Maize root bacteria cooperate to tolerate and metabolise host-secreted plant specialized metabolites

Inaugural dissertation of the Faculty of Science,

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Summary

Plant roots are colonized by microbial communities which can promote growth, provide nutrients and protection against pathogens for the host. Plant specialized metabolites exuded by roots, like benzoxazinoids (BX) produced by maize, can structure root-associated microbial communities. However, the underlying mechanisms are poorly understood. The present thesis aimed to uncover the contribution of i) bacterial tolerance to benzoxazinoids, ii) bacterial metabolisation of benzoxazinoids and iii) the interactions of both for structuring root bacterial communities.

In chapter 1, we established a collection of maize root bacteria and in an extensive high-throughput *in vitro* screening we uncovered that benzoxazinoids inhibit bacterial growth in a strain-dependent and compound-dependent manner. Among the benzoxazinoids tested, 6-methoxybenzoxazolin-2(3H)-one (MBOA), which is the dominant metabolite structuring maize rhizosphere microbiomes, is the most selective compound. Overall gram-positive bacteria were more MBOA-tolerant, indicating that the cell wall properties are one important component defining MBOA tolerance. Tolerance to MBOA correlated positively with the benzoxazinoid-dependent colonisation of maize roots by the bacteria. This study revealed that benzoxazinoids selectively act as antibiotics on members of the maize root microbiome and that their capacity to tolerate benzoxazinoids enhanced root colonisation. We propose that tolerance to secreted antimicrobial compounds presents an important mechanism for structuring the microbial community on plant roots.

In chapter 2, we identified maize root bacteria that metabolise the abundant benzoxazinoid in the rhizosphere, MBOA to 2-amino-7-methoxyphenoxazin-3-one (AMPO). The characteristic red colour of AMPO enabled us to develop a simple plate assay to screen the maize root bacteria strain collection for their ability to metabolise MBOA to AMPO. Few bacterial lineages including *Sphingobium* and *Microbacterium* convert MBOA to AMPO. AMPO-forming bacteria were enriched on roots of BX-producing but not of BX-deficient plants. We utilized the phenotypic diversity within the genus of *Microbacteria* to identify an N-acyl homoserine lactonase (BxdA) as the key enzyme for converting MBOA to AMPO. This study demonstrated the specific recruitment of adapted bacteria on BX-producing roots that can metabolise the host secondary metabolites.

In chapter 3, we designed two synthetic communities (SynComs) differing in their ability to metabolise benzoxazinoids to investigate how benzoxazinoid tolerance and metabolism affect a microbial community. We found that bacteria cooperate to tolerate and metabolise benzoxazinoids. The BX-metabolising SynCom had higher MBOA tolerance than the non-

metabolising SynCom. Further, MBOA structured these SynComs differently, by inhibiting the susceptible strains. The BX-metabolising SynCom bacteria cooperate to metabolise MBOA to form N-(2-hydroxy-4-methoxyphenyl)acetamide (HMPAA), a dominant metabolite that is not formed by single strains. We discovered that HMPAA is formed by the combined activity of an MBOA-degrading *Microbacterium* with a *Pseudomonas* that acetylates the unstable intermediate thereby redirecting the metabolism to HMPAA. This study demonstrated that bacteria on maize roots cooperate to metabolise benzoxazinoids and as a community, they benefitted from an enhanced tolerance to these compounds.

This research reveals microbiological and biochemical mechanisms of how plant specialized metabolites contribute to shape the root microbiota. The ability of maize root bacteria to tolerate and metabolise benzoxazinoids are important traits defining their abundance on BX-producing maize roots. We demonstrate that strains when combined in synthetic communities, they divide labour and cooperate to metabolise and tolerate benzoxazinoids. The deepened understanding of how plant specialized metabolites sculpt community composition provides a tool to selectively steer microbiome structure. Further work is required to understand how bacterial BX-mediated mechanisms affect microbiomes to harness the functions of microbial communities for sustainable agriculture.

General Introduction

Plant root microbiome

Microbiome

Most eukaryotic organisms lived in close association with prokaryotes. Microbes colonise the surfaces and the inside of plant and animal organs and cells (Hickman, 2005). Historically microbes have been primarily studied for their role in causing disease or as symbionts as individual microbes. Still, eukaryotes are typically colonised by diverse microbial communities, which are symptomless at first glance (Bulgarelli et al., 2013). Microbiota members belong to the kingdoms of bacteria, fungi, oomycetes, and protists. The assemblage of microorganisms is termed microbiota, while the sum of its functions, including microbial structural elements and metabolites, is referred to as the microbiome (Berg et al., 2020). The plant or the animal host with all their associated microorganisms are considered as the holobiont while sum of the genetic information of both host and microbiota is referred to as the hologenome. This definition implies that the genetic wealth of diverse microbial symbionts can play an essential role in the adaption and evolution of higher organisms (Zilber-Rosenberg and Rosenberg, 2008). The microbiome affects host evolutionary potential by shifting the mean host phenotype and changing the population's variance in host phenotype (Henry et al., 2021). Since microbiomes are the host's extended genomes, they provide many additional functional capacities. For instance, the microbial communities of plant and animal organs, dedicated to nutrient uptake, greatly increase the metabolic capacity of their host. The plant root microbiome has a similar function to the animal gut microbiome facilitating host nutrition by mobilizing nutrients from the environment (Hacquard et al., 2015). Host-associated microbes are recruited from the environment and partially transmitted between generations (Zilber-Rosenberg and Rosenberg, 2008). Both plant and animal microbiomes are strongly structured by environmental factors of their surrounding environment, i.e. the nutrients present either in soil or the diet in the gut. In addition to such nutritional drivers, microbe-microbe interactions, host genotype, host immune system present well known factors important for microbiota structuring (Hacquard et al., 2015).

Functions of the root microbiome

The root microbiome improves host fitness (Bulgarelli et al., 2013). Through the secretion of organic acids and siderophores, microbes can unlock essential elements such as inorganic phosphate, nitrogen, and potassium to plant-available nutrients and thereby directly promote plant growth (Trivedi et al., 2020). Besides enhancing the bioavailability of micronutrients, the microbiome produces phytohormones such as auxin, gibberellins, and cytokinins, and these phytohormones interfere with the plant hormonal network to stimulate plant growth or

ameliorate abiotic stress (Eichmann et al., 2020). Another important function of the root microbiome is to protect the plant from pathogens and improve plant fitness. In particular root microbiome members directly counteract pathogens by producing antibiotics, lytic enzymes, volatiles and siderophores (Carrión et al., 2019). Direct interactions among microbiome members prevent dysbiosis in the community (Trivedi et al., 2020). Certain microbiome members improve plant defense by triggering induced systemic resistance responses (Haney et al., 2018; Pieterse et al., 2014). In certain plants, this mechanism can depend on the secretion of antimicrobial compounds like coumarins (Stringlis et al., 2018) and benzoxazinoids (Neal and Ton, 2013). Apart from their protective function in biotic stresses, plant microbiomes also improve plant fitness in abiotic stress conditions. In drought, the production of the plant hormone abscisic acid is induced which reduces the immune response and thus shifts the root microbiome to mitigate water stress (Fitzpatrick et al., 2018). Collectively, all these functions of the microbiome related to nutrient provision, hormonal growth promotion, plant protection to biotic and abiotic stressors improve the survival and thus the fitness of the plant host. Plant fitness is therefore a consequence of the entire holobiont (Vandenkoornhuyse et al., 2015).

Composition of the root microbiome

The root microbiota is predominantly recruited from soil, while in some plant species host endophytes are vertically transmitted via seeds (Barret et al., 2015). The root microbiota composition predominantly resembles the surrounding soil microbiome, but multiple other factors define the composition of the root microbiome. The diversity of the microbiome decreases continuously from soil to rhizosphere and roots according to the adaption of the members to host. First the rhizodeposition alters substrate composition and drives a community shift, second the host genotype selects for adapted root endophyte communities (Bulgarelli et al., 2013). The plant microbiota is composed of bacteria, fungi, protists, nematodes, viruses, and archaea. While the soil microbiome harbours various bacterial phyla in similar ratios, certain bacterial phyla namely Pseudomonadota, followed by Bacillota, Actinobacteriota and Bacteriodota, and the fungal phyla Basidiomycetes and Ascomycetes, dominate the root communities. This selective filtering is driven by plant-microbiome coevolution and niche adaption of the microbes (Trivedi et al., 2020).

The major driver of the root microbiome environmental factors such as pH, oxygen levels and temperature because these directly influence the soil microbiome composition (Trivedi et al., 2020). Host factors which differ between plant species, plant genotype and developmental status shape microbiome composition. These host mechanisms like immune responses, root morphology and exudation profiles control pathogenic microbes and favour beneficial microbes to assemble a healthy microbiome (Pascale et al., 2020). Plant defence hormones are important signalling molecules between microbial perception and immune system performance and are

targeted by beneficial and pathogenic microbes. Thus, plant hormones are also responsible for community structuring. For example, salicylic acid can structure the root microbiome to select bacteria that respond positively to salicylic acid (Lebeis et al., 2015). How root exudation impacts root microbiome composition is discussed below. The molecular mechanisms that determine the composition of the root microbiome at the molecular and community level are still not well understood (Trivedi et al., 2020).

Root exudation shaping root microbiome

Roots exude 10-40 % of photosynthetically assimilated carbon into the rhizosphere (Badri and Vivanco, 2008; Haichar et al., 2016; Lynch et al., 1990) creating a very nutritious environment which attracts and feeds soil microbes. Root exudates contain primary metabolites like sugars, amino acids, organic acids, fatty acids, and secondary metabolites (Sasse et al., 2018). The composition of the root exudation shapes the typical root-associated microbial communities (Huang et al., 2019; Sasse et al., 2018; Zhalnina et al., 2018). Thus the metabolic heterogeneity of root exudates may provide a basis for communication and recognition selecting microbial communities tailored to the needs of the host (Trivedi et al., 2020).

