Tukey multiple comparisons of means or a Welch Two Sample t-test for testing two groups. Not normal distributed data was analyzed with a Kruskal-Wallis rank sum test or Wilcoxon rank sum test with continuity correction (a.k.a. Mann–Whitney U test).

4.3. Results

4.3.1. MBOA treatment improves biofilm formation on *Arabidopsis* roots from Ab-V5 but not from Pf-5

Root samples treated with 0.05 mM MBOA showed a thicker and denser Ab-V5 biofilm, covering more surface of the root than was observed in control treatments. Significant amounts of biofilm were found in untreated samples, though not to the same extend as MBOA treated roots (**Figure 7**) (**Supplementary Figure 4**). Even though biofilm was not homogeneous on roots, MBOA treatment exhibited at least around 30 – 40 % of extensive biofilm on the root surface, while this percentage was around 20 % in untreated samples. Pf-5 inoculated roots did not exhibit significant differences in the amount of bacterial biofilm on root surfaces among treatments (**Supplementary Figure 5**, **Supplementary Figure 6**). The same conclusions were drawn from bright field microscopy analysis (**Figure 8**) (**Supplementary Figure 6).**

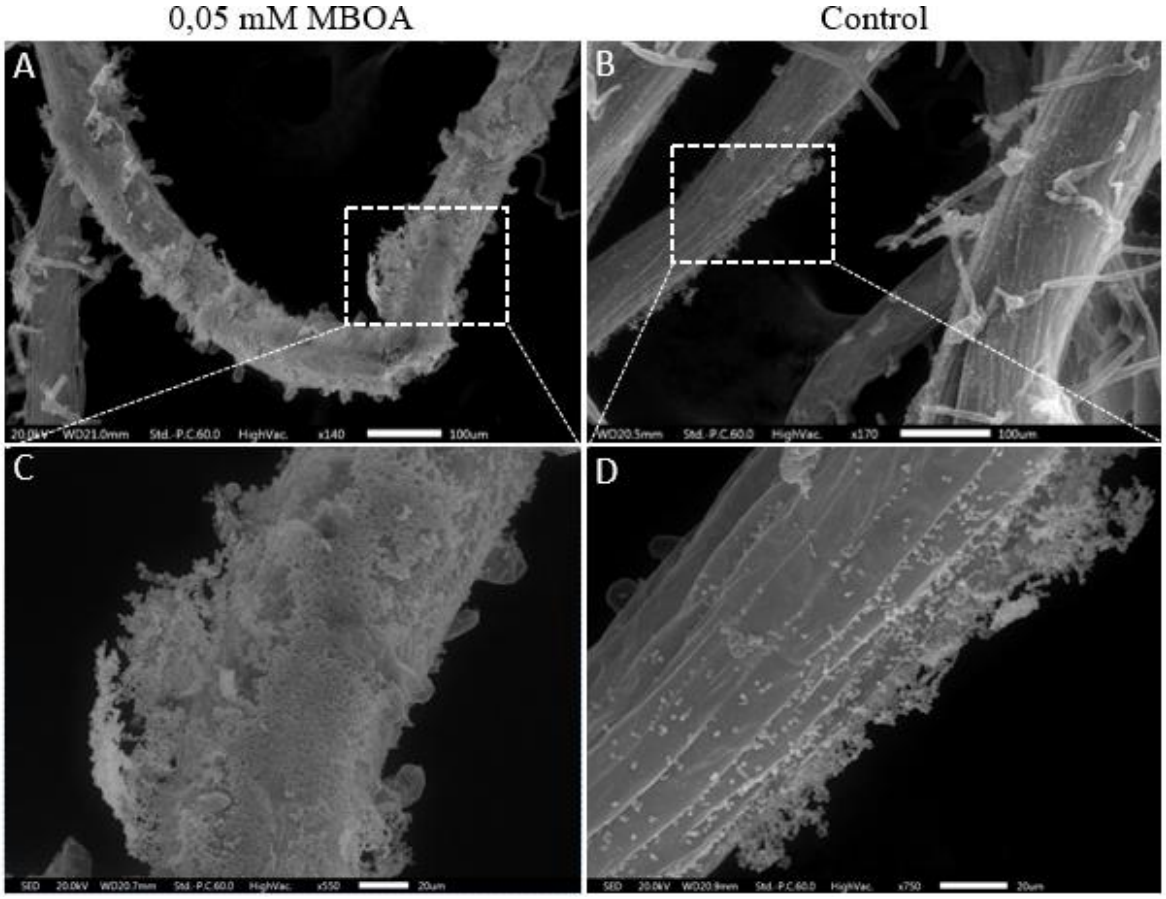

Figure 7: Scanning electron microscopy of Arabidopsis thaliana Col-0 roots inoculated with Ab-V5. Seedlings were grown on ½ MS agar medium for 14 days and incubated for 96 h with Ab-V5 cultures of OD_{600} 0.05, prior to sample preparation. A and C: MBOA treated roots were covered partly with a thick layer of biofilm, covering large areas of the root surface. B and D: Biofilm on mock treatment was produced to a lesser extent. Scale bars indicate 100 µm (A and B) or 20 µm (C and D).

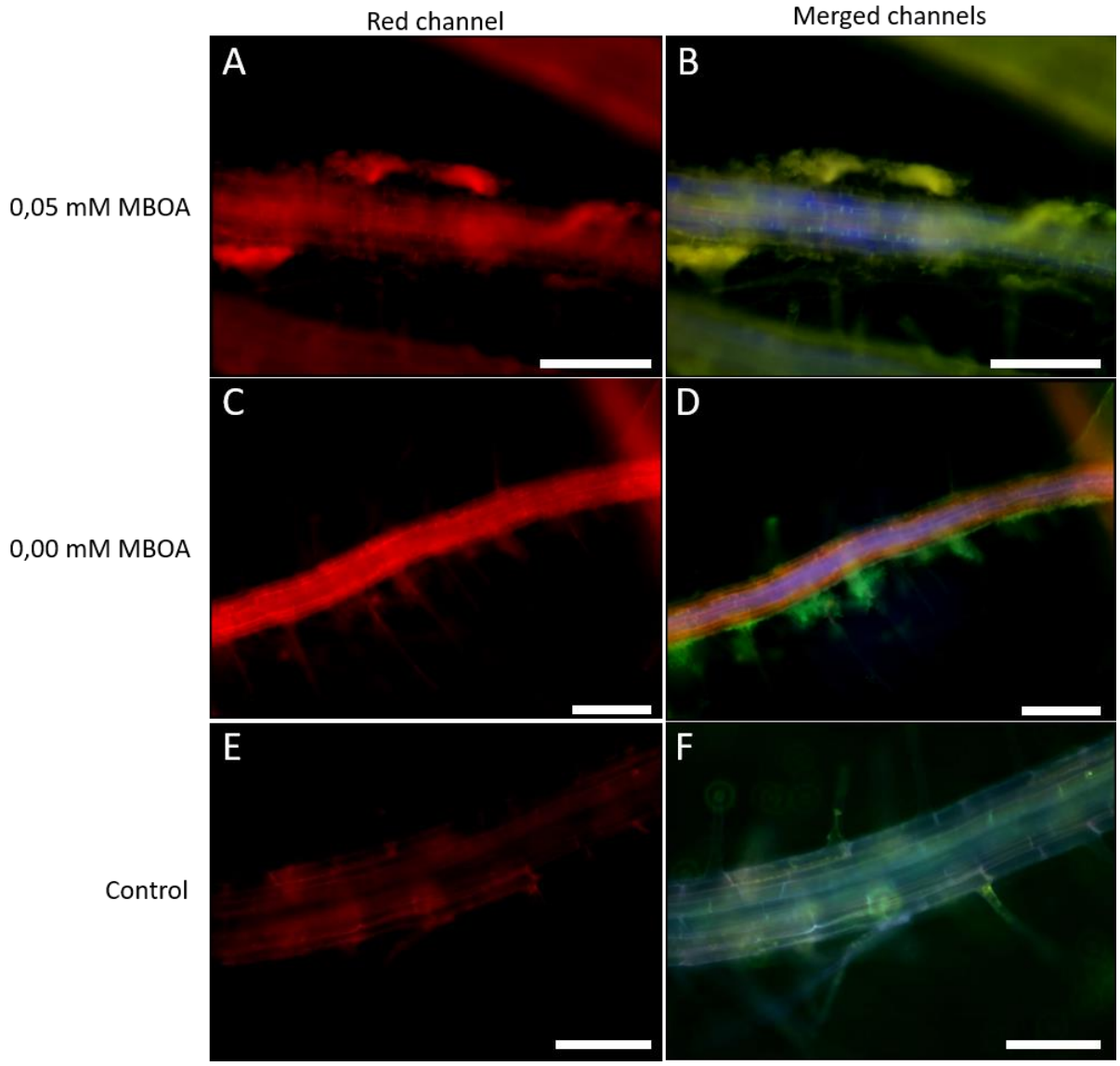

Figure 8: Bright field fluorescence microscopy of Arabidopsis roots inoculated with Ab-V5. Arabidopsis seedlings were grown on ½ MS agar medium for 14 days and incubated for 96 h with Ab-V5 cultures of OD 0.05, prior to sample preparation. A, B: 0.05 mM MBOA treatment. C, D: 0.00 mM MBOA treatment; E, F: control treatments. Seedlings were treated for 1 hour with Nile Red solution which has a peak emission wave length of around 540 nm when bound to neutral lipids and around 650 nm when bound to polar lipids. There was an observable difference in the amount of biofilm on the root surface among the two treatments with 0.05 mM (A and C) accumulating thicker and wider spread biofilm. Scale bars indicate 50 µm (A-D), and 100 µm (E, F).

4.3.2. Ab-V5 and Pf-5 prefer crevices and root hairs as principle colonization sites on the root surface

From analyzing the root surfaces infected by Ab-V5 and Pf-5, we could observed a preference of primary colonization of both bacteria in crevices, protected areas on the root surface and on root hairs. Roots were abundantly colonized in crevices (**Figure 9A and 3B**) and on root hairs (**Figure 9C- 3F**). Furthermore, from fluorescent microscopy images we could observe Ab-V5 and Pf-5 internalizing root tissue via root hairs (**Figure 9C and 3F**). The choice for primary colonization sites was more obvious on Pf-5 treated samples than Ab-

V5, after which both bacteria have the capacity to spread and colonize larger areas on the root surface. The root colonization patterns were not influenced by MBOA treatment, same observations were found in all samples. From the sequencing results of root samples, 16S nucleotide sequences matching with the inoculated bacteria were retrieved (**Supplementary Table 4**: Results of NCBI BLAST searches with the nucleotides sequences from 16S sequencing as input, retrieved from root samples used for bright field fluorescence microscopy.).

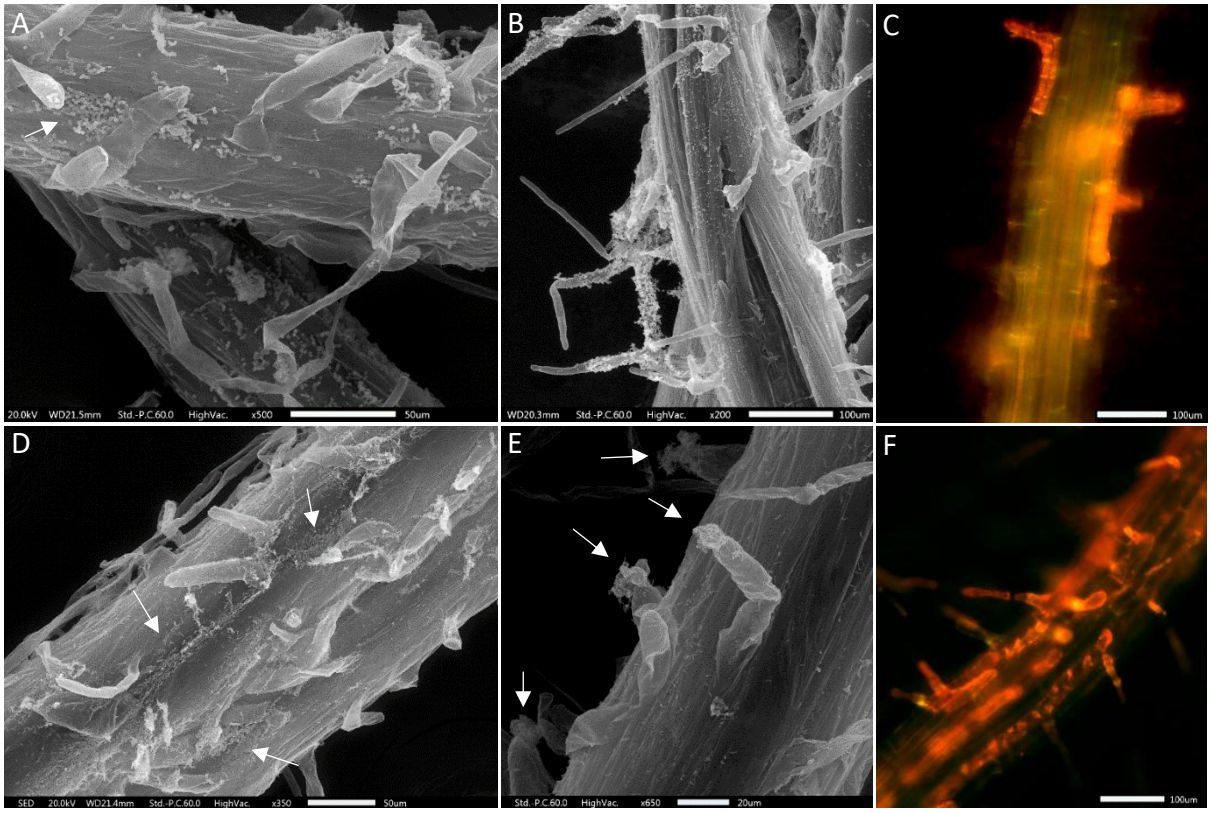

Figure 9: Scanning electron microscopy of Arabidopsis thaliana Col-0 roots inoculated with Ab-V5 (A, B) and Pf-5 (D, E) and bright field fluorescence images of Ab-V5 (C) and Pf-5 (F) inoculated roots. Arabidopsis seedlings were grown on ½ MS agar medium for 14 days and incubated for 96 h with either Ab-V5 or Pf-5 cultures of OD 0.05, prior to sample preparation. For bright field microscopy, seedlings were treated for 1 hour with Nile Red solution which has a peak emission wave length of around 540 nm when bound to neutral lipids and around 650 nm when bound to polar lipids. A and C: Ab-V5 (A) and Pf-5 (C) widely colonize root surfaces along crevices on the root, and on root hairs (B and D). Arrows mark local accumulation of bacteria. Scale bars indicate 50 µm (A and C), 100 µm (B) and 20 µm (D).

4.3.3. Adherence of Ab-V5 to roots and peroxidase activity is unaffected by MBOA treatment

A. thaliana Col-0 seedlings were cultivated as previously described for the microscopic assays, in sterile Petri dishes on ½ MS agar, and inoculated with either Ab-V5 or Pf-5, with or without 0.50 mM MBOA supplement. Control treatments included sterile *A.*

thaliana seedlings with or without 0.50 mM MBOA to assess the potential bias of MBOA in the assays. This bias was not observed in the peroxidase assay, since there was no significant difference between these control treatments. Colonization of the roots by Ab-V5, caused an increase of one and a half times the activity of peroxidases, both with and without treatment of MBOA. MBOA treatment did not influence the peroxidase activity exerted by the *A. thaliana* roots, although there was a significant increase in peroxidase activity when seedlings were inoculated with Ab-V5 (**Figure 10B**). Pf-5 inoculation resulted in a significantly elevated peroxidase activity compared to sterile seedlings, albeit not as pronounced as the increase that was achieved with Ab-V5 inoculation (**Figure 10A**).

Based on results from *in vitro* biofilm assays, we carried out an adherence assay on Ab-V5, which earlier showed improved biofilm after 120 hours of inoculation with 0.05 mM MBOA (Chapter 3). However, there was no significant difference in number of CFU that was retrieved from inoculated *Arabidopsis* roots (**Figure 10C**). Biofilm was previously measured by spectrophotometry of crystal violet stained bacterial cultures that were grown statically for 120 hours. In contrast, the capacity of Ab-V5 bacteria to adhere to *Arabidopsis* roots was evaluated after two hours of incubation under mellow agitation, making the results of the two assays hard to compare.