Bacteria living in the soil sense root exudates such as sugars or organic acids and by chemotaxis they move towards the roots. Signalling molecules such as the polyamines arginine and putrescine inform the microbes of the presence of the plant host and guides them to the roots (Bremont et al., 2014; Liu et al., 2018). Through motility mechanisms such as flagella they move towards the plant and then they attach to the root surface by forming biofilms (Trivedi et al., 2020). Root exudates contain diverse chemicals and generate a chemically distinct environment in the rhizosphere. To colonize this environment, microbes must be adapted to use these metabolites. Rhizobacteria are enriched in genes for carbohydrate metabolisation (Levy et al., 2018), have many transporters (ATP-binding cassettes, phosphotransferase systems and drug and metabolite transporters) which enables them to metabolise, import and export many different compounds (Levy et al., 2018; Trivedi et al., 2020). The synchronization of microbial metabolism with root exudation was demonstrated on oat roots (Zhalnina et al., 2018). The chemical composition of the root exudates changed with plant development and caused simultaneous shifts in the bacterial community. Specifically, the increase of aromatic organic acids led to an increase of bacteria using these compounds for growth. Microbial substrate preferences can be predicted from genome sequences. Slow-growing organisms were found to be good root colonizers because they efficiently use different substrates (Zhalnina et al., 2018). On tomato roots, enriched bacterial genes encode for the metabolism of plant polysaccharides, trehalose, and iron acquisition (Oyserman et al., 2022). Together these findings support the importance of metabolic adaptations of rhizosphere bacteria for root colonization.

Root exudates also contain diverse specialized plant metabolites that govern interactions of plants with the environment, protect the plant against insects and pathogens increase abiotic stress tolerance (Erb and Kliebenstein, 2020). Plant specialized metabolites were shown to structure root-associated microbial communities (Jacoby et al., 2020b). A common approach is to compare the roots or rhizospheres of wild-type plants with biosynthesis mutants defective in the production of specific secondary metabolites. For instance, glucosinolates (Kudjordjie et al., 2021), camalexins (Koprivova et al., 2019), triterpenes (Huang et al., 2019), and coumarins (Harbort et al., 2020; Stringlis et al., 2018; Voges et al., 2019) were shown to shape the microbiota of the model plant *Arabidopsis thaliana*. The saponins tomatine structure the rhizosphere microbiome of tomato (Nakayasu et al., 2021). Maize exudes a variety of plant specialized metabolites that were shown to structure the root microbiome, namely benzoxazinoids (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019; Schütz et al., 2021), diterpenoids (Murphy et al., 2021), zealexins (Ding et al., 2020) and flavonoids (Yu et al., 2021).

Mechanistically plant specialized metabolites like coumarins, flavonoids and benzoxazinoids function as antimicrobials, reduce biofilm formation, inhibit quorum sensing, act as signalling molecules for chemotaxis or affect motility of single microbes (Schulz and Dörmann, 2020; Stassen et al., 2021; Sugiyama, 2021). Here we focus on the antimicrobial activity of plant specialized metabolites and the microbial conversions of these compounds.

The dominant coumarin compound in the *Arabidopsis* rhizosphere, scopoletin inhibits the growth of the fungal pathogens *Fusarium oxysporum* and *Verticillium dahliae* (Stringlis et al., 2018). Various other compounds inhibit the growth of the bacterial pathogen *Ralstonia solanacearum* by breaking the cell membrane integrity (Yang et al., 2016). Certain root microbiome members can tolerate coumarins such as *Pseudomonas* (Voges et al., Stringlis et al.) and certain *Burkholderia* showed strain-level tolerance to fraxetin (Harbort et al., 2020). Another widely distributed class of plant specialized metabolites, the flavonoids exhibit antimicrobial activity against a range of root pathogens in the rhizosphere, and clinical important bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Mierziak et al., 2014; Wu et al., 2019, 2013). The flavonoids are antimicrobial against *Staphylococcus aureus* because they inhibit of DNA gyrase (Wu et al., 2013) and disrupt the function of the membrane. Flavonoids increase bacterial membrane permeability, scatter proton move force and bind to the peptidoglycan (Wu et al., 2019). These mechanisms cause rapid bactericidal activity and leads to low resistance development *in vitro* (Wu et al., 2019). The specialized metabolites exuded by sweetgrasses, the benzoxazinoids have antimicrobial activity due to their ability to intercalate to DNA (Hashimoto and Koichi, 1996). The main benzoxazinoid responsible for maize root microbiome structuring is MBOA (Hu et al., 2018b). The mechanisms of MBOA tolerance were investigated using

experimental evolution, in the endosymbiotic bacterium of entomopathogenic nematodes which belonging to the genus *Photorhabdus*. In MBOA-tolerant strains, acquired mutations located in genes regulating DNA transcription, membrane architecture and membrane channels (Machado et al., 2020). These processes are often related to antibiotic resistance in bacteria (Delcour, 2009; Fernández and Hancock, 2012). In *Photorhabdus*, inactivation of an aquaporin-like channel, AqpZ confers tolerance to MBOA (Machado et al., 2020). These examples demonstrate that coumarins, flavonoids and benzoxazinoids act on cell wall architecture, however the underlying tolerance mechanisms are not yet fully uncovered.

Apart from tolerance, metabolisation of plant specialized metabolites could confer fitness benefits to microbes colonizing the rhizosphere. Members of the *Arabidopsis* root microbiome catabolize triterpenes and use them as carbon source (Huang et al., 2019). Bacteria belonging to the genus *Sphingobium* which are enriched on tomato roots in presence of tomatine and can also use them as carbon source (Nakayasu et al., 2021). The soil bacterium *Pseudomonas mandelii* metabolises the coumarin 7-hydroxycoumarin. The first step of this degradation is catalysed by an alcohol dehydrogenase (HcdE) which is encoded by a gene located in the hydroxycoumarin degradation gene cluster (Krikštaponis et al., 2021). This gene cluster further a monooxygenase (HcdA), a dioxygenase (HcdB), and a putative hydroxymuconic semialdehyde hydrolase (HcdC) which further catalyzes 7-hydroxycoumarin (Krikštaponis et al., 2021, 2018). Various bacteria can degrade flavonoids (Shaw and Hooker, 2008) such as the diazotrophic rhizobacterium *Herbaspirillum seropedicae* which can use the flavonoid naringenin as a carbon source (Marin et al., 2013). In this strain a monooxygenase was identified to catalyse the initial step of the degradation, then followed by a conversion of the dioxygenase and through a meta-clevage pathway oxaloacetic acid is generated which can be metabolised via the tricarboxylic acid cycle (Marin et al., 2016). The specialized endophytic fungus *Fusarium verticillioides* colonizing maize seeds converts the benzoxazinoid BOA to HPMA. The first step in the conversion of BOA to the aminophenol AP is catalysed by a metallo-β-lactamase Mbl1. In a second step, AP is malonylated to malonamic acid HPMA by an arylamine N-malonyltransferase Nat1, which has been identified in the specialized endophytic fungus *Fusarium verticillioides* colonizing maize seeds (Glenn et al., 2016). Degradation genes in operons

Together, these examples show that plant specialized metabolites directly inhibit or promote bacteria. Bacteria evolved mechanisms to cope with plant specialized metabolites such as tolerance or metabolism. While it is well-supported by now that plants structure their root microbiomes by means of exuded compound including secondary metabolites, it is unclear to which extent these microbial mechanisms coping with plant specialiced metabolites, such as tolerance and metabolisation, are defining community composition.

Maize root microbiome

Maize (*Zea mays*) belongs to the family of *Poaceae* and is a globally important cereal crop, grown on over 200 Mio. hectares worldwide (FAO, 2021). It was domesticated from teosinte nearly 14'000 years ago. Besides being a cereal crop, maize is an established model plant for reseach on monocotyledons with the genome sequence and biotechnological protocols available. Recently numerous studies have investigated the composition and the function of the maize root microbiome in response to environmental and biotic factors. These studies reveal that Pseudomonadota dominate the maize microbiome with the main genera *Agrobacterium, Rhizobium Burkholderia, Enterobacter, Pantoea, Pseudomonas*, and *Bradyrhizobium* represented. *Pseudomonadata* show rapid growth upon the release of labile carbon compared to non-rhizospheric microbes, which may explain their abundance in the maize root microbiome. The main factors driving maize microbiota composition include plant genotype, environmental factors, soil characteristics, and agricultural practices (Mehta et al., 2021). For example, soil physiochemical properties (Bourceret et al., 2022) and temperatures structure maize root microbiota in the field (Beirinckx et al., 2020). Plant development and temporal changes alter microbiota composition too, which is correlated with altered root metabolite dynamics (Bourceret et al., 2022; Xiong et al., 2021). Phosphate availability in the soil induces changes in root metabolism which correlates with shifts in the microbiota (Bourceret et al., 2022). While being an important agricultural crop, maize depends on nitrogen fertilization (Edmonds et al., 2013). Modern maize lines recruited fewer microbial taxa belonging to specific N-cycling functional groups thus reducing the capacity of the microbiome to sustain nitrogen uptake for the plant (Favela et al., 2021, 2021). Further plant genetics have a strong influence on microbiome structure, which is more pronounced in nitrogen-limiting conditions, since N insufficiency in maize affects several plant growth traits including root exudation (Meier et al., 2022). Root-exuded flavones enrich for *Oxalobacteracae* in the rhizosphere and they promote maize growth and nitrogen acquisition via specific development of lateral roots (Yu et al., 2021). Even though these studies investigate many different factors shaping the maize microbiota, common to the above listed examples is that the effects are often related to root exudation patterns. Maize produces different plant specialized metabolites as described above, but it is unclear whether these plant specialized metabolites target specific bacterial taxa or if there are shared taxa responding.