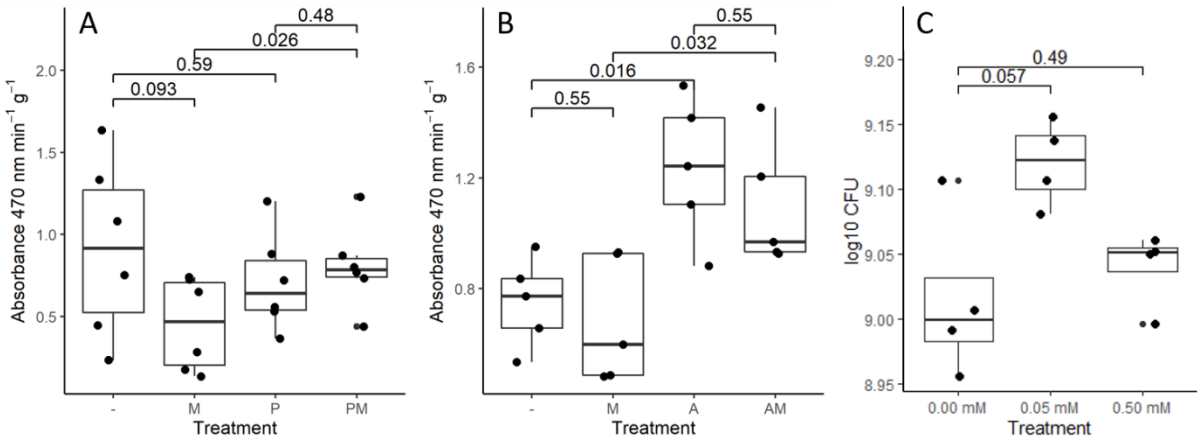

Figure 10: Peroxidase activity of A. thaliana roots is enhanced when plants were inoculated with P. protegens Pf-5 (A) and with A. brasilense Ab-V5 (B). Adherence assay on Ab-V5 inoculated A. thalinana roots (C), does not show significant differences in CFU, when treated with either 0.05 mM or 0.50 mM MBOA. "-": negative control; "M": 0.50 mM MBOA; "P"/"A": Pf-5/ Ab-V5; "PM"/"AM": 0.05 mM MBOA and Pf-5/Ab-V5. P-values indicated in the graph are result of ANOVA tests.

4.4. Discussion

During the first phases of rhizosphere and rhizoplane establishment, PGPB endure strong competition with various commensal microorganisms that dwell in the soil (Whipps

2001). By differences in exudation pattern according to root zones and consequently because of distinct chemotaxis and quorum sensing responses of bacteria, roots are occupied in a non-uniform distribution (Schloter and Hartmann 1998, Vande Broek et al. 1998a, Bloemberg et al. 2000, Gamalero et al. 2004). After colonizing the root surface, endophytic bacteria internalize the plant tissue granting the advantage of a steady supply of nutrients in a protected environment. Penetration however, does not necessarily require active mechanisms (Hardoim et al. 2008), but involves a range of bacterial traits (Compant et al. 2010) and is possible passively via entering through cracks and sites of lateral root emergence (Reinhold-Hurek and Hurek 1998).

Colonization of roots by Ab-V5 we observed was most similar to *A. brasilense* Sp245, which penetrates the root epidermis and internally colonize root hairs and vasculature, as opposed to colonization by *A. brasilense* Sp7 which is limited to the root surface (Schloter and Hartmann 1998, Vande Broek et al. 1998a). Similarly, we found Pf-5 giving preference to root hairs and crevices as primary colonization sites. This colonization pattern is similar to *P. fluorescens* WCS365 which forms a thin biofilm localized around fissures while *P. putida* produces a thick continuous biofilm spreading over the entire root (Bloemberg et al. 2000, Bloemberg and Lugtenberg 2004). The environmental conditions that stimulate biofilm formation in *Pseudomonas* is species specific: where *P. protegens* produces biofilm in nutrient rich environments, *P. fluoresens* and *P. putida* are stimulated to form biofilm in nutrient poor conditions (Ueda and Saneoka 2015). The amount of biofilm formation recorded by SEM and fluorescent microscopy was unaffected by MBOA treatment in Pf-5 inoculated roots. From results that were obtained in earlier studies, the same was concluded: in microtiter plate *in vitro* assays, intermediate concentrations of MBOA (0.05 mM) did not alter biofilm formation of Pf-5 nor affected bacterial growth in liquid cultures.

Concurrently, after 72 hours of inoculation with Ab-V5 and Pf-5, *A. thaliana* seedlings showed elevated peroxidase activity. Since peroxidases keep ROS levels in check and protect cellular homeostasis (Arora et al. 2002, Mittler 2002, Baxter et al. 2014), results may be indicative of a plausible indirect defense mechanism against phytopathogens (M'piga et al. 1997). Fukami *et al.* 2018 showed that treatment of maize plants with *A. brasilense* Ab-V5 stimulated jasmonic acid (JA) and salicylic acid (SA) pathways, leading to activation of induced systemic resistance (ISR) (Fukami et al. 2018b) and expression of defense related genes (Fukami et al. 2017), while ISR by Pf-5 is independent of SA signaling (Pieterse et al. 1996) and marked by increased peroxidase activity (Nandakumar et al. 2001, Suresh et al. 2022), corroborating our results.

We analyzed root colonization patterns of Ab-V5 and Pf-5 on *A. thaliana* Col-0 seedlings by microscopic techniques and studied the absolute effect of MBOA on adherence to the root as well as induction of peroxidase activity. This pioneering study on Ab-V5 and Pf-5 root colonization, shows how MBOA has a significant effect on bacterial ecological behavior by altering the way it occupies the root surface. Colonization of *Arabidopsis* roots by Ab-V5 and Pf-5 stimulated a response in peroxidase activity in the plant, which was not influenced by MBOA treatment. Likewise, MBOA did not alter the outcome in number of adhering bacteria to the roots. Thus, MBOA exerts a subtle influence on bacterial behavior. Taken together, our findings stimulate the thought that MBOA promotes root colonization of *A. thaliana* by Ab-V5 while the colonization process of Pf-5 is not influenced. Since MBOA production by maize peaks around 12 weeks after germination by up to 75 µg per plant (Hu et al. 2018b), MBOA plays an important role in acquisition of PGPB and their establishment within the rhizobiome during early plant development. Bacteria may be stimulated during that time period to disperse in the soil and are attracted to the root, while the diminished production of MBOA in the period that follows allows bacteria to aggregate, produce biofilm and properly colonize the plant roots.

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5. TRANSCRIPTOMICS ON *Azospirillum brasilense* AB-V5 and *Pseudomonas protegens* Pf-5 REVEAL ROLE OF BENZOXAZINOIDS IN EARLY PLANT-MICROBE INTERACTIONS

Abstract

Root colonization by plant growth-promoting bacteria (PGPB) involves recruiting symbiotic partners from a diverse biosphere. Among PGPB, *Azospirillum brazilense* Ab-V5 and *Psuedomonas protegens* Pf-5 are two commercial inoculants renowned for their growth enhancing capacity through production of phytohormones and nitrogen fixation, and by disease suppression respectively. Benzoxazinoids (BXs) have a strong impact on microbiome dynamics in the rhizosphere, therefore we studied the transcriptome of two well characterized PGPB, Ab-V5 and Pf-5 in presence of the relatively stable lactam BX derivative MBOA. We performed RNA sequencing of the total RNA extracts from Ab-V5 and Pf-5, grown statically in liquid cultures for 72 hours in three MBOA concentrations. In Ab-V5, we could reveal the upregulation of a chemotaxis regulatory gene in response to MBOA, representing a key characteristic in PGPB root colonization. Notably, two MBOA concentrations, 0.05 mM and 0.50 mM, impacted cellular respiration and energy metabolism differently. The absence of upregulated chemotaxis genes at 0.50 mM and large alterations in expression profiles of primary metabolism related genes, suggested surplus energy was directed towards metabolic adaptation. Interestingly, symbiosis-related gene downregulation occurred in both treatments, leading to reduced biofilm formation, impaired auxin efflux carriers, and varied nitrogen homeostasis. In contrast, Pf-5 showed very little alterations of gene expression profiles in 0.50 mM MBOA, while no significant differentially expressed genes were found in 0.05 mM MBOA. This study provides insights into how MBOA influences early plant-microbe interactions.

5.1. Introduction

Benzoxazinoids (BX) are secondary metabolites produced by many grasses including rye, wheat and maize (Niemeyer 2009). Set free in the soil by roots, they strongly affect the composition of the rhizomicrobiome, stimulating a vast array of positive features attributed by plant growth promoting bacteria (PGPB), ranging from nutrient acquisition and plant growth to plant defense (Cambier et al. 2000, Niemeyer 2009, Ahmad et al. 2011, Bever et al. 2013, Neal and Ton 2013, Teste et al. 2017, Hu et al. 2018, Cotton et al. 2019, Kudjordjie et al. 2019, Cadot et al. 2021). The growth promoting properties of the well characterized microbial inoculant *A. brasilense* including the strain Ab-V5, primarily stem from its production of plant hormones (Reynders and Vlassak 1979, Tien et al. 1979, Bottini et al. 1989) and to a lesser extent by its nitrogen-fixing abilities (Zimmer et al. 1984). Biosynthesis of plant hormones by *A. brasilense* stimulates the development of lateral roots and root hair (Spaepen et al. 2014, Cohen et al. 2015, de Almeida et al. 2021) leading to improved water and mineral uptake. The PGPB *Pseudomonas protegens* Pf-5 (previously *Pseudomonas fluorescens* Pf-5) on the other hand, is renowned for its large amount of antimicrobial secondary metabolites

and colonizes a wide variety of plant hosts (Budzikiewicz 1993, Khalid et al. 2004, Quecine et al. 2016, Lopes et al. 2018a, 2018b). Therefore, *P. protegens* Pf-5 is of special interest as a biocontrol strain and for conferring disease tolerance (Howell and Stipanovic 1978, 1980, Xu and Gross 1986, Rodriguez and Pfender 1997, Sexton et al. 2017). Inoculated together, *A. brasilense* Sp245 and *P. protegens* CHA0 have complementary functions and show cooperative behavior by formation of structured mixed biofilms (Pagnussat et al. 2016).

Key to root colonization is the directed movement towards colonization sites via chemotaxis. A positive chemo attraction of PGPB has been reported in bacteria towards root exudates (de Weert et al. 2002, Neal et al. 2012, Yuan et al. 2015, O'Neal et al. 2020, Feng et al. 2021, Xie et al. 2022). However, specific studies on chemotaxis to benzoxazinoid (BX) derivatives is limited to 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) isolated from maize roots conducted on *P. putida* (NEAL et al., 2012), which is spontaneously converted into 6-methoxy-benzoxazolin-2-one (MBOA) (Fomsgaard et al. 2004). In an earlier study (Chapter 3), we conducted in vitro assays with MBOA and confirmed the upregulation of a chemotaxis regulatory gene, which had not been previously reported in *A. brasilense* Ab-V5 and *P. protegens* Pf-5.

Intriguingly, *Azospirillum brasilense* and *Pseudomonas fluorescens* establish a positive feedback loop by stimulating BX metabolism of the plant upon root colonization (Walker et al. 2011, Zhou et al. 2020), which renders a species specific readout of BX derivatives in case of *A. brasilense* (Walker et al. 2011, Camilios-Neto et al. 2014). Similarly, maize plants inoculated with *P. fluorescens* MZ05 induce *BX2* and *GLU2* two genes related to BX metabolism and augment BX content in the leaves (Zhou et al. 2020). Consequently, BX exudated in the soil is likely to be of advantage for the PGPB, or might act indirectly as a signaling molecule at the onset of root colonization.

Thus, both *A. brasilense* and *P. protegens* make interesting study objects for analyzing the influence of MBOA on their respective transcriptomes. *P. protegens* Pf-5 is a common root colonizing gram negative bacteria that is distinct from *P. fluorescens* by its production of pyoluterin and 2,4-diacetylphloroglucinol, two potent antimicrobial compounds (Ramette et al. 2011). *P. protegens* has a high metabolic flexibility, with some strains even possessing the capacity to use insects as vectors for dispersal (Flury et al. 2019, Vesga et al. 2021). This versatility is reflected by its extensive genome size of 7.1 Mbp (Paulsen et al. 2005). Specifically the strain Pf-5 is efficient in suppressing *F. verticillioides* mainly owing to the production of rhizoxin, pyrrolnitrin, and 2;4-diacetylphloroglucinol (DAPG) (Quecine et al. 2016) among the wide range of antimicrobial metabolites it releases in the soil (Loper et al.

2007, Gross and Loper 2009) and effective against disease tolerance to *Botryis cinerea* and *Nigrospora* spp. (Balthazar et al. 2022a, 2022b). *A. brasilense* too, exhibits extraordinary genome plasticity characterized by numerous repetitive sequences and origins of replications (Wisniewski-Dyé et al. 2011). The large genome size (6.9 Mbp) and high GC content results from an early duplication event of DNA polymerase dnaE, giving rise to the error-prone DNA polymerase dnaE2 characteristic for terrestrial bacteria (WU et al., 2014). Within the family *Rhodospirillaceae*, where most members are aquatic bacteria, *Azospirillum* is the only genus known to associate with plants (Battistuzzi and Hedges 2009). Related to this, *Azospirillum* acquired around fifty percent of its protein-coding genes through horizontal gene transfer. Most of these genes are associated with rhizosphere adaptation, while conserved ancestral genes perform essential housekeeping functions (Wisniewski-Dyé et al. 2011). Meanwhile, Ab-V5 inoculation significantly improves yield of maize and wheat (Hungria et al. 2010) and confers stress tolerance by stimulation of JA and SA pathways and peroxidase activity (Fukami et al. 2017, 2018).

Considering its crucial role during evolution of plant roots and its strong adaptive potential (Wisniewski-Dyé et al. 2011), the significant impact on the alpha diversity of the root-associated microbiome of Ab-V5 inoculation, comes to no surprise (Ferrarezi et al. 2022). We build on previous findings, which demonstrated the contradicting responsiveness of Ab-V5 and Pf-5 to MBOA treatment in biofilm formation and chemo attraction, to study gene regulation in more depth and unravel regulatory mechanisms explaining those physiological responses that were observed. Furthermore, we discovered from *in planta* microscopic assays that MBOA stimulated biofilm formation of Ab-V5 more when associated with plant roots (Chapter 4), than was measured from *in vitro* assays absent of a live host (Chapter 3). To the best of our knowledge, in contrast to the well-studied influence of BXs on the whole microbiome (Hu et al. 2018, Cotton et al. 2019, Kudjordjie et al. 2019, Cadot et al. 2021) this study represents the first transcriptomic analysis conducted on individual PGPB to investigate the direct impact of BXs on RNA profiles. We found evidence on a molecular level showing how MBOA affects colonization mechanisms of Ab-V5 and Pf-5 that complement and validate what was concluded from microbial and biochemical assays. Given the relevance of both species in plant-microbe interaction, the data we have gathered hold significant potential for unraveling and comprehending colonization behaviors, the conditions for colonization, and inter-species interactions and may be extendible in other conditions. Therefore, it paves the way for further investigations into the remarkable capabilities of Ab-V5 and Pf-5 in enhancing plant growth and optimizing plant-microbe interactions.

5.2. Methods

5.2.1. Bacterial strain and growth conditions

For this study we used the wild type strain *A. brasilense* Ab-V5 (Hungria et al. 2010) which was derived from rhizosphere of maize (*Zea mays*) and *P. protegens* Pf-5 originally isolated from the rhizosphere of cotton seedlings (*Gossypium hirsutum*) (Howell and Stipanovic 1978). Bacterial cultures were stored in 20 % glycerol at -80 °C. At the onset of the experiment, Ab-V5 precultures were grown in DYGS liquid medium (Rodriguez et al. 2004) and Pf-5 in Luria-Bertani (LB) medium (Sambrook et al. 1989) shaking at 28 °C until early log-phase and diluted to Optical Density at 600 nm (OD_{600}) of 0.05 in MSM nitrogen-free liquid medium (Döbereiner and Day 1976) or in Kings medium B (King, Eldora et al. 1954) in case of Ab-5 or Pf-5 respectively. Inoculums were grown in 0.05 mM; 0.50 mM or 0.00 mM MBOA for 72 hours statically at 28 °C in the dark, each treatment containing six biological repetitions.