Benzoxazinoids

Production and root exudation

Benzoxazinoids are plant specialized metabolites produced by members of the *Poaceae* family, which includes important monocot crops such as wheat (*Triticum aestivum*), maize (*Zea*

mays) and rye (*Secale cereale*; Frey et al., 2009; Niemeyer, 2009; Wouters et al., 2016). A few dicotyledons species such as *Acanthaceae, Ranunculaceae, Plantaginaceae,* and *Lamiaceae* also produce benzoxazinoids (Frey et al., 2009). Benzoxazinoids are derived from the shikimic acid pathway, and the first step of the biosynthesis is the conversion of indole-3-glycerol-phosphate to indole which is catalysed by the enzyme benzoxazinless1 (BX1) (Frey et al., 1997). DIMBOA-Glc synthesis from indole is catalyzed by eight sequentially functioning enzymes (BX2-BX9). Maize mutants in benzoxazinoid biosynthesis were obtained in several plant lines in the past and today the most often experimentally used mutant is *bx1* (Frey et al., 1997; Hu et al., 2018b; Maag et al., 2016). Homozygous *bx1* mutant lines still produce small amounts of benzoxazinoids (~10%) through the alternative pathway, where the indole-3-glycerol phosphate lyase (IGL) converts indole-3-glycerol to free indole (Köhler et al., 2015; Maag et al., 2016). Especially young maize seedlings produce the high amounts of benzoxazinoids, but shoots and roots produce them throughout plant growth (Dafoe et al., 2011; Zhou et al., 2018). In maize the methoxylated forms of benzoxazinoids dominate, while rye produces non-methoxylated benzoxazinoids and wheat forms a mixture of both (Belz and Hurle, 2005; Frey et al., 2009). Benzoxazinoids are released from the roots into the rhizosphere through exudation or cell debris. DIMBOA-Glc is the main benzoxazinoid exuded by maize roots (for chemical structures see Figure 1 and for complete names see Table 1) (Hu et al., 2018b). Upon release, the glucosides are hydrolysed by glucosidases for which it is unknown if they are derived from microbes or plants. DI(M)BOA aglucons degrade to the more stable (M)BOA, which is a spontaneous reaction (Macías et al., 2004). In sterile soils, MBOA is stable but in natural soil, microbes convert MBOA to reactive aminophenols (M)AP (Kumar et al., 1993; Niemeyer, 2009; Zikmundová et al., 2002). Aminophenols are further converted to three different metabolite classes via three alternative routes. (I) Aminophenoxazinones A(M)PO may form from a spontaneous reaction in presence of oxygen (Guo et al., 2022) and acetylated to AA(M)PO. (II) Acetamides H(M)PAA form by an acetylation reaction from aminophenol and may be further nitrated to N-(2-Hydroxy-5-nitrophenyl)acetamide and N-(2-hydroxy-3-nitrophenyl)acetamide (Zikmundová et al., 2002). (III) Malonic acids H(M)PMA form by an acylation from aminophenol (Friebe et al., 1998; Nair et al., 1990; Schulz et al., 2013; Schütz et al., 2019; Understrup et al., 2005; Zikmundová et al., 2002). (M)BOA is stable in soil for a few days while the corresponding 2-amino phenoxazine-3-ones are detectable for months (Macías et al., 2004). While the biosynthesis and the chemical dynamics in soil are well characterized, the biochemical mechanisms how benzoxazinoids are degraded in the soil are not yet identified.

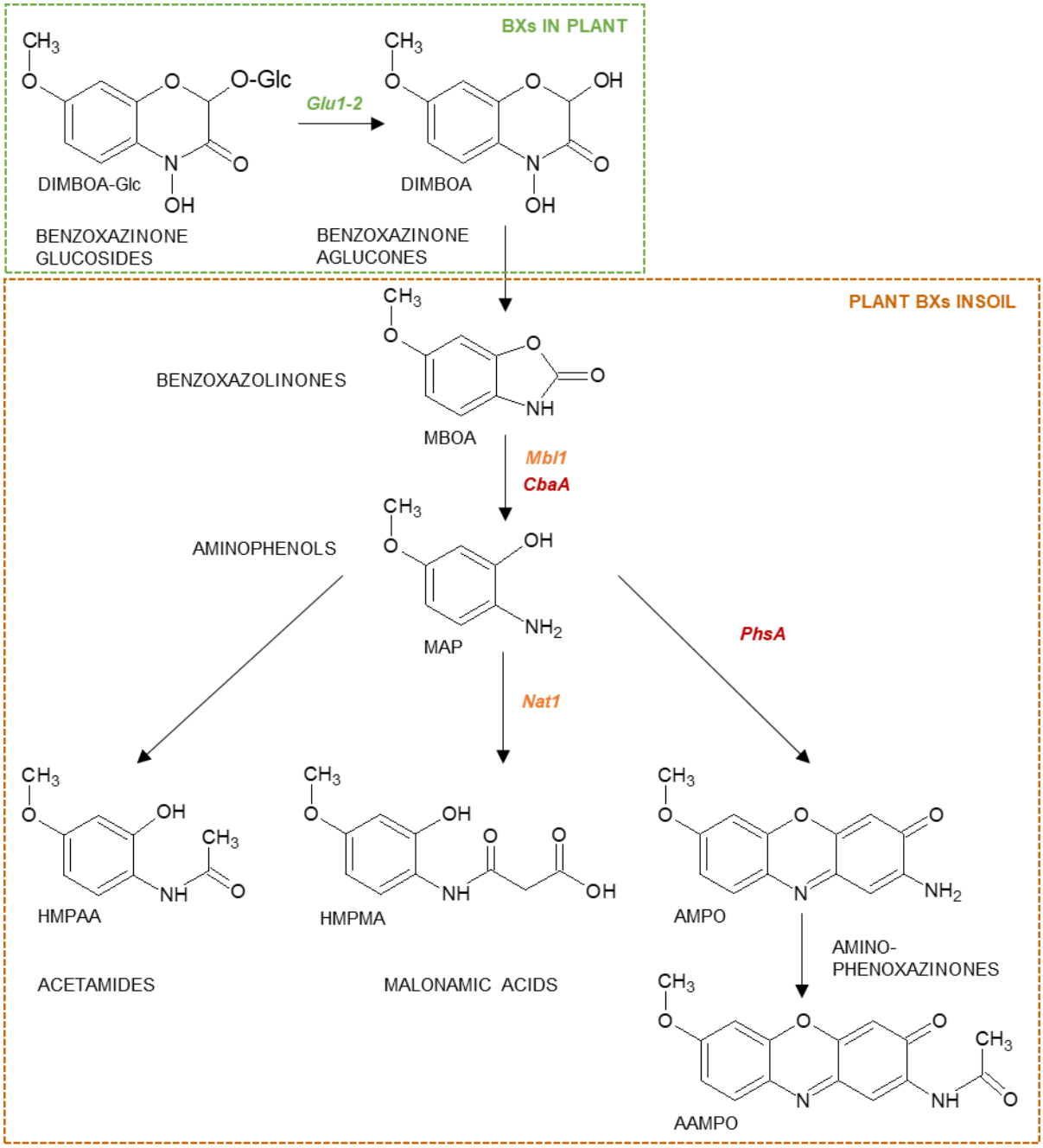

Figure 1: Benzoxazinoid degradation pathways in soil reported in literature. Microbial genes catalysing the conversions are indicated.

Biological functions of benzoxazinoids and aminophenoxazinones

Benzoxazinoids are well-studied multifunctional plant specialized metabolites. On one hand they affect plant metabolism through their functions as phytosiderophores enhancing plant iron uptake (Hu et al., 2018a), by interfering with hormonal pathways and controlling plant defences such as callose deposition in leaves (Ahmad et al., 2011). On the other hand, benzoxazinoids modulate biotic interactions of plants with herbivores, among plants, and with microbes. Leaf herbivory locally induces benzoxazinoid biosynthesis while their metabolic profile in roots is constantly high (Niemeyer, 2009; Robert et al., 2012). Benzoxazinoids have anti-

feeding, anti-digestive or toxic effects against a broad range of insects. The modes of action of benzoxazinoid toxicity is not fully resolved but include neurological effects as well as to the disruption of important metabolic pathways (Wouters et al., 2016). Some specialized insect herbivores like the most damaging insect pest on the planet, the western corn root worm (*Diabrotica virgifera*) circumvent the toxicity of the benzoxazinoids by glycosylating MBOA and accumulating the less toxic MBOA-Glc and HDMBOA-Glc in their body. Interestingly, these sequestered benzoxazinoids render the insect resistant to entomopathogenic nematodes (*Heterorhabtitis bacteriophora*), which are commercially used as biocontrol against the western corn root worm (Robert et al., 2017). Benzoxazinoids also alter nematode abundance by inhibiting most taxa (Sikder et al., 2021).