5.2.2. RNA extraction and sequencing

RNA of bacterial cultures was stabilized by adding two times the culture volume of RNA protect bacterial reagent (Qiagen, Venlo, Netherlands), directly into 15 mL glass tubes containing the bacterial cultures. RNA was isolated using an RNeasy RNA purification kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions, including a cell lysis step with 15 mg/mL lysozyme and 10 mg/mL proteinase K in TE buffer of pH 8 for ten minutes at room temperature. Additionally, we performed an on-column DNA digestion step with an RNase-free DNase set (Qiagen, Venlo, Netherlands). RNA was eluted in two steps with 50 µL RNase-free water in RNase-free micro centrifuge tubes and stored at -80 °C. Quality control of the samples was carried by an Agilent 2100 Bioanalyzer (Agilent, Barueri, Brazil), to select the three best biological repeats per treatment for cDNA library preparation with Illumina Stranded Total RNA prep, and ribosomal depletion with Ribo-Zero plus (Illumina, San Diego, USA). Sequencing of the samples was carried out by a NextSeq (Illumina, San Diego, USA) with a read depth of on average 13 million clusters or 26 million paired-end reads at NGS (Piracicaba, Brazil).

5.2.3. RNA seq data analysis

Initially, the raw read quality was determined using FastQC (Andrews 2010), a commonly used tool for assessing the quality of data generated by RNA sequencing (RNA-seq). After assessing sample quality, Trimmomatic (Bolger et al. 2014) was employed to filter

out low-quality reads and remaining sequencing adapters applying a cut off for Phred quality scores below 25 and removal of Nextera - PE adapters. The filtering of rRNAs from the samples was carried out using RiboDetector (Deng et al. 2022), a specialized tool designed to identify ribosomal RNA (rRNA) sequences and filter them from RNA-seq data which can constitute a significant proportion of the reads obtained during RNA-seq and complicate the analysis of gene expression by misalignment. From the Ab-V5 reads we aligned the trimmed and filtered reads with STAR (Dobin et al. 2013) to the Ab-V5 genome (GenBank accession: GCA_002940725.1) and Pf-5 reads were aligned to the Pf-5 genome (Genbank accession: CP000076), while gene quantification was carried out with HTSeq-count (Anders et al. 2014). Using the R package edgeR (Chen et al. 2016), sample libraries were normalized and analyzed for differential expression among treatments. For functional annotation, DIAMOND (Buchfink et al. 2015) was performed with the non-redundant (nr) NCBI database. Blast2GO suite (Götz et al. 2008) was used to categorize the annotated genes via DIAMOND into functional Gene Ontology (GO) terms.

5.3. Results

5.3.1. MBOA acts as a potential signaling molecule in Ab-V5

Introducing an environmental concentration of 0.50 mM MBOA to *A. brasilense* Ab-V5 caused a wide-scale reprogramming of metabolic regulation. The implications of this augmented concentration were of a larger scale than the alterations that application of 0.05 mM MBOA inflicted on the Ab-V5 transcriptome, which allowed the identification of 69 Differentially Expressed Genes (DEGs) versus 180 in the 0.50 mM MBOA treatment including 54 DEGs in common (**Figure 11**). The 69 DEGs from the 0.05 mM treatment counted 9 upregulated and 60 downregulated DEGs, while the 0.50 mM MBOA treatment consisted of 72 upregulated and 108 downregulated DEGs. Despite deploying several annotation strategies, 109 genes out of 315 unique genes could not be identified (without double counting genes in common) (**Table 1**). Because of the limitation on bacterial transcriptomics involving BX treatment, genes specific for this condition are more difficult to be annotated and represent probably the majority of this group. In addition, a fraction of the unidentified genes was annotated as hypothetical proteins lacking functional information. In contrast, we could not identify any significant DEG when Pf-5 was treated with 0.05 mM MBOA. 0.50 mM MBOA however, led to the identification of a scarce eight DEGs with superficial relative expression values ranging from -1.11 to 1.47 log Fold Change (logFC) (Error! Reference source not found.) (**Supplementary Table 5**).

Table 1: Read library characteristics from illumina NextSeq sequencing. Per treatment, the three best samples were selected and sequenced. The average read count was calculated after trimming with Trimmomatic and filtering with RiboDetector. 109 genes out of 315 unique genes could not be identified in both treatments together including 4 and 7 hypothetical genes in 0.05 and 0.50 respectively.

PGPR	Treatment	Average # reads	# DEGs	# Annotated DEGs	# Unidentified DEGs	% DEGs annotated
Ab-V5	0,05 mM	5025747	110 (81 shared)	69	36 (23 shared)	64
	0,50 mM	2947086	286 (81 shared)	180	96 (23 shared)	66
Pf-5	0,05 mM	11373198	0	0	0	0
	0,50 mM	10240785	8	6	2	75

Upon exposure to MBOA, the transcriptomic profile of Ab-V5 exhibited pronounced alterations primarily in the domains of 'gene regulation,' 'transport,' 'primary metabolism,' and 'signal transduction,' sequentially (**Figure 11A**). This discernible impact suggests that MBOA is conspicuously recognized by the cellular milieu, leading to substantial modifications at the gene regulation tier. This inference is substantiated by the noteworthy number of DEGs identified under the categories of both 'signal transduction' and 'gene regulation,' thereby implying a potential role for MBOA as a signaling molecule. A complete list of DEGs from Ab-V5 RNA-seq is available in APPENDIX C (**Supplementary Table 6**).

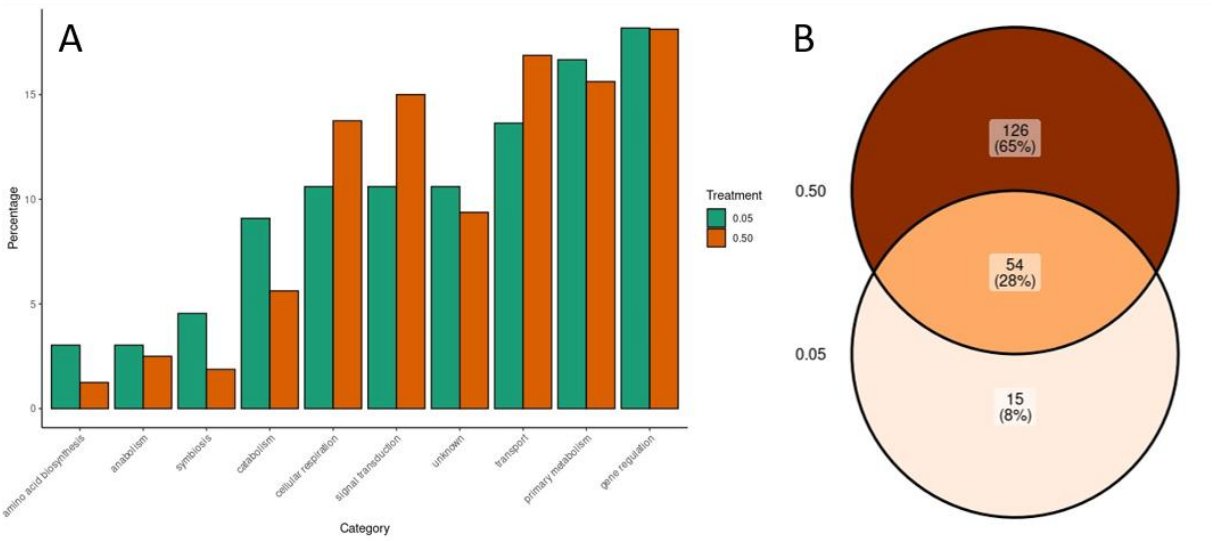

Figure 11: Organization of all common DEGs found in 0.05 and 0.50 mM MBOA treatments of Ab-V5. Treatments are grouped per category (A) and (B) Venn diagram showing unique and common DEGs (p=.05).

5.3.2. Pf-5 is highly tolerant to MBOA

Similarly, Pf-5 cultures were subject to 0.05 mM and 0.50 mM treatment. However, unlike Ab-V5, the transcriptome of Pf-5 suffered few alterations. Besides the 0.05 mM MBOA treatment that rendered no DEGs, 0.50 mM MBOA inflicted a significant change in expression levels of eight DEGs relative to the control treatment (**Figure 12**) (**Supplementary Table 5**). Remarkably, DEGs are principally categorized as belonging to the cellular respiration protein class and have positive logFC values, albeit deviating very little from control treatments.

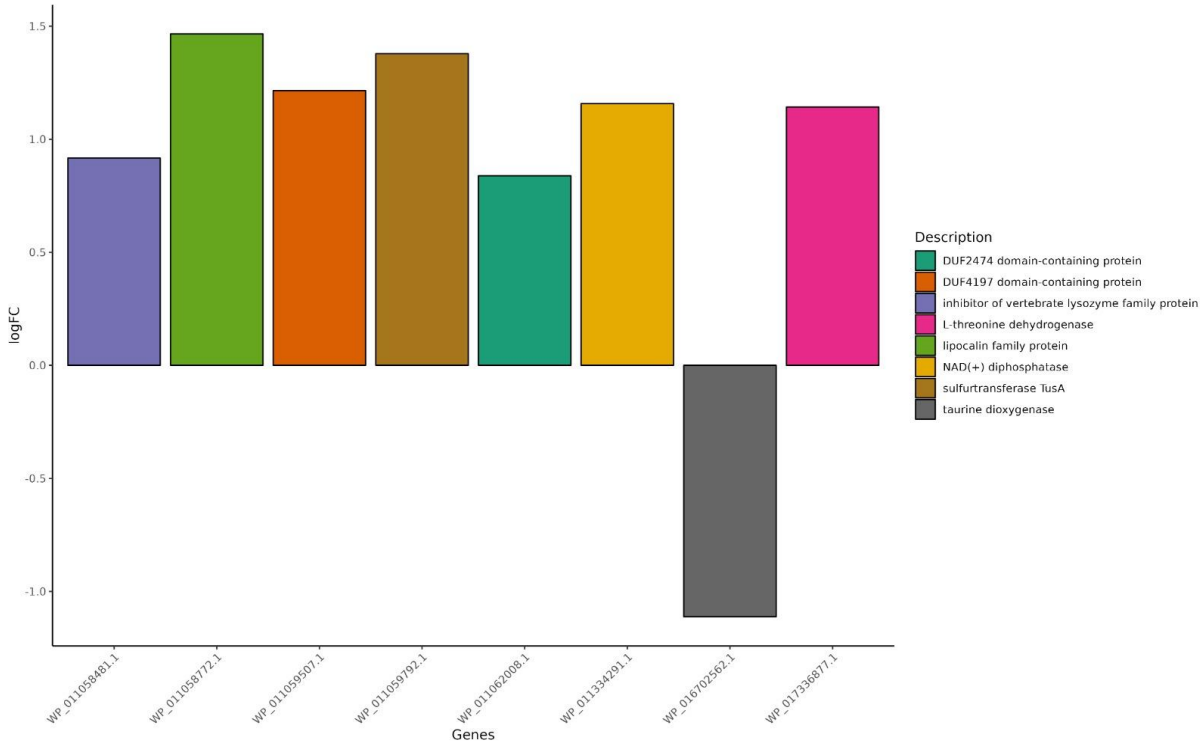

Figure 12: DEGs identified from Pf-5 (=0.05) treated with 0.50 mM MBOA with their respective logFC values.

5.3.3. In Ab-V5, most upregulated genes are found in gene regulation and metabolic processes related to cellular respiration

In concert with significant changes in gene expression within the primary metabolism category, it is noteworthy that a majority of the relatively upregulated DEGs are associated with gene regulation and cellular respiration (**Figure 13A, 13C**). Most notable DEGs displaying the highest logFC values within this category are ‘Ldh family oxidoreductas’ (AHNNBFGK_03305) with a logFC of 7.28; ‘SDR family oxidoreductase’ (AHNNBFGK_02025) with logFC 4.51 and ‘NAD+ synthase’ (AHNNBFGK_00885) with logFC 3.79 among the other 11 upregulated cellular respiration classified DEGs. The direct relationship observed between gene expression and MBOA concentration of

AHNNBFGK_03305 and AHNNBFGK_00885 categorized under cellular respiration, underscores the activation of energy metabolism within the cell (**Figure 14**). AHNNBFGK_03305, is an L-lactate dehydrogenase which is an enzyme known for its role in glycolysis, where it converts pyruvate to L-lactate. Its relative expression was significantly elevated in the 0.05 mM MBOA treatment with the logFC of 3.01 and further increased in the 0.50 mM MBOA treatment to logFC 7.28. The other DEG that showed a correlation between logFC and MBOA concentration within the 'cellular respiration' category, AHNNBFGK_00885, is involved in NAD+ biosynthesis (Nessi et al. 1995, Suda et al. 2003). NAD+ is known to play crucial roles in mediating redox reactions, electron transport, and as a substrate for poly-ADP-ribose polymerases (Stein and Imai 2012) (**Figure 14**).

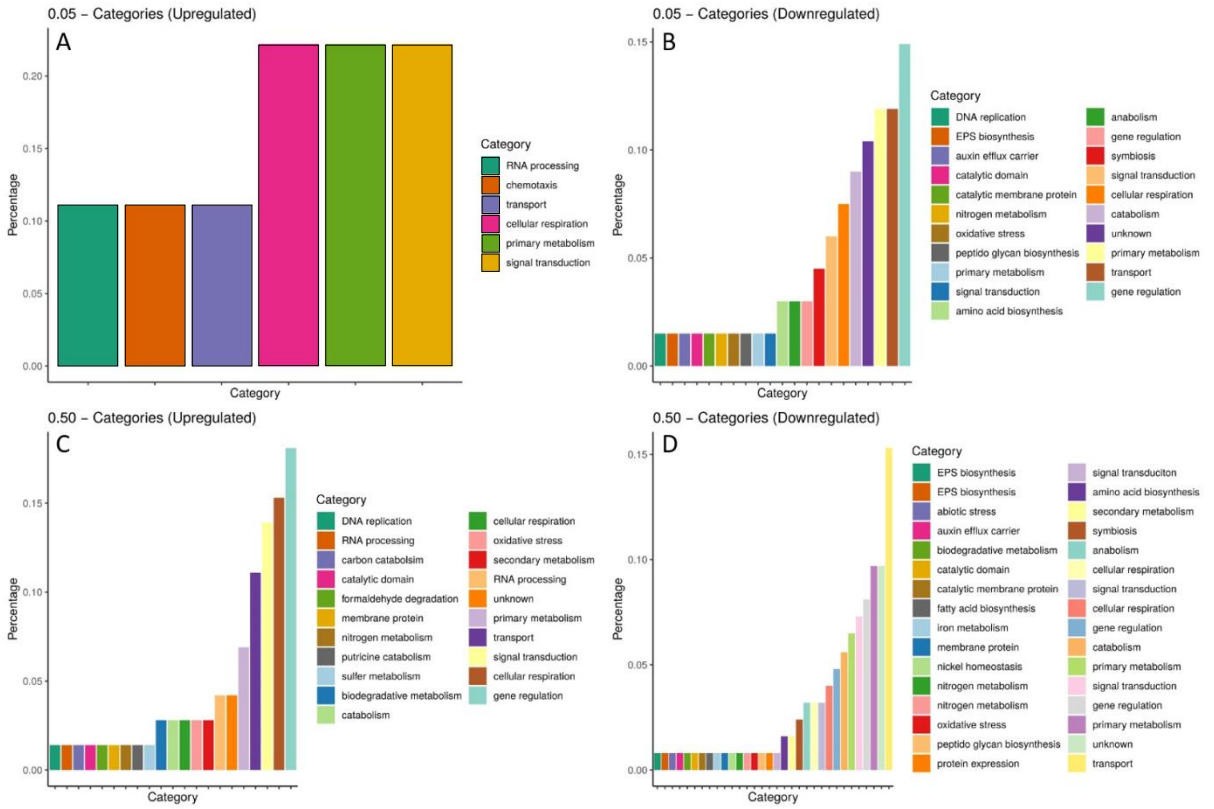

Figure 13: Upregulated Differentially Expressed Genes (DEGs) (A, C) and downregulated DEGs (B, D) identified form Ab-V5, sorted per protein category. Percentage indicated the proportion of total annotated DEGs within that treatment (0.05 or 0.50). Most abundant downregulated protein classes are 'gene regulation', 'signal transduction', 'transport' and 'primary metabolism'. The last three classes proportionally increase according to MBOA concentration increment from 0.05 to 0.50 mM. Upregulated categories are remarkably different by a strong upregulation of DEGs in the 'cellular respiration' class.

Hence, bacteria reside in an active state which allows energy to be spent on cell duplication expanding the bacterial population or on metabolic adaptation. Yet, at least some energy is spent on expelling toxic amounts of MBOA from the cell interior by stimulating gene expression of glutathione S-transferase detoxifying enzymes (**Supplementary Table 6**).

These enzymes render toxic compounds water soluble by transfer of glutathione moieties, facilitating their disposal through ATP-binding cassette transporters (Lu et al. 1997, Sharma et al. 2001) which were upregulated in both treatments (**Supplementary Table 6**).