Root exuded benzoxazinoids are well known to impact plant-plant interactions, specifically by inhibiting the growth of neighbouring plants (Schandry et al., 2020). Especially the microbially formed aminophenoxazinoines are potent allelopathic compounds exceeding the strength of the original precursors (Fomsgaard et al., 2004). Allelopathy can be direct or indirect. Direct allelopathy includes the binding of exuded benzoxazinoids to amino acids, nucleic acids, or metals in the rhizosphere. For example, BOA triggers the production of reactive oxygen species (ROS) in bean (Batish et al., 2006). At cellular level, A(M)PO was found to impair growth of *Arabidopsis thaliana* through inhibiting histone deacetylase (HDAC) activity (Venturelli et al., 2015). Certain plants can detoxify benzoxazinoids such as BOA to malonyl glucoside carbamates (Hofmann et al., 2006). Indirect allelopathy occurs through a reconfiguration of the soil microbiome or altering the nutrient availability in the soil to general resource competition (Robert and Mateo, in press).

Microbes influencing benzoxazinoid dynamics in the soil

As mentioned above, benzoxazinoid metabolisation in the soil depends on microbial activity. In wheat-grown field soil, aminophenoxazinones are the most abundant benzoxazinoid microbial transformation products (Mwendwa et al., 2021). Limited knowledge exists on the mechanism forming aminophenoxazinones in soil except for the need for oxygen and microbial activity. The bacterial isolate *Acinetobacter* sp. was shown to convert BOA to APO *in vitro* (Chase et al., 1991). A more recent study identified a metal-dependent hydrolase CbaA of in the genus *Pigmentiphaga* that catalyses the conversion of 2-chloro-2-benzoxazolinone, a compound related to BOA to 9-chloro-2-amino-2H-phenoxazin-3-one (Dong et al., 2016). Another study showed that supplementation of AP to *Pseudomonas* cultures yielded APO or catechol (Zhao et al., 2000). APO formation was also detected in a co-culture of the fungus *Fusarium verticillioides* with the bacterium *Bacillus mojavensis* when grown on BOA but the underlying biochemical mechanisms were not resolved so far (Bacon et al., 2007). Apart from these bacteria, fungi can also metabolise

benzoxazinoids. As introduced above, *Fusarium verticillioides* converts BOA to HPMA and the enzyme catalyzing these reactions were elucidated (Glenn et al., 2016). Another soil fungus, *Fusarium sambucus* converts BOA to the acetamide HMPAA (Zikmundová et al., 2002). Recently, the bacterial metabolism of DI(M)BOA-Glc, DI(M)BOA and MBOA was studied using liquid cultures of soil bacteria, and the degradation of benzoxazinoids proceeded along the expected sequence of chemical products: Microbes converted first DI(M)BOA-Glc to DI(M)BOA and then further to (M)BOA and A(M)PO. Together these findings prove diverse routes for benzoxazinoid conversions by soil microbes isolated from diverse environments. Together the chemical dynamics of benzoxazinoids in pure bacterial cultures have been studied and key enzymes were identified, however it remains unknown whether root microbiome members of BX-producing host plants can metabolise benzoxazinoids.

Effects of benzoxazinoids on microorganisms

Several benzoxazinoids compounds were reported to have antimicrobial activity. Because of their antimicrobial activity possibly due to their ability to intercalate to DNA (Hashimoto and Koichi, 1996), they are considered as promising chemical lead structures for the development of novel antibiotics that may lead to human medical applications (de Bruijn et al., 2018). For example MBOA was shown to inhibit *Pantoea stewartii*, the bacterium causing Stewart's wilt (Whitney et al., 1961). Further DIMBOA, DIBOA, MBOA and BOA inhibit the human pathogens *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (Bravo et al., 1997). Also the aminophenoxazinone APO inhibits *Helicobacter pylori* (Hanawa et al., 2010), some *Mycobacterium* spp. (Shimizu et al., 2004) and *Chlamydia pneumoniae* (Uruma et al., 2005) *Arabidopsis* root bacteria *in vitro* (Schandry et al., 2021). Besides bacteria, *in vitro* tests revealed that DIMBOA-Glc, DIMBOA and HDMBOA-glc also inhibit the growth of fungal pathogens (Ahmad et al., 2011; Couture et al., 1971; Niemeyer, 2009). The fungal endophyte in maize seeds, *Fusarium verticillioides*, overcomes benzoxazinoid toxicity by detoxifying BOA to HMPMA. Genes encoding this enzyme were horizontally transferred to other fungal species. This indicates that plant specialized metabolites exert evolutionary pressure on these fungi (Glenn et al., 2016). Apart from their antimicrobial activity, DIMBOA-Glc triggers a chemotactic attraction of the beneficial rhizobacterium *Pseudomonas putida* to the maize rhizosphere (Neal et al., 2012). HDMBOA-Glc inhibits the virulence of the pathogenic *Agrobacterium tumefaciens* (Maresh et al., 2006). Using an experimental evolution experiment with bacteria from the genus *Photorhabdus*, the endosymbiotic bacterium of entomopathogenic nematodes, it was shown that MBOA tolerance, relies on several mechanisms. One of them is mediated by an aquaporin-like channel gene *aqpZ* (Machado et al., 2020). Although it is apparent that benzoxazinoids directly affect bacterial

growth of single isolates, it is unknown if their antimicrobial activity has a role in shaping root microbiomes.

Benzoxazinoids structure maize root microbiomes

Exuded from maize roots, benzoxazinoids structure the root-associated microbial communities of maize grown in natural soil (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019). In presence of benzoxazinoids a few taxonomic groups like the *Methylophilacae* have been found to positively respond to benzoxazinoids (Cotton et al., 2019) while some bacterial families were systematically depleted, namely the *Flavobacteraceae* and *Comamonadaceae* (Cadot et al., 2021a) and in another study *Xanthomonadaceae* (Kudjordjie et al., 2019). Further it was shown that APO shapes synthetic communities composed of *Arabidopsis* root bacteria grown *in vitro.* Interestingly, isolates belonging to *Flavobacteraceae* and *Comamonadaceae* were depleted in APO treated communities (Schandry et al., 2021). Further in a recent study was shown that supplementing natural soils with BOA also structures the soil microbial community (Schütz et al., 2021). Even though many effects of benzoxazinoids on bacteria were described, the mechanisms underlying the structuring of microbial communities by benzoxazinoids are complex and not yet fully uncovered.

Microbiomics

The study of microbiome composition and functioning functions via culture-dependent and culture independent approaches. Traditionally microbiome research was based on culture-dependent approaches until the development of next generation sequencing technologies made it possible to characterize und improve the understanding of plant microbiomes (Fitzpatrick et al. 2020). Current state-of-the-art is to combine both culture-independent and culture-dependent methods to assess microbial functioning on strain and community level. The cultivation of microbes is needed for functional studies and manipulative experiments. Sequencing experiments are descriptive but important to build hypothesis (Vorholt et al., 2017). Methods used to study microbiomes either focus on the community level or on the strain level (Table 1). To investigate the composition of microbiomes, culture-dependent methods such as microscopy or culture independent methods like metagenomics of marker genes can be employed. The latter method can be extended to full genomes (metagenomics) and will give a further insight to the metabolic and genetic potential of a community based on the genetic material. To elucidate microbial function on a community level, methods based on the active metabolic pathways such as metatranscriptomics, metaproteomics and metabolomics are employed (Berg et al., 2020). For functional understanding of microbiomes, ecological assays testing for specific traits of microbiomes can be performed. One option is to perform plant-soil feedback experiments, where the function of a conditioned microbiome is tested by assessing the growth of a plant on a

conditioned soil (Hu et al., 2018b). On the strain level, strains need to be cultivated and phenotyped in large scale to assess their potential. The genetic basis encoding for these functions can then be identified by comparative genomics. Comparative genomics requires strong phenotypes and a dataset of closely related strains with a heterogenous distribution of phenotypes (Sheppard et al., 2018). To assess microbial function, single strains can be exposed to certain treatments and then be subjected to transcriptomics, proteomics and metabolomics (Berg et al., 2020). For the functional characterization of genetic mechanisms, the identified genes can be knocked out in natural strains, or the genes can heterologously expressed in non-host strains (Song et al., 2021). Using natural strains with a specific knock-outs of a selected gene, simple bioassays and reconstitution experiments can be performed to investigate the function of certain genes for their role in a microbial community or for the host (O'Banion et al., 2020).

Table 1: Overview methods used to investigate microbiomes are focused on composition and function on community level and on strain level based on cellular material, genetic material or active mechanisms.

Community level	*Strain level*
WHO?	
Microbial potential based on cellular material	
• Microscopy	• Culturomics • Phenotyping strains
WHAT?	
Metabolic and genetic potential based on genetic material	
• Metagenomics • Marker genes / Complete genomes	• Comparative genomics
Microbial function based on active metabolic pathways	
• Metatranscriptomics • Metaproteomics • Metabolomics	• Transcriptomics • Proteomics • Metabolomics
WHY?	
Ecological function based on active mechanisms	
• Ecological assays for microbiome function	• Reconstitution experiments
Genetic basis based on active mechanisms	
	• Mutants • Purified protein assays

Microbiome decomposition for reconstruction

To study functions of microbiomes and mechanisms of microbe-microbe interactions and microbe-host interactions beyond next-generation sequencing technologies, reductionist systems were developed (Fig. 2). First microbiomes from a given environment are deconstructed by isolating single members to establish strain collections. Then the systems microbes are introduced to a reductionist system as single strains or synthetic microbial communities

(SynCom). SynComs are composed by a defined mix of a strains from a strain collection (Vorholt et al., 2017). This approach allows the detailed study of microbiome components under controlled conditions and permits the establishment of causal links between phenotypes and genotypes. Although, these reduced systems do not accurately represent nature, they allow the replication of phenotypes mediated by the microbiome (Vorholt et al., 2017). SynComs approaches are widely applied to disentangle mechanisms in plant-microbiome assembly (Song et al., 2021). To remove one level of complexity it is also possible to grow the microbial consortia on agar plates (Berendsen et al., 2018) or in liquid cultures (Schandry et al., 2021) to study molecular mechanisms in microbial interactions in different conditions. Understanding plant-microbe interactions at different levels of complexity is a crucial gap that needs to be filled to identify mechanisms that control the assembly of the host-associated microbiomes.