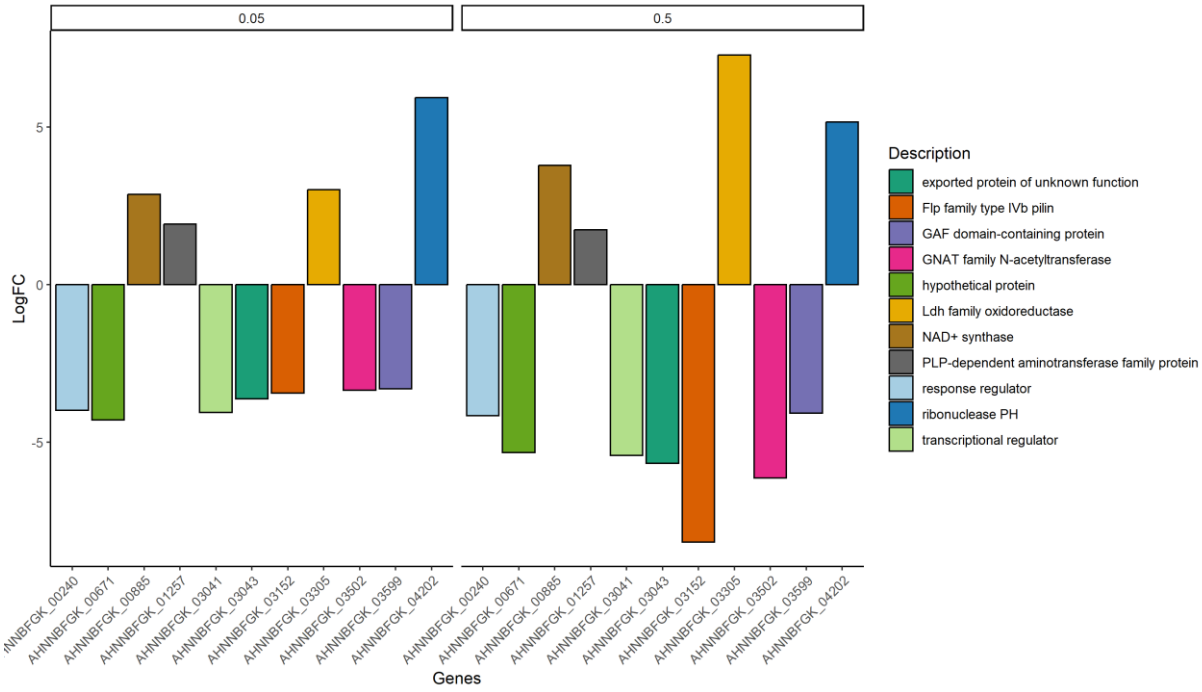

Figure 14: Common DEGs (p=0.05) in treatments 0.05 and 0.50. DEGs from Ab-V5 exhibiting either positive or negative correlations with MBOA concentration. Downregulated DEGs were selected with a cut-off logFC value of -3.

5.3.4. 0.05 mM MBOA stimulates chemotaxis in Ab-V5, while in general symbiosis related processes are downregulated

In our investigation, we systematically grouped DEGs associated with symbiotic processes, recognizing the complexity of symbiosis as a phenomenon governed by intricate interplays. In the 0.05 mM MBOA treatment, this grouping encompasses eight DEGs categorized under extracellular polymeric substance (EPS) biosynthesis, symbiosis, nitrogen metabolism, auxin homeostasis, and chemotaxis (**Figure 15**). The chemotaxis regulator CheZ (AHNNBFGK_04641), which exhibited a logFC of 2.29, is a specific phosphatase for CheY-P and plays a pivotal role in modulating the flagellar motor complex. Consequently, it influences the direction of bacterial movement and the frequency of tumbling events (Huang and Stewart 1993, Bren et al. 1996, Wadhams and Armitage 2004). Thus, CheZ upregulation promotes chemotaxis by reducing tumbling events and stimulating longer uninterrupted runs (Kuo and Koshland 1987, Huang and Stewart 1993).

It's noteworthy that the majority of DEGs associated with symbiosis displayed downregulation. This includes the "Flp family type IV pilin" (AHNNBFGK_03152) and the

enzyme responsible for processing precursor subunits for pilin assembly, known as "prepilin peptidase" (AHNNBFGK_03151) (Nunn and Lory 1991) with logFC values of -3.44 and -7.17 in 0.05 and the logFC values -8.16 and -3.06 in 0.50 respectively (**Figure 15**). Pili are proteinaceous, polymeric appendages distinct from flagella, serving various symbiosis-related functions (Schreiber and Donnenberg 2002). Biofilm formation, another crucial feature associated with symbiosis and cell adherence, involves the production of extracellular polymeric substances (EPS) (Ramey et al. 2004, Danhorn and Fuqua 2007, Ueda and Saneoka 2015, Flemming et al. 2016, Viruega-Góngora et al. 2020). In both the 0.05 and 0.50 treatment, an EPS biosynthesis protein transcript (AHNNBFGK_05273) exhibited a slight relative downregulation with a logFC of -1.86 and -1.60 respectively (**Figure 15**), while in the 0.50 mM MBOA treatment an additional EPS biosynthesis protein transcript (AHNNBFGK_05278) with a logFC value of -2.61 was differentially expressed (**Figure 15**).

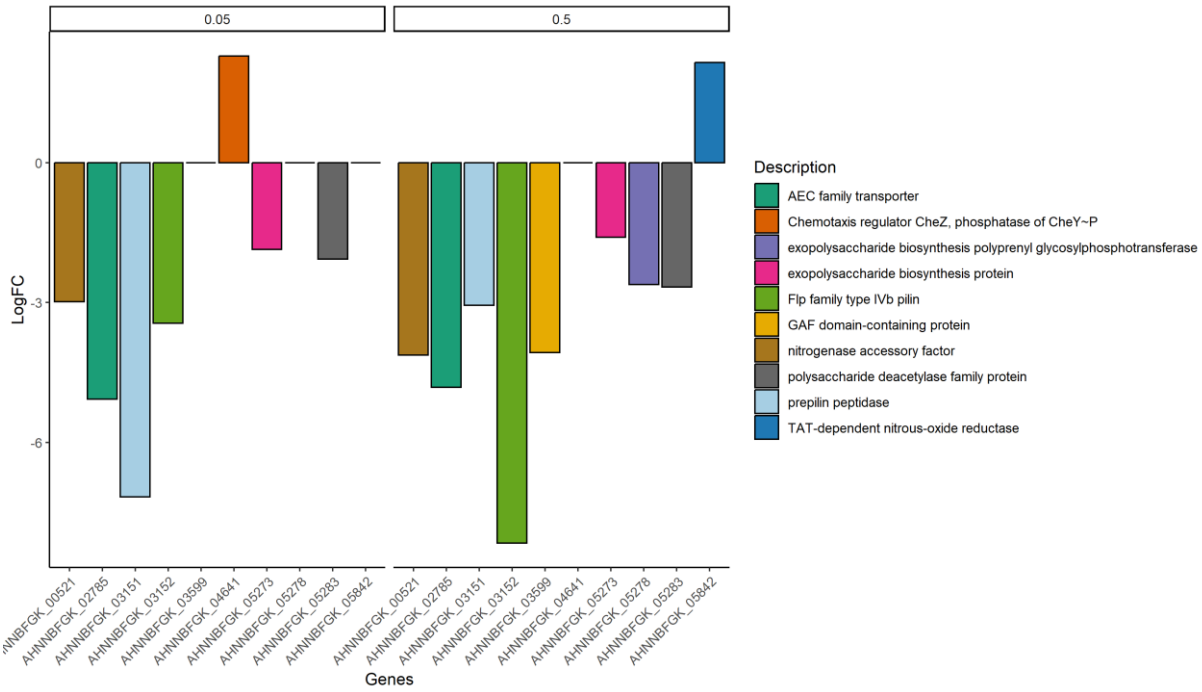

Figure 15: Symbiosis related DEGs (p = .05) with logFC values for the treatments 0.05 mM MBOA and 0.50 mM MBOA from Ab-V5.

Our findings indicate that both the 0.05 and 0.50 mM MBOA treatments significantly repressed the gene expression of the "auxin efflux carrier protein" (AHNNBFGK_02785), with logFC values of -5.07 and -4.82, respectively (**Figure 15)**. *A. brasilense*'s pivotal role in plant growth promotion is largely attributed to the production of indole-3-acetic acid (IAA), the most common form of auxin (Dobbelaere et al. 1999). Biosynthesis of plant hormones by *A. brasilense* stimulates the development of lateral roots and root hairs, leading to enhanced water and mineral uptake (Spaepen et al. 2014, Cohen et al. 2015). This alteration in root

architecture is intricately linked to auxin production (Spaepen et al. 2014, Méndez-Gómez et al. 2021). This suggests that the export of IAA produced by *A. brasilense* is reduced under these conditions when compared to the control treatment.

Interestingly, our study reveals that the expression of nitrogen accessory proteins is increasingly suppressed with a rise in MBOA concentration from 0.05 to 0.50 mM, with logFC values of -2.98 and -4.13, respectively (**Figure 15**). Notably, only in the 0.50 mM treatment, the gene expression of "TAT-dependent nitrous-oxide reductase" exhibits a relative increase with a logFC of 2.15 (**Figure 15**). This enzyme catalyzes the final step in denitrification, reducing nitrous oxide (N2O) to dinitrogen (N2). In summary, MBOA treatment does not directly promote symbiosis related mechanisms other than chemotaxis.

5.4. Discussion

At the onset of root colonization by PGPB, potential symbionts must be recruited from the biosphere that harbors diverse microorganisms (O'Sullivan and O'Gara 1992, de Weert et al. 2002, Feng et al. 2021). Given the overwhelming amount of metabolites secreted from root systems in the soil, calling for specific symbionts is not straightforward. Attraction by primary metabolites such as sugars and amino acids is short-lived and unspecific, because of the enormous number of microorganisms that harbor the potential to metabolize those compounds (Traoré et al. 2000, Kawasaki et al. 2016). MBOA however, is a relative stable component (Etzerodt et al. 2008) with a sustained release in the soil (Cambier et al. 2000, Hu et al. 2018). Intriguingly, only in the lower concentration (0.05 mM) we tested, a chemotaxis regulatory gene CheZ was upregulated, while in the higher concentration (0.50 mM), which corresponds to an area in close proximity to roots, no such DEG was found. Hence, Ab-V5 seems to be more sensitive to lower concentrations of MBOA, which possibly enables bacteria to be attracted over longer distances. This makes sense, since the number of bacteria and potential symbionts in the soil multiplies by the third power with distance from the source, considering the soil environment as a homogeneous three dimensional space.

When treated with MBOA, the transcriptome of Ab-V5 was most affected within the categories 'gene regulation', 'transport', 'primary metabolism' and 'signal transduction' in that respective order. Hence, MBOA is clearly perceived by the cell and inflicts substantial alterations on the gene regulation level, considering the amount of DEGs found under both categories 'signal transduction' and 'gene regulation' and potentially acts as a signaling molecule. As a result there of, genes categorized as 'primary metabolism' and 'transport' contain most members with modified expression profiles after 'gene regulation', signifying

the adaptation of the bacterial population to the altered environmental conditions. Most probably, the findings suggest that Ab-V5 undergoes a profound genetic reprogramming when exposed to MBOA-enriched environments, with potentially wide-ranging physiological implications. In contrast, significant alterations of the Pf-5 transcriptome were limited in the 0.50 mM MBOA treatment and not detected when subjected to the lowest concentration of MBOA (0.05 mM). This inert character of Pf-5 is in line with its response to MBOA treatment in growth curves and biofilm formation assays (Chapter 3).

The contrasting effects of the 0.05 mM and 0.50 mM MBOA treatments on *A. brasilense* are evident in their impact on cellular respiration and energy metabolism (5 downregulated and 2 upregulated DEGs in 0.05 vs. 7 downregulated and 13 upregulated DEGs in 0.50 mM MBOA). The 0.50 mM treatment notably stimulated cellular respiration, through glycolysis and by promoting oxidoreductases involved in the electron transport chain. The absence of upregulated chemotaxis genes in the 0.50 mM treatment suggests that the increased energy generated through cellular respiration is probably not allocated to bacterial locomotion. Instead, this surplus energy is more likely directed toward metabolic adaptation and global transitions in bacterial metabolic processes, allowing the bacteria to respond to varying environmental cues. This adaptive response highlights the remarkable versatility and resilience of Ab-V5 when faced with different environmental conditions to seek favorable surroundings and metabolically prepare for colonization.

In general it seems that properties relating to symbiotic interactions are relatively inactive under MBOA regime. Notably, biofilm biosynthesis related genes were negatively correlated with MBOA concentration, and IAA release was diminished by relative downregulation of auxin efflux carriers, however, biosynthesis is therefore not necessarily ceased in Ab-V5 bacteria. Curiously, nitrogen homeostasis was ambiguously affected. In both treatments the nitrogenase accessory factor was relatively downregulated, which impedes the reduction of dinitrogen to ammonium, while nitrous-oxide reductase was relatively upregulated in 0.50 mM treatment, which may lead to a local buildup of nitrogen. Nitrogen however, is unreactive and safe for the cell to store in large amounts (Holland 2020). Nitrogen, typically in the form of ammonium, is often a limiting nutrient for plant growth (Rosenblueth et al. 2018). Additionally, atmospheric dinitrogen is not directly suitable for plant metabolism. Hence, plants largely depend on biological nitrogen fixation by diazotrophic bacteria for their metabolic needs, which reduce dinitrogen to ammonium through the nitrogenase enzyme system (Ormeño-Orrillo et al. 2013). This enzyme system requires accessory proteins for the biosynthesis of metallocenters, which are crucial for its

proper functionality (Curatti et al. 2005, Burén and Rubio 2018, Nonaka et al. 2019). We therefore surmise that MBOA treated cells might prepare for root colonization by accumulating symbiosis related metabolites such as IAA and nitrogen, the latter as substrate for ammonium metabolism.

BX inflict major changes in microbial organization of the rhizosphere (Hu et al. 2018, Cotton et al. 2019, Kudjordjie et al. 2019, Cadot et al. 2021), yet the specific mechanisms by which BX act are a compelling point of debate. Our results show how MBOA acts in the first stages of symbiosis, being in signal perception, chemotaxis and metabolic preparation. In higher concentrations of MBOA (0.50 mM) or hypothetically in closer proximity to the roots, where MBOA is formed, Ab-V5 experiences a metabolic reprogramming and prepares for transitioning to a symbiotic lifestyle. Considering the untreated Ab-V5 bacteria as the reference physiological state, energy homeostasis is strongly upregulated, allowing for a reallocation of energy for altering transport and rerouting metabolic networks. Nitrogen metabolism is in that stage partly induced, perhaps to stockpile substrate for nitrogen fixation. Similarly, release of IAA by auxin efflux carriers is slowed in comparison to MBOA-free Ab-V5.

Considering its ecological impact and its individual growth promoting properties, cereals specifically benefit from acquiring *A. brasilense* (Tien et al. 1979, Steenhoudt and Vanderleyden 2000, Fukami et al. 2017, 2018, Oliveira et al. 2017, Ferrarezi et al. 2022, 2023). Stable secondary metabolites that are not convenient microbial fermentation substrates, are therefore suitable compounds for targeting PGPB of particular interest. While small progress is being made in uncovering molecular mechanics of bacterial recognition and on revealing the physiological implication that BX substrates impose on PGPB, this study can be considered as a step in the direction of revealing the molecular function of MBOA.

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6. CONCLUSIONS

The investigations presented in this research shed light on the intricate processes involved in the root colonization by PGPB, highlighting the roles of chemotaxis and biofilm formation in this essential ecological niche. These findings collectively contribute to a deeper understanding of the nuanced interactions that govern the plant-microbe-environment interface. That brings us back to the to the initial research hypothesis:

"Conditioning of the soil with MBOA improves environmental conditions and increases the success rate of microbial inoculation in crop cultivation."

To assess the 'success rate of microbial inoculation' we evaluated 1) successful establishment of PGPB in the rhizosphere and 2) plant root colonization by PGPB. Establishment in the rhizosphere was studied by testing the tolerance of both individual PGPR and pathogenic *Fusarium* to MBOA, and by evaluating chemotactic response of PGPR.

In summary, we found out that MBOA exerted a significant negative effect on bacterial growth of Ab-V5, while other PGPR strains we tested were less affected. We surmise that this negative effect we observed most likely stems from applying relatively high concentrations of MBOA rather than being a limiting factor for Ab-V5 symbiosis in the rhizobiome. Ab-V5 is frequently associated with BX producing grass plant species and hence may be expected to be tolerant to MBOA (Michiels et al. 1991, Croes et al. 1993, Vande Broek et al. 1998b, Housh et al. 2021). Interestingly, Ab-V5 at the same time displayed a chemotactic response, which promotes rhizosphere establishment. In contrast, Pf-5 *in vitro* growth and biofilm production was not affected as much. One way to interpret these results, could be that the sensitivity of Ab-V5 to MBOA enables recruitment of these PGPR by the plant through MBOA production. While Ab-V5 might be recruited from the bulk soil, Pf-5 is restricted to its occurrence in close proximity of the root and can better withstand high concentrations of MBOA produced during early stages of maize embryo development (Klun et al. 1970, Cambier et al. 2000, Hu et al. 2018b). Taken together, when comparing the sensitive Ab-V5 with the inert Pf-5, it is hard to draw conclusions on which bacteria would be more successful in the MBOA concentrations that were tested.

Another facet of creating favorable conditions for crop cultivation is biocontrol of pest species. We tested therefore how ectopically applied MBOA suppresses growth conditions for *Fusarium* strains by scoring conidia germination and biomass. We inferred from the responses of different host isolates that *Fusarium* adapted to a BX-producing host such as maize, was tolerant to MBOA while strains from non-producing hosts are not and exhibited significant reduction of germinating conidia. In agreement with being a necrotrophic broad

spectrum pathogen, *Fusarium* demonstrated the resilience to overcome all-round fungistatic plant defense metabolites when adapted to BX metabolism of the host plant. Accordingly, for cultivating non-BX producing crops, applying MBOA is an interesting line of thought for controlling host-specific *Fusarium* strains that are susceptible for BX. For instance, co-cultivating the BX producing crop wheat with watermelon suppresses *F. oxysporum* f. sp. *niveum* in the soil and *Fusarium* wilt infections on watermelons (Xu et al. 2015).

So germination of *Fusarium* conidia were suppressed by MBOA treatment, which limits disease and is advantageous for antagonistic endophytes such as Pf-5, *Burkholderia ambifaria*, *Bacillus mojavensis*, *Paenibacillus polymyxa* and *Citrobacter* (Bacon et al. 2007, Mousa et al. 2015, Quecine et al. 2016, Simonetti et al. 2018). Furthermore, from *in vitro* chemotaxis experiments in Chapter 3 and transcriptomic data from Chapter 5 it was inferred that Ab-V5 is attracted to MBOA. We could relate the positive chemotactic response that was measured by accumulation of Ab-V5 CFUs in MBOA treatment with stimulation of a regulatory protein CheZ within the Che1 chemotaxis signal transduction pathway. Interestingly, the regulatory protein CheZ, at the same time promotes clustering and adherence (Bible et al. 2008, 2012, Siuti et al. 2011), though our transcriptomic analysis points out that biofilm synthesis is downregulated. The latter fact was also confirmed from biofilm measurements in microtiter assays. Initially we related the diminished biofilm with a reduced growth rate recorded from growth curves. At the same time, transcriptomics showed that the highest number of DEGs was associated with cellular respiration. This lead us to believe that the diminished growth rate underpins the role of MBOA in stimulating the motile bacterial lifestyle, since energy spent on growth and duplication events is allocated instead to chemotaxis, motility and an increased cellular respiration.

PGPB can either reside in a motile physiological state that promotes dispersion and locomotion, or in a sedentary state by adhering and clustering while engaging in symbiosis. Therefore, chemotaxis and biofilm formation represent two distinct but interrelated mechanisms in the PGPB's toolkit for root colonization. Chemotaxis is primarily stimulated in the motile form of PGPBs and is particularly crucial during the early stages of rhizosphere integration prior to root colonization. It enables the PGPBs to navigate toward favorable environments and root exudates, facilitating their initial contact with host roots. In contrast, biofilm formation comes into play when PGPBs adhere to root surfaces and transition to a sessile lifestyle. This biofilm represents a protective and nutrient-rich microenvironment that contributes to the establishment of PGPBs in the rhizosphere.

Interestingly, bacterial appendixes used for motility such as the polar flagella and TAD pili are important factors for adherence, root colonization and infection (Wisniewski-Dyé et al. 2011, Shelud'ko et al. 2019, Cai et al. 2021). Therefore, chemotaxis, motility and biofilm are highly intertwined and antagonistically regulated (Guttenplan and Kearns 2013, Besharova et al. 2016, Prüß 2017). For example, the highly conserved Che1 chemotaxis signal transduction pathway regulates rotation direction of the flagellar motor (Wuichet and Zhulin 2010). By removing phosphate from CheY, CheZ promotes longer runs in a certain direction by diminishing the amount of turns. Apart from chemotaxis, the Che1 pathway regulates flocculation and cell adhesion (Bible et al. 2008, 2012, Siuti et al. 2011). Mutants in the response regulator CheY, clump and flocculate more and produce more biofilm which enhances attachment on wheat roots (Bible et al. 2008, 2012, Siuti et al. 2011). Hence, upregulation of the suppressor CheZ, phosphatase of CheY, has a double-faced positive response on both chemotaxis and cell adherence.

The discrepancy we discovered between both upregulation of CheZ which promotes besides chemotaxis also clustering and adherence, and downregulation of biofilm related genes at the same time, may be of a more complex nature. For instance, post-translational modifications can alter the affinity and specificity of substrates to certain receptors or simply serve as a mark for degradation, which is a faster and more versatile regulatory mechanism than transcriptional regulation (Vadyvaloo and Martínez 2014, Cain et al. 2014, Vanheule et al. 2018). Possibly, CheZ might initially regulate chemotaxis and after the bacteria has attached to the root, swiftly switch to stimulating biofilm formation as a result of post-translational modifications.

Concerning the impact on rhizosphere establishment of PGPB, as first part of the research hypothesis, we conclude that MBOA stimulates the motile form and has the potential to attract Ab-V5. Germination of conidia originating from *Fusarium* associated with no BX producing hosts is suppressed which is favorable for both host plants and PGPB. So in general, conditions for PGPB establishment in the rhizosphere may be improved taking in to account the parameters that were analyzed as described above.

Secondly, we examined the influence of MBOA on root colonization as a second part of MBOA promoting successful inoculation using Ab-V5 and Pf-5 as study objects. To this end we therefore studied root colonization *in vitro,* by varying growth medium composition; time points during biofilm maturation, as well as biofilm production on *Arabidopsis thaliana* root surfaces. At last, we evaluated peroxidase activity and adherence to *Arabidopsis* roots, results of both assays were not influenced by MBOA.

An intriguing observation form MBOA treatment is the delay in root colonization mechanisms such as biofilm formation. While the *in vitro* biofilm assays might lack essential plant tissue components, the *in planta* assays appear to promote root colonization and biofilm production more effectively: the exceeding biofilm accumulation in MBOA treatment observed by *in vitro* biofilm assays after 120 hours was recorded after 96 hours by *in planta* microscopic assays. This implies that the presence of host-specific factors are a determining factor for the effect of MBOA on bacterial biofilm formation. Additionally, whereas a decay in biofilm was observed from *in vitro* cultures which may correspond to depletion of nutrients in the culture medium, biofilm can be maintained on plant roots that can provide nutrients for symbiotic bacteria. Thus, it is not taken for granted that biofilm on plant roots decay at the same rate as in host-free conditions, if they do at all. On top of that, the ability of PGPBs to harness host factors is even essential for their long-term survival and interaction with the host plant (de Weert et al. 2002, Neal et al. 2012, Yuan et al. 2015, O'Neal et al. 2020, Feng et al. 2021, Xie et al. 2022).

Initially, we related the delay in biofilm production by Ab-V5 with the decreased growth rate in liquid medium since biofilm is a quorum sensing regulated mechanism, which depends on cell density (Ding et al. 2011). At the same time, none of the PGPB we tested showed a linear correlation between biofilm and MBOA concentration, underscoring that MBOA influences biofilm formation in a more complex manner rather than exerting a direct negative effect by chemical interaction. We then observed a negative correlation between MBOA concentration and the number of DEGs related to biofilm biosynthesis that were identified by RNA-seq. Those results were similar to *in vitro* studies when biofilm was measured by crystal violet staining after 72 hours. Hence, this all lead us to conclude that during early root colonization biofilm production is suppressed by MBOA on a transcriptional level.

We also found positive correlation between MBOA concentration and DEGs related to energy metabolism and nitrogen metabolism. Apparently, bacteria reside in an active state which allows allocation of energy to cell duplication expanding the bacterial population, locomotion or to metabolic adaptation. Bearing in mind the negative effect of 0.50 mM MBOA on both the optical density of Ab-V5 cultures and the absence of the DEG CheZ, we surmise that energy may rather be allocated to metabolic adaptation. This conclusion is also based on the majority of DEGs being situated in the categories 'primary metabolism', 'gene regulation', 'transport' and 'signal transduction'. Further results from RNA-seq indicate that MBOA treatment negatively influences symbiosis related mechanisms by relative

downregulation of DEGs associated with biofilm and pilin biosynthesis, nitrogen metabolism and IAA export. On the other hand, 0.50 mM MBOA stimulates nitrous-oxidase reduction which is part of nitrogen fixation while 0.05 mM MBOA stimulates chemotaxis. Thus, Ab-V5 is more probable to be attracted to 0.05 mM than to 0.50 mM MBOA, which we can relate to remoteness of the source of exudation. Since the soil environment is a three dimensional space, the MBOA concentration diminishes with the third power of distance. This implies that at a certain distance away from an MBOA source (host plant roots), Ab-V5 becomes attracted to move to the source, until the concentration of MBOA is so high – presumably at the roots of the host plant - that Ab-V5 is no longer attracted and will switch to sedentary state and settle on the root system.

Taken together, when considering mechanisms related to root colonization we found that several are repressed under MBOA regime, such as biosynthesis of pili and biofilm for proper root attachment. In contrast, it causes significant alterations in cellular respiration, signal transduction and primary metabolism, in line with motility towards the source of MBOA, while specifically at the higher concentration of MBOA, nitrogen metabolism is partly turned on which allows stockpiling of substrates for nitrogen fixation, in line with a sedentary state and settling on the roots. Hence, we conclude that MBOA does not directly stimulate root colonization, but causes alterations of Ab-V5 metabolism that may indicate a transitioning to a symbiotic physiology.

In this doctoral study we aimed at gauging the impact of MBOA on different trophic levels, being fungal pathogenic species and PGPB, to explore its ecological impact on the complex soil microbiome. Albeit having analyzed a selected few individual microbial species, we revealed the versatile and selective character of MBOA on different PGPB and *Fusarium* isolates. Needless to say, the root microbiome is an eternally complex environment with countless factors manipulating symbiotic interactions. However, this pioneering work opens new avenues for research on the effect of MBOA on the microbiome. One interesting area to further explore could encompass metagenomics analysis to reveal how MBOA affects the diversity of the root microbiome or extend this research on other promising PGPB.

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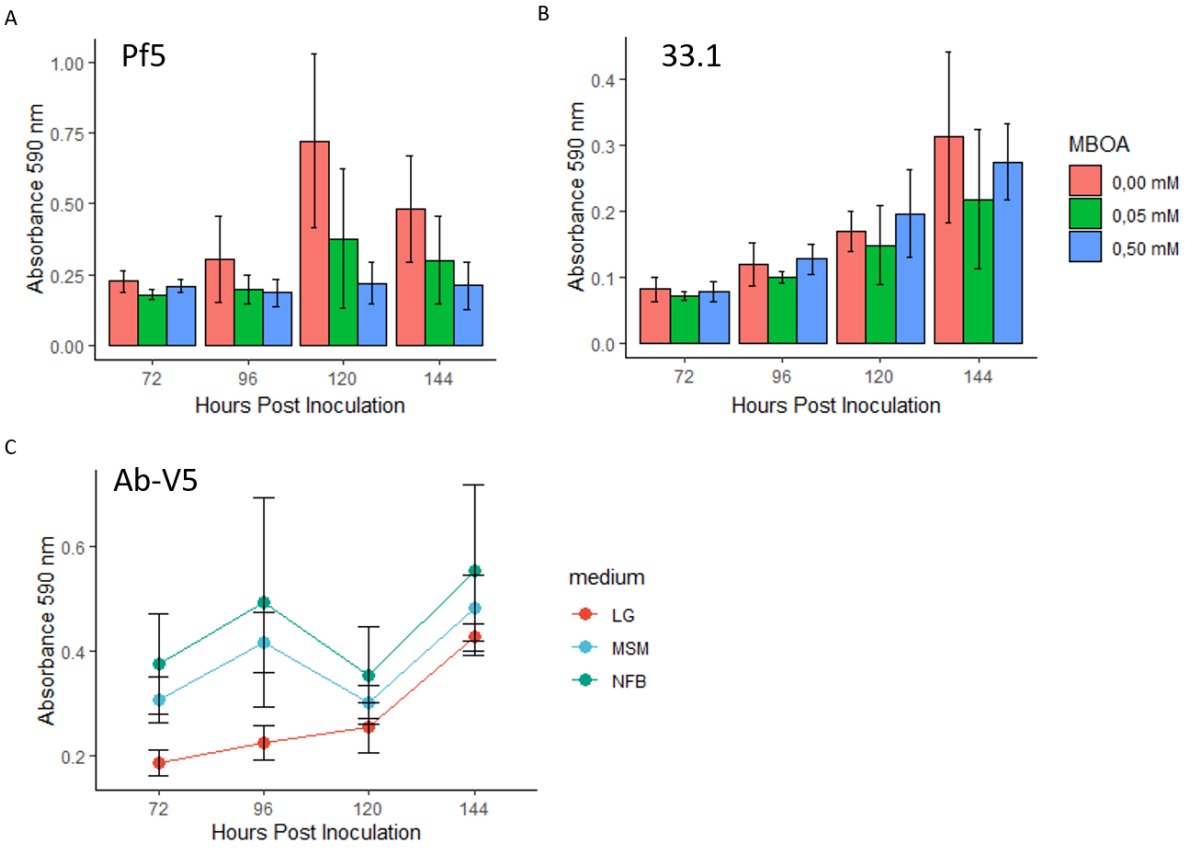
APPENDICES

Appendix A – Supplementary data of chapter 3

Supplementary Figure 1

Biofilm



Supplementary Figure 1: Preliminary experiments for determining the optimal time points for biofilm production of Pseudomonas protegens Pf5 (A), Pantoea agglomerans 33.1 (B) and for determining suitable growth media in Azospirillum brasilense Ab-V5 (C). Biofilm was determined from liquid cultures of static growth in microtiter plates at 28°C after the indicated time of incubation by spectrophotometry using crystal violet staining.

Supplementary Table 1

Chemotaxis

Supplementary Table 1: CFU counted on DYGS agar plates by ImageJ via automated counting. Bacteria were collected from syringes containing MBOA solution inserted in OD 0.05 Ab-V5 cultures after incubation for 15 minutes.

Sample	0,00 mM	0,05 mM	0,50 mM
1.1	368	2597	1935
1.2	738	2196	1973
1.3	699	1955	2179
1.4	722	1733	3125
1.5	1161	1860	2664
total	**3688**	**10341**	**11876**
2.1	272	2385	4214
2.2	152	1975	3271
2.3	143	1830	2984
2.4	100	1888	2154
2.5	110	2235	2487
total	**777**	**10313**	**15110**
3.1	810	2502	2774
3.2	699	2544	3879
3.3	799	1787	3096
3.4	1421	1932	3465
3.5	764	1691	3218
total	**4493**	**10456**	**16432**
4.1	538	1486	664
4.2	601	1640	798
4.3	684	1736	763
4.4	780	1609	811
4.5	616	1640	843
total	**3219**	**8111**	**3879**
5.1	1294	1936	1920
5.2	762	1825	1422
5.3	799	2037	1004
5.4	1077	2390	1147
5.5	719	2743	1145
total	**4651**	**10931**	**6638**

STATISTICAL ANALYSIS

ANOVA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
treatment	2	4.913	2.4566	42	8.16e-13 ***
Residuals	72	4.211	0.0585		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

TukeyHDS post-hoc test

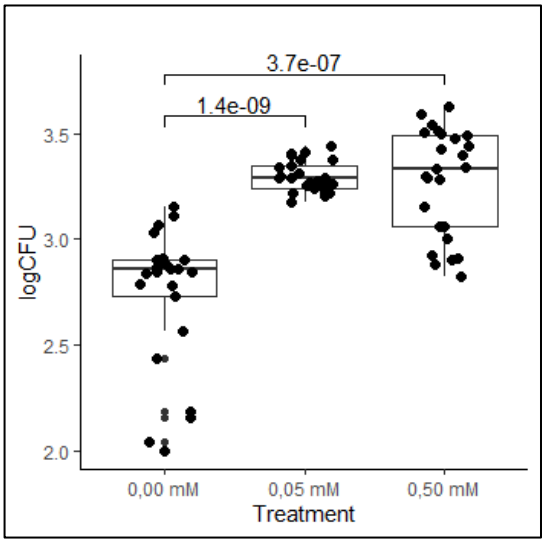
Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = logcfu ~ treatment, data = data)

$treatment

	diff	lwr	upr	p adj
0,05 mM-0,00 mM	0.55560666	0.3919048	0.7193085	0.0000000
0,50 mM-0,00 mM	0.52933806	0.3656362	0.6930400	0.0000000
0,50 mM-0,05 mM	-0.02626859	-0.1899705	0.1374333	0.9220226



Supplementary Table 2

Chemotaxis

Supplementary Table 2: CFU counted on DYGS agar plates by ImageJ via automated counting. Bacteria were collected from syringes containing MBOA solution inserted in OD 0.05 Ab-V5 cultures after incubation for 15 minutes.