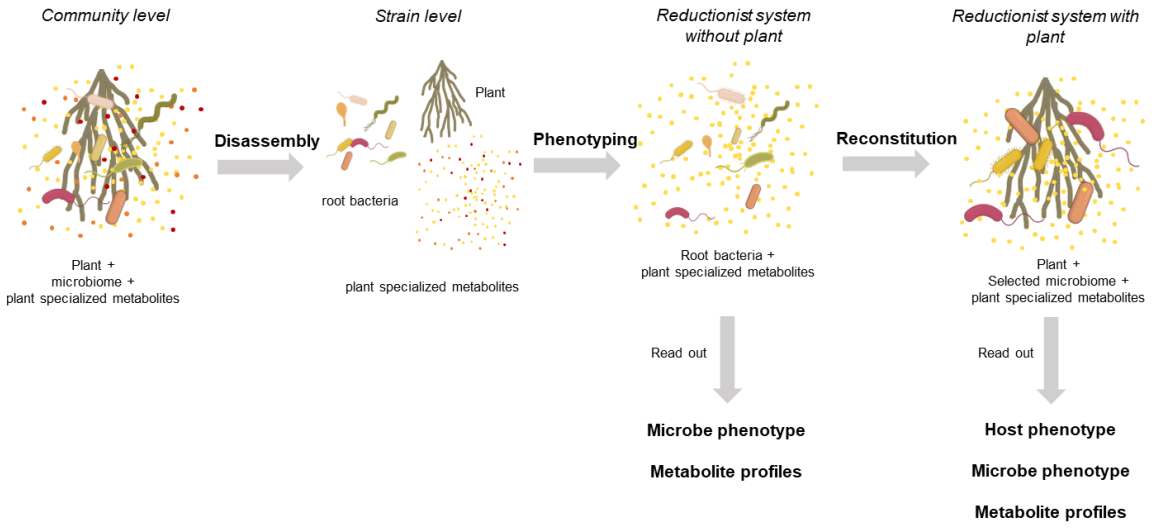

Figure 2: Decomposition and reconstruction of plant microbiomes. On community level, natural microbiome live in association with the plants which produce plant specialized metabolites. Strain collections are isolated from plants and characterized, then bacteria are tested in pure culture in purified plant specialized metabolites. Based on the research questions, bacteria are tested in pure culture or as synthetic communities on their effect on plant growth in axenic plant systems. Adapted from Vorholt et al. 2017, Fig. 2

Strain collections and SynComs

A collection of bacterial strains from a specific environment are prerequisite to study microbiomes in reductionist systems. They are limited to cultivable microbes, however as technology advances, more cultivation techniques are developed and enable the cultivation of more microbes. The culturable fraction of the total bacterial community in the rhizosphere is about 40%–70% whereas in soil it is much lower with 0.1%–1.0% (Dazzo et al., 2019). Having microbes in cultures makes it possible to screen for desired phenotypes of single strains. The microbes must be isolated and cultured from root samples of plants grown in the same environment (e.g. the same soil type) to study the functions of the root microbiota in a particular

environment. Microbes adapt genetically and functionally to specific habitats and hosts and thus differentiate. Therefore, new research questions require the establishment of new culture collections and are the basis for reductionist experiments. Several comprehensive culture collections were established for plant species including *Arabidopsis thaliana* (Bai et al., 2015) , clover (Hartman et al., 2017), rice (Zhang et al., 2019), *Lotus japonicus* (Wippel et al., 2021) and maize (Beirinckx et al., 2020; Niu et al., 2017). New high-throughput approaches using limiting dilutions to facilitate the establishment of strain collections are developed (Zhang et al., 2021).

Synthetic communities

Studying single strains gives limited insights into possible functions in natural systems, since interactions within the microbial community modulates their functions. In community context microbes can complement each other or can cooperate, both mechanisms contributing to community traits not seen in mono-associations. The current state-of-the-art to study mechanisms governed by microbial communities is the use of reduced and rationally composed SynComs. They may be selected for taxonomic composition or due to their individual functions. Resulting SynComs may be either tested for their ability to respond to certain factors, e. g. the tolerance to certain compounds or their ability to metabolise compounds or SynComs may be applied to axenic plant systems to test their effect on plant phenotypes (Vorholt et al., 2017). The big advantage of the synthetic community approach is that organisms can be added, eliminated, or substituted at the strain level and their function can be manipulated directly (O'Banion et al., 2020). The consequences of perturbations can be monitored in the SynComs at different levels and thus enables the exploration of the role of individual organisms in the community. Explorative experiments can be initially performed with larger SynComs, reflecting higher complexities of communities, followed reduced approaches to narrow down interesting phenotypes or the combination of strains required for this phenotype can be tested. Either certain strains can be removed from communities and drop-out communities can be tested if they lose a phenotype or pairs of strains can be tested for certain phenotypes (Fitzpatrick et al., 2020; Liu et al., 2018). Recently the host-specific assembly of root microbiomes in *Arabidopsis thaliana* and *Lotus japonicus* was tested with SynComs (Wippel et al., 2021). To investigate the role of plant specialized metabolites exuded by *Arabidopsis* on the root microbiome was studied by inoculating SynComs to Arabidopsis plants and respective coumarin mutant lines (Voges et al., 2019). These examples demonstrate that a reductionist approach helps to identify the mechanisms of how plant specialized metabolites can structure microbiomes.

High-throughput in *vitro* systems

Since microbiomes are extremely diverse and are composed of thousands of microbes, comprehensive strain collections also compromise a high number of isolates. Thus high-

throughput phenotyping systems have been developed. In these systems, bacteria are grown in liquid cultures in multiple multi-well plates in a robot which can handle up to 25 plates at once. Bacterial growth is monitored regularly over the growth phase by optical density in a spectrophotometer, then the various growth parameters can be calculated (Maier et al., 2018; Schandry et al., 2021). One phenotype which is suitable to screen in this system is the growth of microbes in different chemical compounds and investigating whether they inhibit or promote the growth of microbes. Such a high-throughput system has been employed to screen the response of gut microbes to antibiotics (Maier et al., 2018) and was adopted to screen a collection of *Arabidopsis* root microbes on their response to benzoxazinoids (Schandry et al., 2021). This system can also be used to screen for metabolisation of certain compounds by microbes. These high-throughput screening systems allow to screen many strains in a relatively short time and in a cost-efficient way yielding a lot of data for many strains (Maier et al., 2018). These phenotypic data collected are good resource to study the genetic basis of certain traits in bacteria by comparative genomics. For comparative genomics to work efficiently and yield good results, having a collection of closely related strains is a key perquisite. Having a big collection of strains which are closely related but show heterogeneity in a desired trait makes it possible to efficiently predict genetic features responsible for certain traits (Sheppard et al., 2018). One approach to find genetic features responsible for certain phenotypes is the orthogroup approach (Emms and Kelly, 2019). It compares the genomes of strains where a phenotype is present with the genomes of strains lacking the phenotypes and identify orthologous groups of genes present in the positive genomes (Emms and Kelly, 2019). This approach was successfully employed to identify key symbiosis genes in Rhizobia (Garrido-Oter et al., 2018). Another approach is to study the transcriptome of a given microbe in a given environment, either in pure culture or when grown in presence of the host comparing two conditions (Westermann and Vogel, 2021). Comparative transcriptomics were used for example to identify the enzymes involved responsible for the degradation of halogenated aromatic compounds by bacteria (K. Chen et al., 2018). The combination of high-throughput *in vitro* phenotyping with comparative genomics and transcriptomics is thus a promising approach to unravel the genetic basis responses of bacterial plant microbiome members to plant specialized metabolites.

Axenic plant growth systems

To demonstrate the effects of microbes on the plant host, a microbe-fee reference is needed to compare to the effect of inoculated microbes. This requires the possibility to grow the plant in an axenic growth system. For *Arabidopsis* agar-based plat and peat-based gnotobiotic plant growth systems have been developed, which enable to investigate mechanisms underlying plant growth promotion, microbial community establishment, indirect pathogen protection or

mineral nutrition of the host (Kremer et al., 2021; Ma et al., 2022). For maize plant growth systems to study the effect of single microbes or synthetic microbial communities were described, too. Maize seedlings have been grown in agar with a synthetic community isolated from soil (Niu et al., 2017). In another study, maize seedlings were grown in sterilized perlite (Wagner et al., 2021). However, these systems are limited to a short growth period of due to size restrictions of maize.

Aims of this study

The motivation of this study was to investigate the mechanisms through which the benzoxazinoids structure the maize root microbiomes. Maize is an ideal study system since it is a well-studied crop, has multifunctional plant specialized metabolites of which the biosynthetic pathway is well studied, and mutants are available. As introduced above, bottom-up experimental approaches give the opportunity to study the direct effects of benzoxazinoids on maize root microbiome members. Further it allows the identification of the genetic basis of traits by genomics. The big advantage of the use of root bacteria isolated from benzoxazinoid producing roots is that they specifically adapted to the host secondary metabolites, and they evolved mechanisms to cope with the benzoxazinoids. Possible mechanisms to dealing with benzoxazinoids is (i) to tolerate them, (ii) to fully degrade them and utilizing these molecules as carbon source and (iii) that it is metabolised to other compounds which are less toxic or can inhibit the growth of other microbiome members. Furthermore, the compounds may either be metabolised alone or through cooperation with other members of the maize root microbiome members.