Sample	0,00 mM	0,05 mM	0,50 mM
1.1	2196	2828	2825
1.2	2441	3096	3000
1.3	2159	2277	2336
1.4	2315	1945	3147
1.5	2206	3601	1578
total	**11317**	**13747**	**12886**
2.1	1804	1567	2385
2.2	2094	1957	2220
2.3	2133	2071	2051
2.4	1086	2419	1715
2.5	1389	3392	1319
total	**8506**	**11406**	**9690**
3.1	1313	633	769
3.2	859	990	601
3.3	850	893	370
3.4	1385	1385	109
3.5	1824	992	363
total	**6231**	**4893**	**2212**
4.1	2741	2153	864
4.2	2731	3320	472
4.3	2780,25	2268	287
4.4	2680	2812	47
4.5	2969	1772	130
total	**13901,25**	**12325**	**1800**
5.1	889	1578	2668
5.2	931	1359	1845
5.3	872	1970	2167
5.4	1598	1920	2967
5.5	1349	2730	1869
total	**5639**	**9557**	**11516**

STATISTICAL ANALYSIS

ANOVA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
treatment	2	0.4087	0.20436	11.04	0.000101 ***
Residuals	52	0.9625	0.01851		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

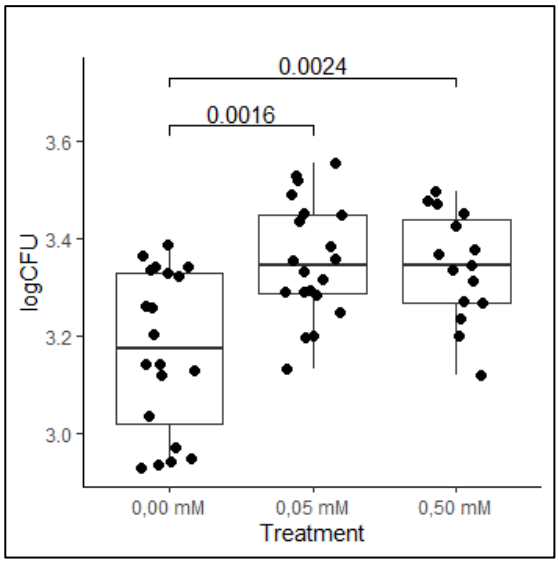
TukeyHSD post-hoc test

Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = logcfu ~ treatment, data = data)

$treatment

	diff	lwr	upr	p adj
0,05 mM-0,00 mM	0.18410125	0.08030524	0.2878973	0.0002350
0,50 mM-0,00 mM	0.17203734	0.05992483	0.2841498	0.0014793
0,50 mM-0,05 mM	-0.01206391	-0.12417642	0.1000486	0.9635551



Supplementary Table 3

Chemotaxis

Supplementary Table 3: CFU counted on DYGS agar plates by ImageJ via automated counting. Bacteria were collected from syringes containing MBOA solution inserted in OD 0.05 Ab-V5, RZ2MS9, 33.1 or Pf-5 cultures after incubation for 15 minutes.

Bacteria	MBOA	logcfu
Ab-V5	0,00 mM	1,778151
Ab-V5	0,00 mM	2,079181
Ab-V5	0,00 mM	1,662758
Ab-V5	0,00 mM	1,84003
Ab-V5	0,50 mM	3,056905
Ab-V5	0,50 mM	3,053078
Ab-V5	0,50 mM	2,929419
Ab-V5	0,50 mM	2,690196
RZ2MS9	0,00 mM	3,318063
RZ2MS9	0,00 mM	3,49693
RZ2MS9	0,00 mM	3,619093
RZ2MS9	0,00 mM	3,426511
RZ2MS9	0,50 mM	3,733999
RZ2MS9	0,50 mM	3,61595
RZ2MS9	0,50 mM	3,659916
RZ2MS9	0,50 mM	3,514548
33.1	0,00 mM	3,198657
33.1	0,00 mM	3,176091
33.1	0,00 mM	3,033424
33.1	0,00 mM	3,369216
33.1	0,50 mM	3,292256
33.1	0,50 mM	3,506505
33.1	0,50 mM	3,380395
33.1	0,50 mM	3,342423
Pf-5	0,00 mM	2,342423
Pf-5	0,00 mM	2,633468
Pf-5	0,00 mM	3,271842
Pf-5	0,00 mM	3,222716
Pf-5	0,50 mM	3,320146
Pf-5	0,50 mM	2,982271
Pf-5	0,50 mM	2,919078
Pf-5	0,50 mM	3,540329

STATISTICAL ANALYSIS

Ab-V5

Shapiro-Wilk normality test

data: mod1$residuals

W = 0.96822, p-value = 0.8836

Wilcoxon rank sum exact test

data: logcfu by MBOA

W = 0, p-value = 0.02857

RZ2MS9

Shapiro-Wilk normality test

data: mod1$residuals

W = 0.97104, p-value = 0.906

Wilcoxon rank sum exact test

data: logcfu by MBOA

W = 2, p-value = 0.1143

33.1

Shapiro-Wilk normality test

data: mod1$residuals

W = 0.95253, p-value = 0.7367

Wilcoxon rank sum exact test

data: logcfu by MBOA

W = 2, p-value = 0.1143

Pf-5

Shapiro-Wilk normality test

data: mod1$residuals

W = 0.8874, p-value = 0.2213

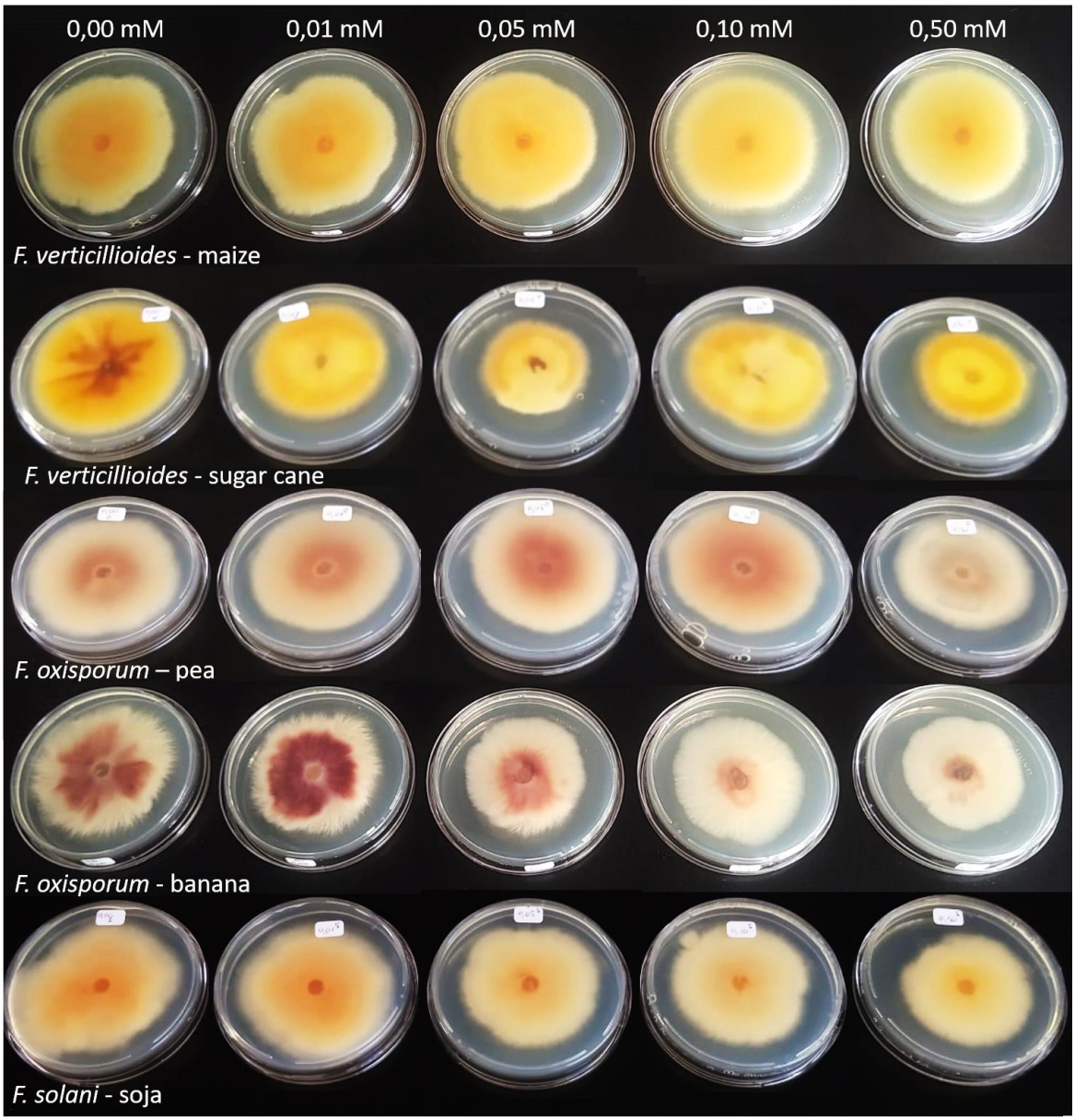
Wilcoxon rank sum exact test

data: logcfu by MBOA

W = 4, p-value = 0.3429

Supplementary Figure 2

Fusarium



Supplementary Figure 2: Various Fusarium isolates cultivated on freshly prepared PDA plates containing the concentration of MBOA indicated in the figure. An agar plug of fungal culture was placed in the middle of the plate and the cultures' diameter was measured after 7 days of inoculation.

Appendix B – Supplementary data of chapter 4

Supplementary Table 4

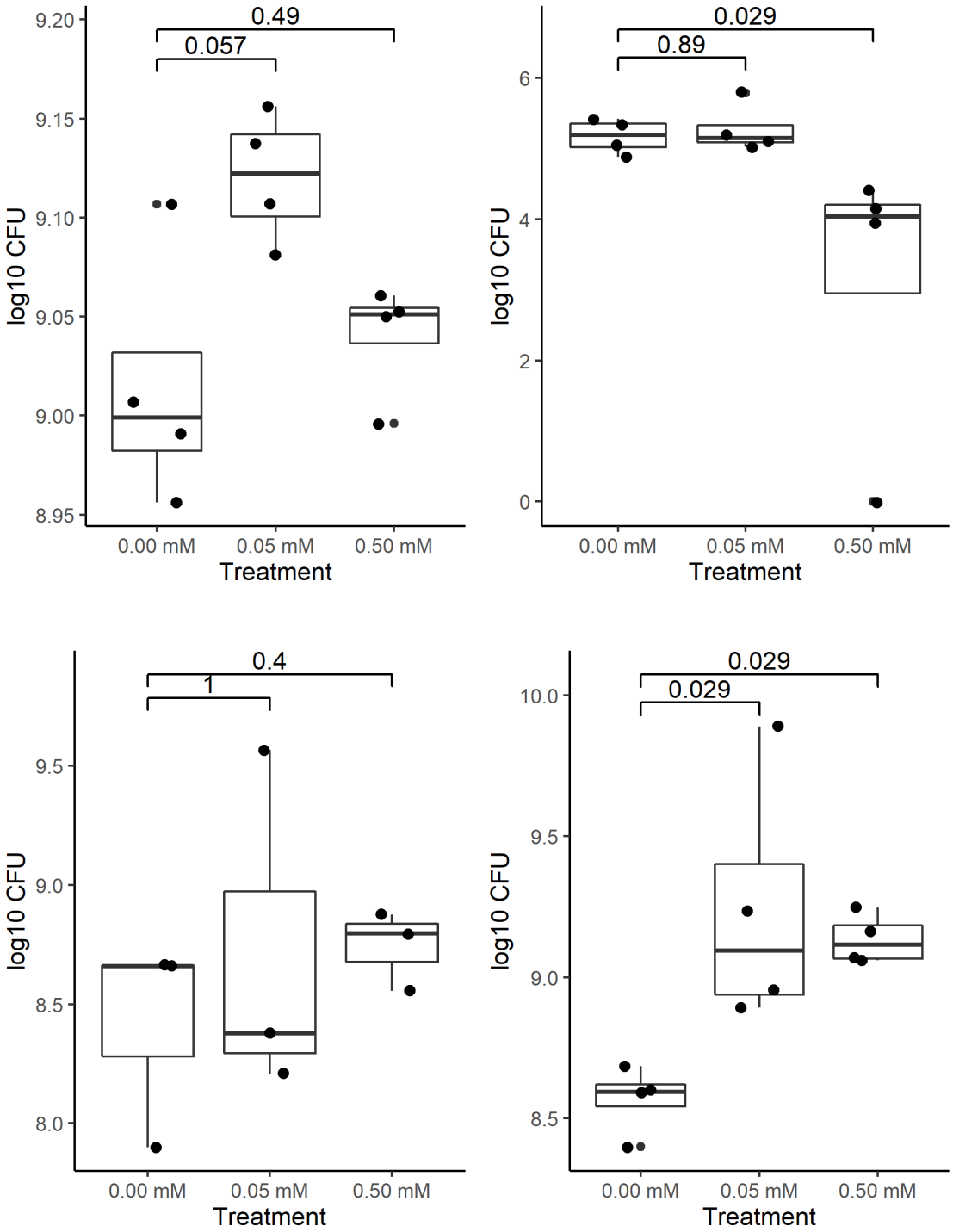
Sequencing results

Supplementary Table 4: Results of NCBI BLAST searches with the nucleotides sequences from 16S sequencing as input, retrieved from root samples used for bright field fluorescence microscopy.