In chapter 1 we established a collection of maize root bacteria from benzoxazinoid producing roots. We tested this strain collection in a high-throughput *in vitro* growth system on tolerance to different benzoxazinoids. To investigate the ability of maize root bacteria to metabolise benzoxazinoids, we performed targeted metabolomics of pure cultures and screened for benzoxazinoid metabolisation using a simple plate assay (chapter 2). To identify the genetic basis of benzoxazinoid metabolisation by maize root bacteria we applied comparative genomics combined with transcriptomic experiments to identify the key enzyme in MBOA metabolisation. To confirm the function of the identified genetic locus, we performed heterologous expression of the candidate enzyme in a non-host bacterium and based on in vitro protein assay (chapter 2). We made use of the synthetic community approach to study the effect of benzoxazinoid metabolisation and tolerance of single strains in controlling community size, structuring communities, and microbial cooperation in benzoxazinoid metabolisation (chapter 3).

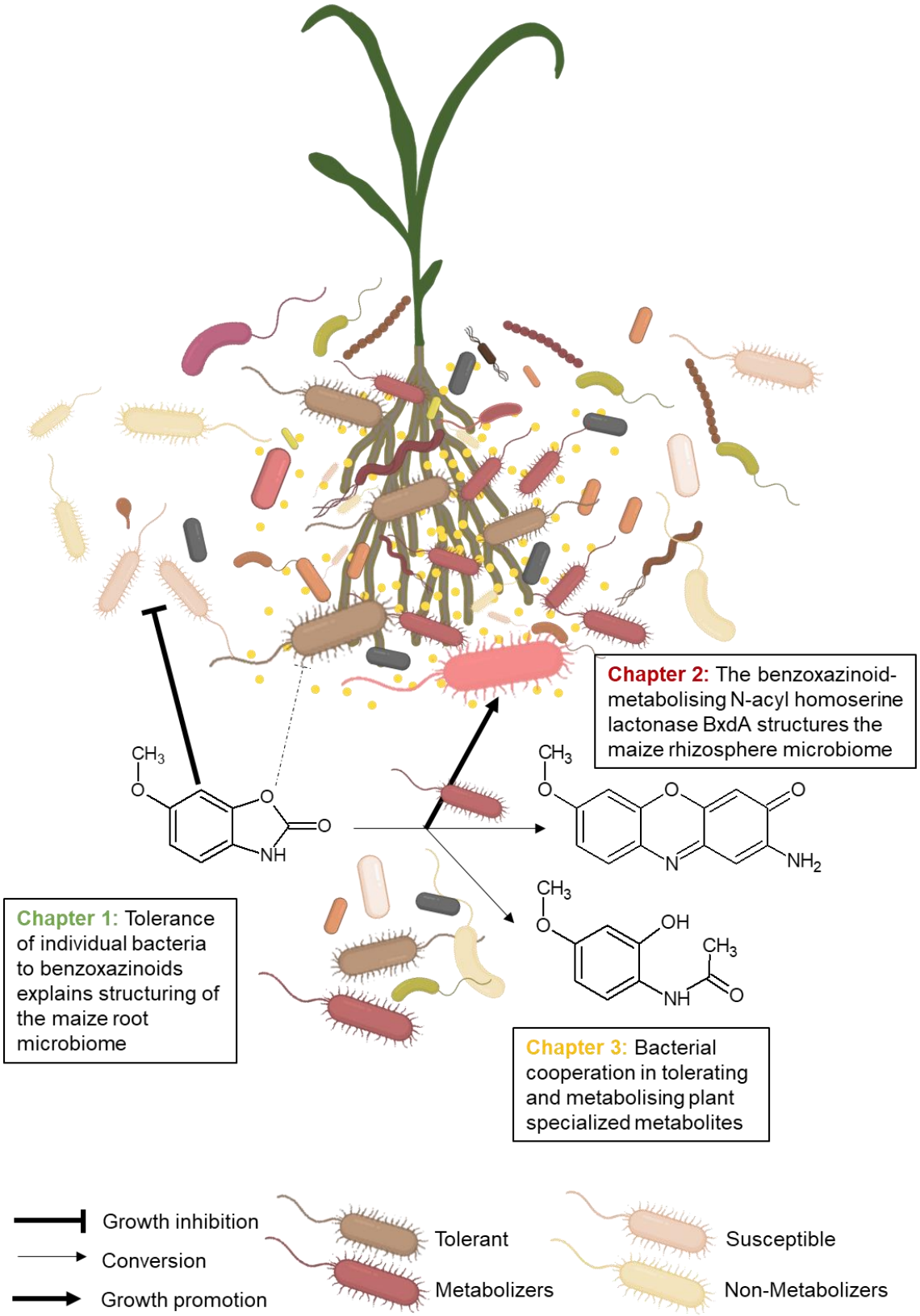

Figure 3: Thesis Outline illustrating benzoxazinoid tolerance and benzoxazinoid metabolisation of single strains and bacterial cooperation as mechanisms of how maize root microbiome members cope with benzoxazinoids.

Table 1: Abbreviation benzoxazinoid metabolites

Abbreviation	**Full name**	**Class**	**Mass** [g/mol]	**Formula**
HMPAA	N-(2-hydroxy-4-methoxyphenyl) acetamide	Acetamides	181.19	C9H11NO3
HPAA	N-(2-hydroxyphenyl) acetamide	Acetamides	151.16	C8H9NO2
AP	2-aminophenol	Aminophenols	109.13	C6H7NO
MAP	2-amino-5-methoxyphenol	Aminophenols	139.15	C7H9NO2
AAMPO	2-acetylamino-7-methoxy-phenoxazin-3-one	Aminophenoxazine	284.27	C15H12N2O4
AAPO	2-acetylamino-3H-phenoxazin-3-one	Aminophenoxazine	254.24	C14H10N2O3
AMPO	2-amino-7-methoxy-phenoxazin-3-one	Aminophenoxazine	242.23	C13H10N2O3
APO	2-amino-3H-phenoxazin-3-one	Aminophenoxazine	212.21	C12H8N2O2
NHAAPO	2- (N-hydroxy) acetylamino 3H-phenoxazin-3-one	Aminophenoxazine	270.24	C14H10N2O4
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one	Benzoxazinone	181.15	C8H7NO4
DIM2BOA	2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one	Benzoxazinone	241.20	C10H11NO6
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one	Benzoxazinone	211.17	C9H9NO5
HDMBOA	2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one	Benzoxazinone	225.20	C10H11NO5
HMBOA	2-Hydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one	Benzoxazinone	195.17	C9H9NO4
NMBOA	6-methoxy-4-nitro-benzoxazolin-2(3H)-one	Benzoxazinone	210.14	C8H6N2O5
DIBOA-Glc	4-hydroxy-2-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxy-1,4-benzoxazin-3-one	Benzoxazinone glucoside	343.29	C14H17NO9
DIM2BOA-Glc	2-(2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one)-b-D-glucopyranose	Benzoxazinone glucoside	403.34	C16H21NO11
DIMBOA-Glc	4-hydroxy-7-methoxy-2-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxy-1,4-benzoxazin-3-one	Benzoxazinone glucoside	373.31	C15H19NO10
HMBOA-Glc	2-O-Glucosyl-7-methoxy-1,4(2H)-benzoxazin-3-one	Benzoxazinone glucoside	357.31	C15H19NO9
BOA	benzoxazolin-2(3H)-one	Benzoxazolinone	135.12	C7H5NO2
HBOA	2-Hydroxy-3,4-dihydro-2H-1,4-benzoxazin-3-one	Benzoxazolinone	165.15	C8H7NO3
MBOA	6-methoxybenzoxazolin-2(3H)-one	Benzoxazolinone	165.15	C8H7NO3
CHBT	6-chloro-2-benzoxazolinone	Benzoxazolinone derivate	169.56	C7H4ClNO2
HDM2BOA-Glc	2-(2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one)-β-d-glucopyranose	Benzoxazolinone glucoside	417.4	C17H23NO11
HDMBOA-Glc	4,7-dimethoxy-2-{[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxy}-3,4-dihydro-2H-1,4-benzoxazin-3-one	Benzoxazolinone glucoside	387.34	C16H21NO10
MBOA-Glc	3-β-D-Glucopyranosyl-6-methoxy-2-benzoxazolinone	Benzoxazolinone glucoside	195.17	C14H18NO8
HMPMA	N-[2-hydroxy-4-methoxyphenyl] malonamic acid	Malonamic acids	225.20	C10H11NO5
HPMA	N-[2-hydroxyphenyl] malonamic acid	Malonamic acids	195.17	C9H9NO4
NBOA-6-OH	6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one	NA	196.12	C7H4N2O5

Chapter 1

Tolerance of individual bacteria to benzoxazinoids explains structuring of the maize root microbiome

Unpublished work

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Abstract

Plant specialized metabolites exuded by roots can structure root-associated microbial communities. However, the underlying mechanisms are poorly understood. We established a strain collection of maize root bacteria to test the tolerance of individual strains to benzoxazinoids and assess to what extent strain-level tolerance can explain community restructuring. We find that *in vitro*, benzoxazinoids inhibit bacterial growth in a strain-dependent and compound-dependent manner. Bacterial tolerance to the benzoxazinoid breakdown product MBOA explains benzoxazinoid-dependent colonization of corresponding taxonomic units on maize roots in the field. In particular, MBOA-tolerant strains are more abundant on benzoxazinoid-producing roots and rhizosphere than MBOA-susceptible strains. Thus, benzoxazinoids structure maize root microbiota by acting as selective antimicrobials. Conversely, tolerance to plant specialized metabolites can increase root colonization of adapted taxa. We propose that tolerance to secreted antimicrobial compounds is an important mechanism that determines microbial community composition in the field.

Introduction

Plant roots are colonized by diverse microorganisms, including bacteria, fungi, oomycetes, and protists (Bulgarelli et al., 2013). Root-associated microbial communities or single microbes provide several benefits to their host plants. They can improve plant growth through the production of plant hormones (Lugtenberg and Kamilova, 2009), improve plant nutrient uptake (Fabiańska et al., 2019; Tao et al., 2019; van der Heijden et al., 2015), and protect plants against pathogens (Durán et al., 2018). Root microbial communities are mainly recruited from soil and thus its composition resemble on the surrounding soil microbiome (Bulgarelli et al., 2013; Hacquard et al., 2015). Plants shape the composition of their root microbial communities by plant morphological traits (Rodriguez et al., 2019; Yu et al., 2021), plant immune responses (Teixeira et al., 2019) and the exudation of a diverse cocktail of chemicals (Sasse et al., 2018). Through this root exudation, plants release up to 25% of their assimilated carbon to the surrounding soil (Massalha et al., 2017; van Dam and Bouwmeester, 2016). Since they serve the microbes as growth substrates (Huang et al., 2019; Zhalnina et al., 2018) they attract them to the rhizosphere. Root exudates contain primary metabolites such as sugars, amino acids, or organic acids, phytohormones, and plant specialized metabolites.

Plant specialized metabolites are important govern interactions of plants with the environment (Erb and Kliebenstein, 2020). Plant specialized metabolites were shown to shape the root-associated microbial communities (Jacoby et al., 2020b; Lareen et al., 2016; Sasse et al., 2018). Often, studies compare the microbial community composition on roots or rhizospheres of wild-type plants with the respective biosynthesis mutants defective in the production of specific secondary metabolites. Specifically glucosinolates (Kudjordjie et al., 2021), camalexin (Koprivova et al., 2019), triterpenes (Huang et al., 2019), and coumarins (Harbort et al., 2020; Stringlis et al., 2018; Voges et al., 2019) can structure microbial community composition of the model plant *Arabidopsis thaliana*. Similarly, secondary metabolites were shown to structure root communities for the important staple crop maize (*Zea mays*), namely benzoxazinoids (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019; Schütz et al., 2021), diterpenoids (Murphy et al., 2021), zealexins (Ding et al., 2020) and flavonoids (Yu et al., 2021). While it becomes evident that specialized plant metabolites present key drivers for community assembly, the underlying mechanisms remain largely unknown.

Benzoxazinoids (BXs) are specialized compounds produced by *Poaceae,* including important crops such as maize, wheat and rye (Frey et al., 2009; Niemeyer, 2009). Benzoxazinoids are alkaloids derived from indole and are especially abundant in young seedlings where they account for up to 1% of plant dry weight (Dafoe et al., 2011; Glauser et al., 2011; Köhler et al., 2015; Zhou et al., 2018). Benzoxazinoids are multifunctional compounds that, in addition to

structuring root and rhizosphere microbiomes, have well known defence activities against insect pests (Dafoe et al., 2011; Niemeyer, 2009; Robert et al., 2012; Zhou et al., 2018) and pathogens (Couture et al., 1971; Niemeyer, 2009), defence signalling functions (Ahmad et al., 2011; Meihls et al., 2013) and act as phytosiderophore improving plant nutrition through chelation of iron (Hu et al., 2018a).

The main benzoxazinoid, which is exuded form maize roots to the surrounding rhizosphere, DIMBOA-Glc (Hu et al., 2018b). After deglycosylation to DIMBOA, a rapid conversion to MBOA takes place in the rhizosphere and stays stable for days (Macías et al., 2004). In soil, a more stable benzoxazinoid metabolisation product AMPO accumulates that remains detectable in soil for months (Kumar et al., 1993; Macías et al., 2004), but this conversion requires microbial activity in the soil. Such aminophenoxazinones were shown to suppress the growth of neighbouring plants, indicating they have an allelopathic function (Macías et al., 2004; Niemeyer, 2009; Schulz et al., 2013; Venturelli et al., 2015). While maize primarily produces methoxylated benzoxazinoids, other Poaceae species like rye or barley produce non-methoxylated analogues like DIBOA-Glc (Belz and Hurle, 2005; Frey et al., 2009), which are deglycosylated and then converted to BOA and the aminophenoxazinone APO in soil (Macías et al., 2004). To which extent, which benzoxazinoid compound impacts members of bacterial communities, remains to be investigated.

The benzoxazinoid degradation product MBOA was identified as a dominant metabolite in soils where benzoxazinoid-producing maize plants grew and where rhizosphere structuring by benzoxazinoids was observed (Hu et al., 2018b). Therefore, it is possible that direct effects of MBOA on maize rhizosphere bacteria play an important role in the observed mechanisms. Relatively little is known about how root and rhizosphere bacteria can cope with toxic benzoxazinoids in root exudates of their host plant. The first evidence was gained from tests with a few selected microbes where pure benzoxazinoid compounds like MBOA, were shown to inhibit bacterial growth (Bravo et al., 1997; Machado et al., 2020; Neal et al., 2012; Schandry et al., 2021). Besides growth inhibition, the range of biological activities include chemotactic attraction, e.g., of the beneficial rhizobacteria *Pseudomonas putida* (DIMBOA) (Neal et al., 2012) or the manipulation of virulence of the pathogenic bacterium *Agrobacterium tumefaciens* (HDMBOA) (Maresh et al., 2006). A few bacteria were also shown to have the ability to convert benzoxazinoids to different degradation products (Schulz et al., 2018; Schütz et al., 2019). Tolerance to the structurally similar non-methoxylated benzoxazinoids BOA and APO varies across a collection of root bacteria. Using a high-throughput phenotyping platform, they screened 180 taxonomically diverse collection of bacteria isolated from *Arabidopsis* roots (Schandry et al., 2021). Tolerance to MBOA in the genus *Photorhabdus*, the endosymbiotic bacterium of

entomopathogenic nematodes, was studied using experimental evolution. It was shown that MBOA tolerance, relies on several mechanisms, one of them is mediated by an aquaporin-like channel gene *aqpZ* (Machado et al., 2020). Although it is apparent that benzoxazinoids directly affect bacterial physiology in a variety of ways, the mechanisms that control responses to benzoxazinoids in communities are still largely unknown.

A powerful approach to study functions and molecular mechanisms in microbiomes is to combine culture-independent (amplicon sequencing) with culture-dependent methods based on strain collections (Vorholt et al., 2017). Several comprehensive culture collections were established for plant species including *Arabidopsis thaliana* (Bai et al., 2015) , clover (Hartman et al., 2017), rice (Zhang et al., 2019), *Lotus japonicus* (Wippel et al., 2021) and maize (Beirinckx et al., 2020; Niu et al., 2017). The numerous successful examples of using reductionist approaches prompted us to build our own culture collection of maize root bacteria to extend our present work (Cadot et al., 2021b; Hu et al., 2018b) for more in-depth mechanistic studies for instance how benzoxazinoids structure the maize root microbiota. It is known that benzoxazinoids have different effects on single bacterial strains, but the differential effects of benzoxazinoids on different community members remain largely unknown. To fill this knowledge gap and to investigate the mechanisms steering shaping of maize root microbial communities by benzoxazinoids, we sought to test single root microbiome members on benzoxazinoid tolerance. Different levels of tolerances of the strains to benzoxazinoids and differential levels of tolerances to the different benzoxazinoids may explain their abundance on BX-producing maize roots. Further we thought to test whether benzoxazinoids may not only shape root microbial communities but also control the total community size on roots. These results would help to understand the mechanisms of plant secondary metabolite mediated shaping of microbial communities on roots.

Given that benzoxazinoid exudation results in an accumulation of MBOA around plant roots (Hu et al., 2018b), that benzoxazinoids generally function to structure the maize root and rhizosphere microbial communities (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019) and that a broad range of tolerances of diverse bacteria to benzoxazinoids were found (Schandry et al., 2021), we hypothesized that the tolerance of individual bacteria to benzoxazinoid compounds like MBOA explains at least in part the BX-dependent microbiome structure on maize roots. To test this hypothesis, we established a strain collection of maize root bacteria, screened them for tolerances against pure benzoxazinoids (DIMBOA-Glc, MBOA) and the aminophenoxazinone AMPO. Among the 50 isolates tested, we found a broad gradient of tolerance to MBOA without a strong phylogenetic signal. Mapping these strains to root microbiome profiles, revealed that tolerance to MBOA largely explained their BX-dependent

abundance on maize roots. Together these findings demonstrate that the antimicrobial activity of benzoxazinoids is an important trait for the plant to select a beneficial root microbiome.

Materials and Methods

Establishment of culture collection

The culture collection of maize root bacteria (MRB) was built with strains isolated in five independent isolation events (Table S1). Most strains were isolated from wild-type maize plants (inbreed line B73) in greenhouse pot experiments with Changins soil (Table S3), i.e. the same soil where we first demonstrated the microbiome structuring activity of benzoxazinoids (Hu et al., 2018b). A small subset of strains were isolated from BX-deficient *bx1* (B73) plants (isolation event 3). Most strains were isolated from 'dirty roots' where the loose soil was shaken off and 10 cm long root fragments (corresponding to the depth of -1 to -11 cm in the soil) including the firmly attached rhizosphere soil were harvested (marked as "root_rhizo" in Table S1) and prepared as root extracts as described above. For plating, the root, rhizosphere, and soil extracts which were prepared as described above were diluted from $1:10^{-3}$ to $1:10^{-6}$ in 10 mM $MgCl_2$ depending on the isolation event and the isolation medium and 50 µl was spread with a delta cell spreader (Sigma-Aldrich, St. Louis, USA) on square agar plates (12 x 12 cm, Greiner bio-one, Kremsmünster, Austria) containing different media with a final density of 100-300 CFU per plate. Strains were isolated on Flour medium (6 g/l Starch, 0.3 g/l Yeast extract, 0.3 g/l Sucrose, 18 g/l Agar, 10 mg/ml Cycloheximide), Pseudomonas agar (Difco, Le pont de Claix, France) or 10% TSB supplemented with MBOA (200 mg/l; Sigma-Aldrich, St. Louis, USA) or DMSO (2 ml/l; Sigma-Aldrich, St. Louis, USA). All media contained the fungistatic cycloheximide (10 mg/l; Sigma-Aldrich, St. Louis, USA). The plates were incubated at room temperature (22 - 24 °C) for 5-10 days, single colonies were picked and repeatedly re-streaked on full-strength TSB or LB media until the bacterial isolates yielded visibly pure colonies.