Sample	Hit	Identities
A116	Ab-V6	916/987(93%)
A216	Ab-V6	800/937(85%)
A316	Ab-V6	800/937(85%)
AM116	Ab-V6	916/987(93%)
AM126	Ab-V6	844/928(91%)
AM216	Ab-V6	865/973(89%)
AM226	Ab-V6	815/924(88%)
AM316	Ab-V6	818/894(91%)
C216	Draba incana chloroplast, armoracia rusticana chloroplast, Descurainia sophia chloroplast	
C226	Armoracia rusticana chloroplast, Descurainia sophia chloroplast, Rorippa apetala chloroplast	
C227	Armoracia rusticana chloroplast, Descurainia sophia chloroplast, Rorippa apetala chloroplast	
P116	Pf-5	881/997(88%)
P127	Pf-5	951/1026(93%)
P216	Pf-5	872/992(88%)
P227	Pf-5	849/956(89%)
PM116	Pf-5	1007/1010(99%)
PM127	Pf-5	952/1030(92%)
PM216	Pf-5	1022/1029(99%)
PM316	Pf-5	919/997(92%)

Supplementary Figure 3

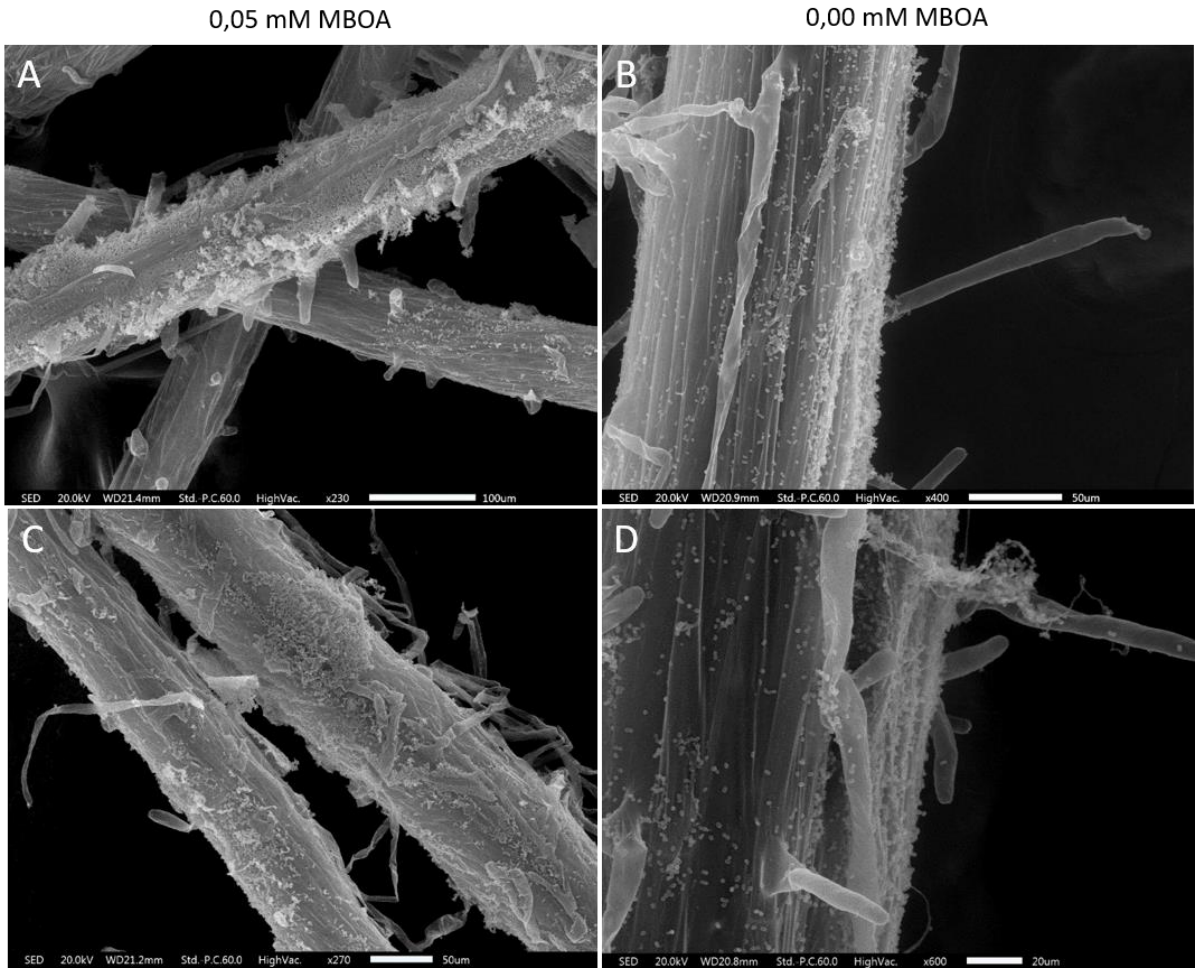
Adherence assay *Arabidopsis* roots with Ab-V5



Supplementary Figure 3: Results of the adherence assay. MBOA treatment did not have a significant influence on the number of bacteria isolated from roots. *Arabidopsis* were grown on ½ MS agar plates and inoculated with OD 0.05 Ab-V5 treated with 0.00 mM; 0.05 mM or 0.50 mM MBOA for 3 h under a 120 RPM agitation regime. Roots were washed and ground, diluted in PBS and plated for enumeration. CFU: Colony Forming Units. P-values displayed in the graphs were calculated by ANOVA tests.

Supplementary Figure 4

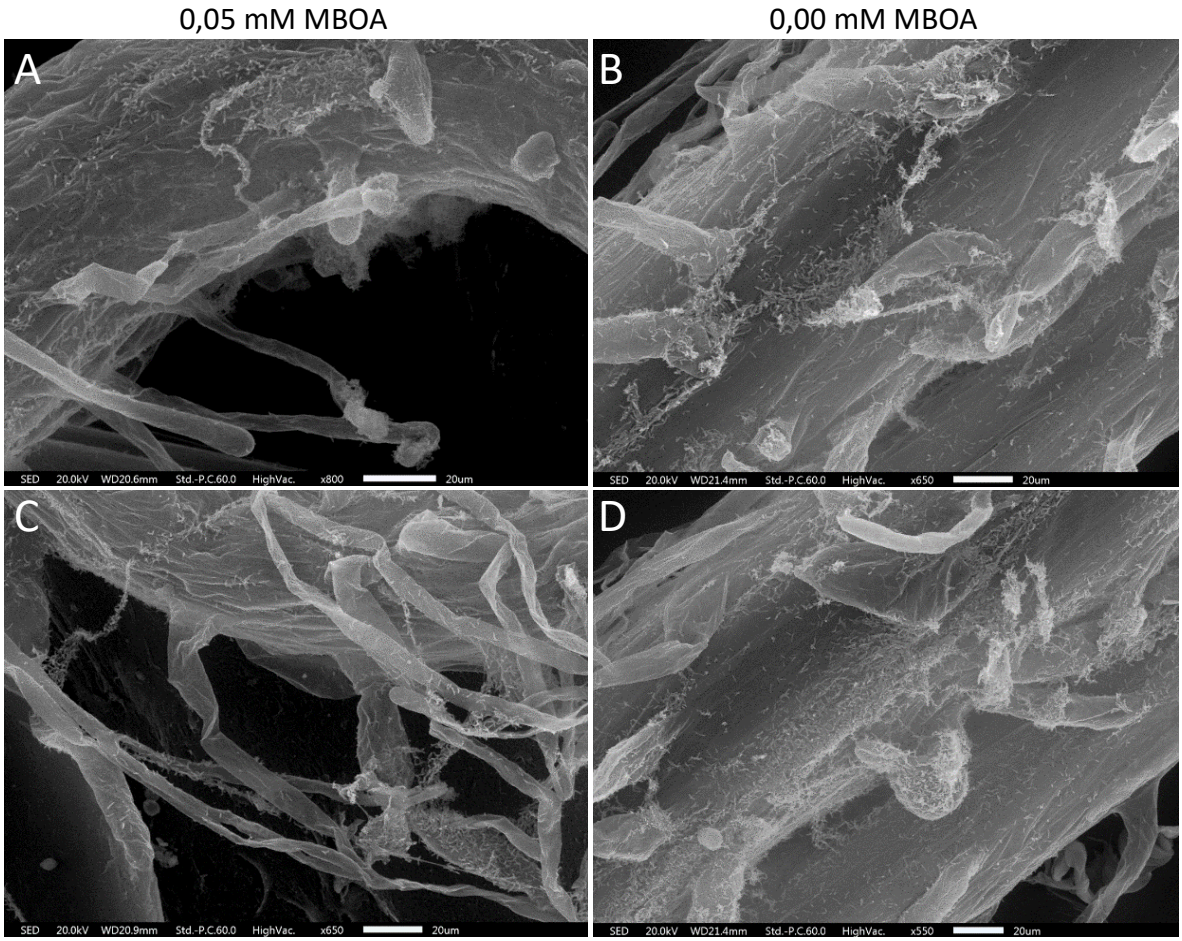
Scanning Electron Microscopy of *Arabidopsis* roots with Ab-V5



Supplementary Figure 4: Scanning electron microscopy of *Arabidopsis* roots inoculated with Ab-V5. *Arabidopsis* seedlings were grown on ½ MS agar medium for 14 days and incubated for 96 h with Ab-V5 cultures of OD 0.05, prior to sample preparation. A and C: 0.05 mM MBOA treatment. B and D: 0.00 mM MBOA treatment. There was an observable difference in the amount of biofilm on the root surface among the two treatments with 0.05 mM (A and C) accumulating thicker and wider spread biofilm. Scale bars indicate 100 µm (A), 50 µm (B, C) and 20 µm (D).

Supplementary Figure 5

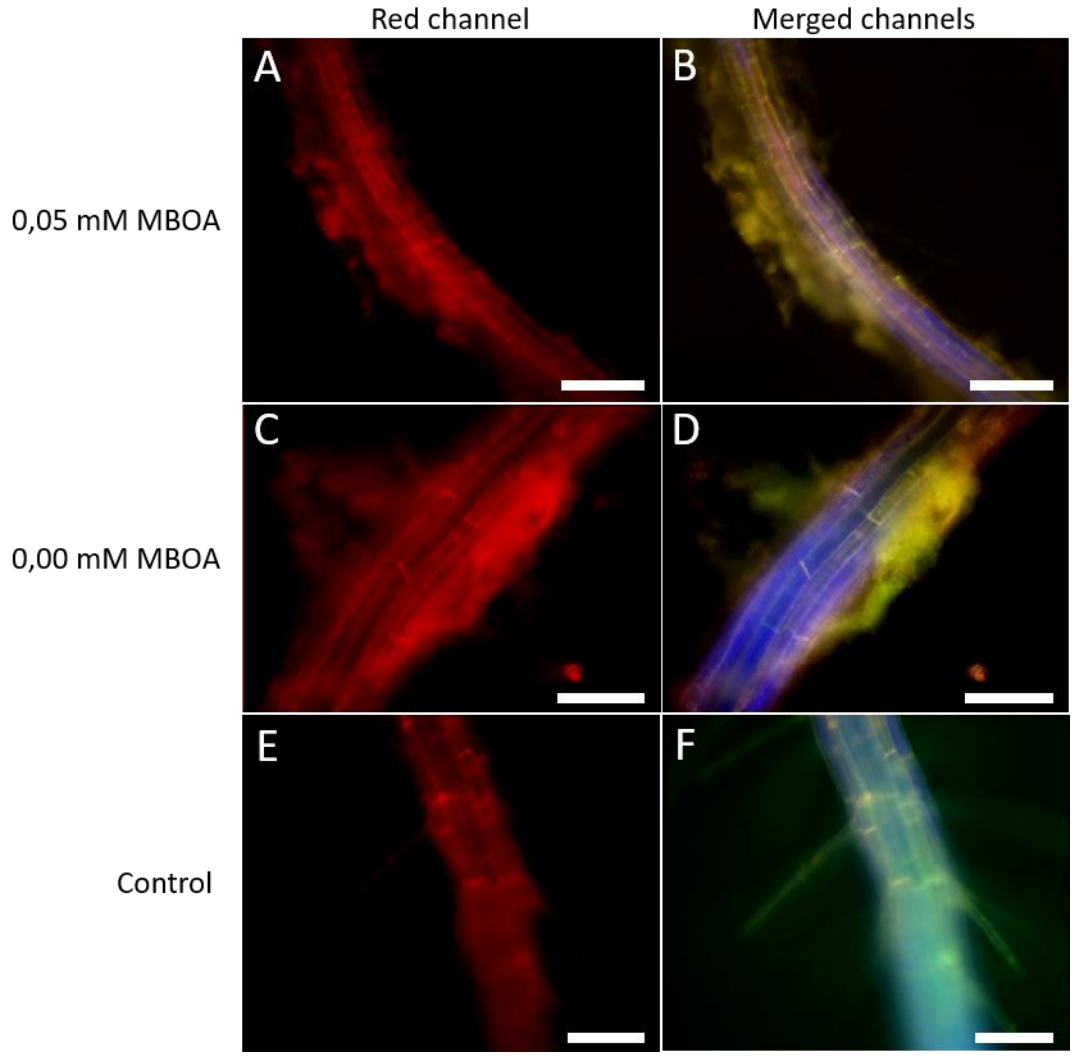
Scanning Electron Microscopy of *Arabidopsis* roots with Pf-5



Supplementary Figure 5: Scanning electron microscopy of *Arabidopsis* roots inoculated with Pf-5. A and C: 0.05 mM MBOA treatment. B and D: 0.00 mM MBOA treatment. *Arabidopsis* seedlings were grown on ½ MS agar medium for 14 days and incubated for 96 h with Pf-5 cultures of OD 0.05, prior to sample preparation. There was no observable difference in the amount of biofilm on the root surface among the two treatments. Scale bars indicate 20 µm.

Supplementary Figure 6

Bright Field fluorescence microscopy of *Arabidopsis* roots with Pf-5



Supplementary Figure 6: Bright field fluorescence microscopy of *Arabidopsis* roots inoculated with Pf-5. *Arabidopsis* seedlings were grown on ½ MS agar medium for 14 days and incubated for 96 h with Pf-5 cultures of OD 0.05, prior to sample preparation. A, B: 0.05 mM MBOA treatment; C, D: 0.00 mM MBOA treatment; E, F: control treatments. Seedlings were treated for 1 hour with Nile Red solution which has a peak emission wave length of around 540 nm when bound to neutral lipids and around 650 nm when bound to polar lipids. There was no observable difference in the amount of biofilm on the root surface among the two treatments (A, B and C, D). Scale bars indicate 50 µm (A, B) and 100 µm (C – F).

Appendix C – Supplementary data of chapter 5

Supplementary Tables 5

Sequencing statistics

RNA-seq on Pf-5

Supplementary Table 5: DEGs identified from 0.50 mM MBOA treatment on Pf-5 (p = 0.05).

Genes	Description	Category	LogFC
WP_011334291.1	NAD(+) diphosphatase	cellular respiration	1,16
WP_016702562.1	taurine dioxygenase	cellular respiration	-1,11
WP_017336877.1	L-threonine dehydrogenase	cellular respiration	1,14
WP_011058481.1	inhibitor of vertebrate lysozyme family protein	housekeeping enzyme	0,92
WP_011059792.1	sulfurtransferase TusA	sulfer metabolism	1,38
WP_011058772.1	lipocalin family protein	transport	1,47
WP_011059507.1	DUF4197 domain-containing protein	unknown	1,22
WP_011062008.1	DUF2474 domain-containing protein	unknown	0,84

Supplementary Table 6

RNA-seq on Ab-V5

Supplementary Table 6: Overview of all DEGs identified in both 0.05 mM and 0.50 mM MBOA treatments (p = 0.05).

Genes	Description	Category	logFC 0,05	logFC 0,50
AHNNBFGK_03470	histidine biosynthesis protein	amino acid biosynthesis	-2,34	-2,72
AHNNBFGK_00590	formyltetrahydrofolate deformylase	amino acid biosynthesis	-2,87	-3,84
AHNNBFGK_03476	riboflavin biosynthesis protein RibD	anabolism	-2,80	-4,01
AHNNBFGK_00938	D-glycero-beta-D-manno-heptose-7-phosphate kinase	anabolism	-4,34	-3,71
AHNNBFGK_02785	AEC family transporter	auxin efflux carrier	-5,07	-4,82
AHNNBFGK_05533	threonine/serine dehydratase	catabolism	-2,36	-2,67
AHNNBFGK_02037	alpha/beta fold hydrolase	catabolism	-2,59	-3,31
AHNNBFGK_00345	DUF1624 domain-containing protein	catabolism	-2,61	-3,14
AHNNBFGK_02106	2-hydroxychromene-2-carboxylate isomerase	catabolism	-3,81	-2,52
AHNNBFGK_04590	DUF4743 domain-containing protein	catalytic domain	-2,29	-2,07
AHNNBFGK_00685	ATP-binding protein	catalytic membrane protein	-5,20	-4,64
AHNNBFGK_03305	Ldh family oxidoreductase	cellular respiration	3,01	7,28
AHNNBFGK_00885	NAD+ synthase	cellular respiration	2,86	3,78
AHNNBFGK_01223	SDR family oxidoreductase	cellular respiration	-1,54	-2,10
AHNNBFGK_05453	NADH-quinone oxidoreductase subunit NuoF	cellular respiration	-1,99	-1,96
AHNNBFGK_06212	alanine dehydrogenase	cellular respiration	-2,17	-1,79
AHNNBFGK_02723	NAD(P)H-hydrate dehydratase	cellular respiration	-2,28	-2,07
AHNNBFGK_00074	Ldh family oxidoreductase	cellular respiration	-2,50	-2,39
AHNNBFGK_05273	exopolysaccharide biosynthesis protein	EPS biosynthesis	-1,86	-1,60
AHNNBFGK_04555	RNA methyltransferase	gene regulation	-2,10	-2,44
AHNNBFGK_02618	LysR family transcriptional regulator	gene regulation	-2,10	-2,31
AHNNBFGK_00239	response regulator transcription factor	gene regulation	-2,28	-1,99
AHNNBFGK_04248	IS5/IS1182 family transposase	gene regulation	-2,35	-2,42
AHNNBFGK_05050	response regulator transcription factor	gene regulation	-2,62	-3,47
AHNNBFGK_04633	response regulator transcription factor	gene regulation	-2,67	-3,30
AHNNBFGK_02659	response regulator transcription factor	gene regulation	-2,68	-2,30
AHNNBFGK_03502	GNAT family N-acetyltransferase	gene regulation	-3,35	-6,13
AHNNBFGK_03041	transcriptional regulator	gene regulation	-4,05	-5,41
AHNNBFGK_03501	GNAT family N-acetyltransferase	gene regulation	-4,09	-3,82
AHNNBFGK_00521	nitrogenase accessory factor	nitrogen metabolism	-2,98	-4,13
AHNNBFGK_06193	endolytic transglycosylase MltG	peptido glycan biosynthesis	-1,81	-2,13

Continous

Genes	Description	Category	logFC 0,05	logFC 0,50
AHNNBFGK_01257	PLP-dependent aminotransferase family protein	primary metabolism	1,92	1,74
AHNNBFGK_02823	2-keto-4-methylthiobutyrate aminotransferase	primary metabolism	-1,89	-2,30
AHNNBFGK_06222	xanthine dehydrogenase family protein molybdopterin-binding subunit	primary metabolism	-2,03	-1,60
AHNNBFGK_06062	50S ribosomal protein L31	primary metabolism	-2,46	-3,27
AHNNBFGK_01002	aspartate aminotransferase family protein	primary metabolism	-2,48	-2,50
AHNNBFGK_03675	glycoside hydrolase	primary metabolism	-2,80	-2,36
AHNNBFGK_01483	carboxylating nicotinate-nucleotide diphosphorylase	primary metabolism	-2,91	-3,28
AHNNBFGK_05280	glycosyltransferase	primary metabolism	-3,71	-3,14
AHNNBFGK_02796	glycosyltransferase	primary metabolism	-7,49	-3,57
AHNNBFGK_04202	ribonuclease PH	RNA processing	5,93	5,15
AHNNBFGK_03828	serine/threonine-protein kinase	signal transduction	-2,22	-2,21
AHNNBFGK_00686	HAMP domain-containing protein	signal transduction	-2,92	-2,54
AHNNBFGK_03599	GAF domain-containing protein	signal transduction	-3,30	-4,07
AHNNBFGK_00240	response regulator	signal transduction	-3,98	-4,16
AHNNBFGK_05283	polysaccharide deacetylase family protein	symbiosis	-2,07	-2,67
AHNNBFGK_03152	Flp family type IVb pilin	symbiosis	-3,44	-8,16
AHNNBFGK_03151	prepilin peptidase	symbiosis	-7,17	-3,06
AHNNBFGK_01446	MFS transporter	transport	-2,59	-3,11
AHNNBFGK_00994	DHA2 family efflux MFS transporter permease subunit	transport	-2,71	-2,83
AHNNBFGK_00964	microcin ABC transporter ATP-binding protein	transport	-2,82	-3,36
AHNNBFGK_02170	cobalt transporter	transport	-2,92	-3,83
AHNNBFGK_05150	Na/Pi cotransporter family protein	transport	-3,26	-3,21
AHNNBFGK_02107	potassium channel protein	transport	-4,81	-4,37
AHNNBFGK_01499	tetratricopeptide repeat protein	unknown	-2,79	-2,64
AHNNBFGK_01737	family Rossman fold protein	unknown	-3,20	-2,54
AHNNBFGK_03451	hypothetical protein	unknown	-3,32	-2,99
AHNNBFGK_03043	exported protein of unknown function	unknown	-3,62	-5,67
AHNNBFGK_00671	hypothetical protein	unknown	-4,29	-5,32

Conitnuation

Genes	Description	Category	logFC 0,05	logFC 0,50
AHNNBFGK_05480	hypothetical protein	unknown	-4,52	-3,77
AHNNBFGK_00758	alpha/beta hydrolase	catabolism	-3,27	
AHNNBFGK_02937	alpha/beta hydrolase	catabolism	-2,06	
AHNNBFGK_04641	Chemotaxis regulator CheZ, phosphatase of CheY~P	chemotaxis	2,29	
AHNNBFGK_01133	DNA polymerase III subunit epsilon	DNA replication	-1,91	
AHNNBFGK_03107	GNAT family N-acetyltransferase	gene regulation	-3,65	
AHNNBFGK_04632	transcriptional activator, LuxR/FixJ family	gene regulation	-2,22	
AHNNBFGK_05389	pyrroloquinoline quinone biosynthesis protein PqqE	oxidative stress	-2,90	
AHNNBFGK_00736	glycosyltransferase family 1 protein	primary metabolism	-2,45	
AHNNBFGK_04979	aspartate carbamoyltransferase catalytic subunit	primary metabolism	2,00	
AHNNBFGK_03203	histidine kinase (plasmid)	signal transduction	1,41	
AHNNBFGK_04279	DUF4880 domain-containing protein	signal transduction	2,91	
AHNNBFGK_05265	hybrid sensor histidine kinase/response regulator	signal transduction	-2,42	
AHNNBFGK_03149	type II and III secretion system protein family protein	transport	-3,65	
AHNNBFGK_04404	MFS transporter	transport	2,60	
AHNNBFGK_05590	ABC transporter ATP-binding protein	transport	-6,12	
AHNNBFGK_03549	hypothetical protein	unknown	-6,07	
AHNNBFGK_04770	alpha,alpha-trehalose-phosphate synthase (UDP-forming)	abiotic stress		-1,78
AHNNBFGK_03471	ATP-grasp domain-containing protein	anabolism		-5,24
AHNNBFGK_05366	aminotransferase	anabolism		-1,86
AHNNBFGK_00988	glutathione S-transferase	biodegradative metabolism		-2,22
AHNNBFGK_01001	glutathione S-transferase	biodegradative metabolism		1,54
AHNNBFGK_05412	glutathione S-transferase family protein	biodegradative metabolism		1,91
AHNNBFGK_05326	glycerol dehydrogenase	carbon catabolsim		2,08
AHNNBFGK_02106	2-hydroxychromene-2-carboxylate isomerase	catabolism		-2,52
AHNNBFGK_02998	alpha-galactosidase	catabolism		-1,91
AHNNBFGK_04102	DUF1206 domain-containing protein	catabolism		2,41
AHNNBFGK_06116	D-amino acid dehydrogenase	catabolism		-2,12

Continuation

Genes	Description	Category	logFC 0,05	logFC 0,50
AHNNBFGK_00661	DUF4347 domain-containing protein	catalytic domain		2,94
AHNNBFGK_00070	acetoin dehydrogenase dihydrolipoyllysine-residue acetyltransferase subunit	cellular respiration		-2,67
AHNNBFGK_00188	cytochrome c oxidase subunit I	cellular respiration		-1,78
AHNNBFGK_00361	formate dehydrogenase subunit gamma	cellular respiration		2,80
AHNNBFGK_00725	4-hydroxybenzoate octaprenyltransferase	cellular respiration		2,84
AHNNBFGK_00799	NAD(P)/FAD-dependent oxidoreductase	cellular respiration		2,66
AHNNBFGK_01240	energy transducer TonB	cellular respiration		1,38
AHNNBFGK_01707	succinate dehydrogenase flavoprotein subunit	cellular respiration		1,79
AHNNBFGK_02025	SDR family oxidoreductase	cellular respiration		4,51
AHNNBFGK_02251	NAD(P)/FAD-dependent oxidoreductase	cellular respiration		1,76
AHNNBFGK_02473	SDR family oxidoreductase	cellular respiration		1,67
AHNNBFGK_02615	Gfo/Idh/MocA family oxidoreductase	cellular respiration		-6,56
AHNNBFGK_04526	cytochrome c	cellular respiration		2,20
AHNNBFGK_04675	NAD(P)-dependent oxidoreductase	cellular respiration		2,12
AHNNBFGK_05452	NADH-quinone oxidoreductase subunit G	cellular respiration		1,88
AHNNBFGK_05920	cytochrome c oxidase subunit II	cellular respiration		-1,57
AHNNBFGK_01721	ATP-dependent DNA helicase	DNA replication		2,41
AHNNBFGK_05278	exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase	EPS biosynthesis		-2,61
AHNNBFGK_01464	3-oxoacyl-ACP reductase	fatty acid biosynthesis		-2,46
AHNNBFGK_02843	GFA family protein	formaldehyde degradation		2,07
AHNNBFGK_00642	LysR family transcriptional regulator	gene regulation		-1,76
AHNNBFGK_01092	pirin family protein	gene regulation		1,99
AHNNBFGK_01239	response regulator transcription factor	gene regulation		1,99
AHNNBFGK_01719	XRE family transcriptional regulator	gene regulation		3,55
AHNNBFGK_02366	LysR family transcriptional regulator	gene regulation		2,88
AHNNBFGK_02566	LysR family transcriptional regulator	gene regulation		-2,23
AHNNBFGK_02799	winged helix-turn-helix transcriptional regulator	gene regulation		2,39
AHNNBFGK_03042	response regulator transcription factor	gene regulation		-2,28

Continuation

Genes	Description	Category	logFC 0,05	logFC 0,50
AHNNBFGK_03410	N-acetyltransferase	gene regulation		-2,34
AHNNBFGK_04004	class I SAM-dependent RNA methyltransferase	gene regulation		1,83
AHNNBFGK_04282	IS6 family transposase	gene regulation		3,47
AHNNBFGK_05158	transcriptional repressor	gene regulation		-6,25
AHNNBFGK_05175	Crp/Fnr family transcriptional regulator	gene regulation		2,62
AHNNBFGK_05510	GntR family transcriptional regulator	gene regulation		1,58
AHNNBFGK_05822	LysR family transcriptional regulator	gene regulation		-1,99
AHNNBFGK_05828	Crp/Fnr family transcriptional regulator	gene regulation		5,95
AHNNBFGK_05980	GNAT family N-acetyltransferase	gene regulation		1,67
AHNNBFGK_05992	GNAT family N-acetyltransferase	gene regulation		1,84
AHNNBFGK_01932	ferritin-like domain-containing protein	iron metabolism		-2,66
AHNNBFGK_01036	membrane protein	membrane protein		1,91
AHNNBFGK_04629	YccF domain-containing protein	membrane protein		-2,00
AHNNBFGK_05026	hydrogenase maturation nickel metallochaperone HypA	nickel homeostasis		-1,65
AHNNBFGK_04727	peptidase	nitrogen metabolism		-2,60
AHNNBFGK_05842	TAT-dependent nitrous-oxide reductase	nitrogen metabolism		2,15
AHNNBFGK_00723	glutamate--cysteine ligase	oxidative stress		1,65
AHNNBFGK_03666	rubrerythrin	oxidative stress		-3,35
AHNNBFGK_05844	rubrerythrin family protein	oxidative stress		6,50
AHNNBFGK_00030	CCA tRNA nucleotidyltransferase	primary metabolism		1,71
AHNNBFGK_00830	16S rRNA (guanine(966)-N(2))-methyltransferase RsmD	primary metabolism		-2,09
AHNNBFGK_00837	dihydroorotase	primary metabolism		-2,01
AHNNBFGK_01485	UDP-galactopyranose mutase	primary metabolism		-3,22
AHNNBFGK_02249	glycosyltransferase	primary metabolism		-1,59
AHNNBFGK_02791	nucleotide sugar dehydrogenase	primary metabolism		-2,49
AHNNBFGK_02803	MBL fold metallo-hydrolase	primary metabolism		-2,09
AHNNBFGK_02809	sigma-70 family RNA polymerase sigma factor	primary metabolism		-2,03
AHNNBFGK_02852	isochorismatase family protein	primary metabolism		-2,51

Continuation

Genes	Description	Category	logFC 0,05	logFC 0,50
AHNNBFGK_04706	HPF/RaiA family ribosome-associated protein	primary metabolism		-1,74
AHNNBFGK_04846	glycosyltransferase family 61 protein	primary metabolism		1,65
AHNNBFGK_05149	NAD-dependent epimerase/dehydratase family protein	primary metabolism		-2,36
AHNNBFGK_05530	HAD family hydrolase	primary metabolism		5,30
AHNNBFGK_06100	phosphomethylpyrimidine synthase ThiC	primary metabolism		-1,89
AHNNBFGK_06293	RNA polymerase sigma factor RpoH	primary metabolism		-1,35
AHNNBFGK_01858	aminoacyl-tRNA hydrolase	protein expression		-4,87
AHNNBFGK_04163	gamma-glutamyl-gamma-aminobutyrate hydrolase family protein	putricine catabolism		2,58
AHNNBFGK_02072	RNA pseudouridine synthase	RNA processing		1,60
AHNNBFGK_05037	ribonuclease HII	RNA processing		2,84
AHNNBFGK_05179	RNA helicase	RNA processing		1,57
AHNNBFGK_01149	cobalamin biosynthesis protein	secondary metabolism		1,86
AHNNBFGK_02721	type II toxin-antitoxin system ParD family antitoxin	secondary metabolism		-1,71
AHNNBFGK_03147	CobQ/CobB/MinD/ParA nucleotide binding domain-containing protein	secondary metabolism		-3,57
AHNNBFGK_04049	antibiotic biosynthesis monooxygenase	secondary metabolism		2,00
AHNNBFGK_02344	response regulator	signal transduciton		-2,72
AHNNBFGK_00550	PAS domain S-box protein	signal transduction		1,40
AHNNBFGK_00942	methyltransferase domain-containing protein	signal transduction		2,39
AHNNBFGK_01214	class I SAM-dependent methyltransferase	signal transduction		-2,21
AHNNBFGK_01538	PAS domain S-box protein	signal transduction		2,01
AHNNBFGK_01557	two-component sensor histidine kinase	signal transduction		-2,39
AHNNBFGK_01892	methyltransferase	signal transduction		-2,09
AHNNBFGK_01898	PAS domain S-box protein	signal transduction		-1,98
AHNNBFGK_02283	class I SAM-dependent methyltransferase	signal transduction		1,41
AHNNBFGK_02697	response regulator	signal transduction		1,71
AHNNBFGK_02754	PAS domain S-box protein	signal transduction		1,89
AHNNBFGK_02862	adenylate/guanylate cyclase domain-containing protein	signal transduction		1,68
AHNNBFGK_03307	HAMP domain-containing histidine kinase	signal transduction		3,55

Continuation

Genes	Description	Category	logFC 0,05	logFC 0,50
AHNNBFGK_03964	histidine kinase	signal transduction		2,51
AHNNBFGK_04592	PrkA family serine protein kinase	signal transduction		-1,59
AHNNBFGK_05275	class I SAM-dependent methyltransferase	signal transduction		-4,16
AHNNBFGK_05542	response regulator	signal transduction		-3,52
AHNNBFGK_05950	response regulator	signal transduction		1,55
AHNNBFGK_05961	HAMP domain-containing histidine kinase	signal transduction		-4,07
AHNNBFGK_04061	sulfurtransferase TusA family protein	sulfer metabolism		1,57
AHNNBFGK_00277	sulfite exporter TauE/SafE family protein	transport		-3,05
AHNNBFGK_00309	TatABCE protein translocation system subunit (plasmid)	transport		-3,43
AHNNBFGK_00461	Trk system potassium transporter TrkA	transport		-2,41
AHNNBFGK_00836	ion transporter	transport		1,51
AHNNBFGK_00860	MFS transporter	transport		2,35
AHNNBFGK_01145	sulfite exporter TauE/SafE family protein	transport		1,72
AHNNBFGK_01340	MFS transporter	transport		-2,76
AHNNBFGK_01595	amino acid ABC transporter permease	transport		-2,06
AHNNBFGK_01889	lysine transporter LysE	transport		-2,36
AHNNBFGK_02047	ABC transporter substrate-binding protein	transport		-2,00
AHNNBFGK_02730	TrkH family potassium uptake protein	transport		-1,94
AHNNBFGK_02745	DctP family TRAP transporter solute-binding subunit	transport		1,91
AHNNBFGK_02940	manganese efflux pump	transport		-2,41
AHNNBFGK_03005	aspartate-alanine antiporter	transport		-1,48
AHNNBFGK_03304	C4-dicarboxylate ABC transporter permease	transport		2,84
AHNNBFGK_03658	ABC transporter permease	transport		1,89
AHNNBFGK_04179	branched-chain amino acid ABC transporter substrate-binding protein	transport		2,24
AHNNBFGK_04828	ABC transporter ATP-binding protein	transport		2,13
AHNNBFGK_04919	type VI secretion system protein TssA	transport		-1,86
AHNNBFGK_06101	oxalate/formate MFS antiporter	transport		-1,91
AHNNBFGK_06225	TRAP transporter permease	transport		-1,44

Continuation

Genes	Description	Category	logFC 0,05	logFC 0,50
AHNNBFGK_03561	DUF2312 domain-containing protein	unknown		-1,97
AHNNBFGK_05926	DUF1328 domain-containing protein	unknown		-3,53
AHNNBFGK_02652	hypothetical protein	unknown		1,63
AHNNBFGK_03704	hypothetical protein	unknown		-2,74
AHNNBFGK_03705	hypothetical protein TSH58_00630	unknown		-3,38
AHNNBFGK_05365	hypothetical protein	unknown		1,66
AHNNBFGK_06256	hypothetical protein	unknown		1,88
AHNNBFGK_02793	right-handed parallel beta-helix repeat-containing protein	unknown		-1,78

Conclusion