Bacterial cultures and media

Bacterial strains were routinely sub-cultured at 25 °C – 28°C in tryptic soy broth (TSB, Sigma-Aldrich, St. Louis, USA) or Luria-Bertani medium (LB, Carl Roth, Karlsruhe, Germany) liquid or solid medium amended with 15 g/l agar (Sigma-Aldrich, St. Louis, USA). For cryopreservation, single colonies were inoculated in full-strength liquid TSB or LB medium, grown for two days at 28°C with 180 rpm shaking, and then mixed with the same volume of 40% sterile glycerol (Sigma-Aldrich, St. Louis, USA) in single screw cap microtubes (Sarstedt, Nürnbrecht, Germany). The resulting 20% glycerol stocks were slowly frozen down and stored at -80°C.

The same liquid cultures, of which the glycerol stocks were prepared, were used for sequence-based isolate identification using the 16S rRNA gene. Liquid cultures were diluted 1:10 or 1:100 in sterile water and used as a DNA template for the PCR. The PCR reactions were set up as follows: 15 µl sterile water, 15 µl 2x DreamTaq buffer (Thermo Fisher Scientific, Waltham,

USA), 1.5 µl of each primer (stock concentration 10 µM, 27f and 1492r; sequences in Table S5) and 2 µl of the diluted liquid culture as DNA template. For some bacteria, the DNA was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, USA). PCR was performed in a Biometra T-advanced cycler according to the following program: 95°C for 3 min, 30 cycles with 95°C for 15 s, 55°C for 15 s and 72°C for 45 s followed by final elongation at 72°C for 5 min. PCR products were verified on an agarose gel (1 %, Sigma-Aldrich, St. Louis, USA) and sent for Sanger sequencing with the primers 1492r and/or 27f (Microsynth, Balgach, Switzerland). Sanger sequences were blasted against the NCBI database (National Center for Biotechnology Information, Rockville Pike, USA) for species identification. All metadata, sequences, and taxonomies of the maize root bacteria culture collection are listed in Table S1 and the strain collection maintained in the laboratory of the authors.

Mapping maize root bacteria isolates to microbiome profiles

We mapped the 16S rRNA gene sequences of our maize root bacteria strains obtained by Sanger sequencing to the 16S rRNA gene sequences of published maize root community profiles (i.e., to the OTUs or ASVs of these microbiota datasets) for three purposes. First, to investigate how abundant community members corresponding to our maize root bacteria strains are in the microbiota profiles of the maize roots from where the maize root bacteria strains were isolated from. Second, to detect community members, which correspond to our maize root bacteria strains, in root microbiota profiles of maize grown in the field and in other soils and quantify their abundance. Third, to determine difference in the abundance of maize root bacteria in the root bacteria profiles of BX-producing vs BX-deficient plants. For the first purpose, we mapped the maize root bacteria strains to the ASV data of the feedback experiment described in Hu et al. 2018. For the second purpose, the sequences of the maize root bacteria (Table S1) were mapped against the ASVs datasets of the field experiment of Hu et al. 2018, Cotton et al. 2019 and Cadot et al. 2021. For the third purpose, the field data of Hu et al. (2018) was used again since it also includes microbiota profiles of the *bx1* mutant maize line, but here the OTUs clustered dataset was used. In brief, we trimmed the strain's 16S rRNA gene sequences to overlap with the gene region of the microbiota analyses (primers 799F and 1193R). The sequence information upstream of 799F was trimmed using the function *matchPattern* (package Biostrings; (Pages et al., n.d.), a 360 bp long sequence downstream of 799F was kept for alignment to the microbiota datasets using the function *AlignSeqs* (package DECIPHER, (Wright, 2016). Then a distance matrix was calculated for all strain sequences to the identified operational taxonomic units (OTUs) or the amplicon sequence variants (ASVs) of the tested microbiota datasets using the function *DistanceMatrix* (package DECIPHER). The most closely mapping community members (OTU or ASV) and its sequence similarity to the tested strain were listed in Table S1. We did not consider mappings

<97 % sequence similarity, the typical threshold for defining OTUs. For strains mapping to several different ASVs (within 97 %), their relative abundance was summed up. Since some strains mapped to several OTUs, the most identical and most abundant OTU were selected per strain.

Phylogenetic tree

The phylogenetic tree was constructed from 16s sequences obtained by sanger sequencing as described above. First, the 16s sequences were concatenated and then aligned using MAFFT v. 7.475 (Katoh et al., 2002) with default options. The aligned sequences were then used as input to RAxML v. 8.2.12 (Stamatakis, 2014). The multi-threaded version ` raxmlHPC-PTHREADS` was used with the options ` -f a -p 12345 -x 12345 -T 23 -m GTRCAT` with 1000 bootstrap replicates. The phylogenetic tree was visualized and annotated in R using the package ggtree (Yu et al., 2017).

In vitro growth assays

Before the actual testing of maize root bacteria for their tolerance against different benzoxazinoid compounds, we prepared liquid pre-cultures in a 96-well format from fresh petri plates. Pre-cultures from plates were prepared with freshly picked isolates and inoculated with an inoculation needle (Greiner bio-one, Kremsmünster, Austria) to 1 ml of 50 % liquid TSB in 2 ml 96-well deep-well plates (Semadeni, Ostermundigen, Switzerland), covered with a Breathe-Easy membrane (Diversified Biotech, Dedham, USA) and grown until stationary phase for 4 days at 28°C and 180 rpm.

Assays were set up by inoculating 4 µl of the pre-cultures to fresh 50 % liquid TSB in 200 µl 96-well microtiter plates (Corning, Corning, USA) containing the compounds and concentrations to be tested: DIMBOA-Glc (2500 µM), MBOA, BOA (each at 250, 500, 625, 1’250, 2’500 and 5’000 µM), AMPO (10, 25 and 50 µM) or APO (10, 25, 50 and 100 µM). These treatments solutions were prepared by mixing their stock solutions to 50 % liquid TSB. Stock solutions were prepared in the solvent DMSO (Sigma-Aldrich, St. Louis, USA) in different concentrations depending on the solubility of the compounds (Table S5) and the DMSO concentration was kept constant in each treatment including the control. All reactions and replicated plates were pipetted using a liquid handling system (Mettler Toledo, Liquidator 96™, Columbus, USA). All plates were piled up with lid and inserted to a stacker (BioStack 4, Agilent Technologies, Santa Clara, United States), which was connected to a plate reader (Synergy H1, Agilent Technologies, Santa Clara, United States). Using this system, the optical density (OD600, absorbance at 600 nm) of every culture was recorded every 100 min continuously (depending on the number of plates processed) over 68 hours. Prior to each measurement, the plates were shaken for 120 s. In each plate, wells

with 'no bacteria controls' were included and in addition in each run one plate containing only media was run in parallel to monitor eventual contaminations (Fig. S7a).

We set up separate runs for the different compounds and in one run, we always tested all concentrations of a compound against all 52 strains split to two plates with 3 replicates per strain and an empty media control plate resulting in total 23 plates per run. (e.g. 6 concentrations of MBOA + 3 concentrations of AMPO + 2 control treatments * 2 plate types with 3 replicates of 52 strains and no bacteria control (stock plate A and B) + media plate without bacteria = 2208 single reactions per run). Every compound was repeated in at least 2 runs. Data were exported from the software (Gen 5, Agilent Technologies, Santa Clara, United States) to excel. Using R studio statistical software, the area under the growth curve (x-axis for time and y-axis for OD600) was calculated.

Quantification of bacterial community size with plating

We quantified the size of the bacterial community on roots of B73 and *bx1*(B73) (Maag et al., 2016) plants by plating the cultivable bacteria of root, rhizosphere, and soil extracts of 7-week-old plants (same experiment as isolation event 4, Table S1), and root extracts from 6-week-old plants. For the root extracts, 10 cm root fragments were washed twice in the 50 ml tubes with sterile ddH_2O (vigorously shaking the tubes 30 times), chopped into small pieces with a sterile scalpel, transferred into a 50 ml Falcon tube and mixed with 10 ml sterile magnesium chloride buffer containing Tween20 (10 mM $MgCl_2$ + 0.05 % Tween; Sigma-Aldrich, St. Louis, USA)and homogenized with a laboratory blender (Polytron, Kinematica, Luzern, Switzerland) for 1 minute at 20'000 rpm, followed by vortexing for 15 s. The pellet of the root washing step was resuspended in 5 ml $MgCl_2$Tween and represented the rhizosphere compartment. Soil extracts were prepared by mixing approximately 5 g of soil from the pot experiment with 5 ml $MgCl_2$Tween and vortexing for 15 s. Extracts were serially diluted and 20 µl were plated on 10 % TSB agar plates containing cycloheximide (10 mg/l). Plates were tilted to spread the drops for counting, then incubated for six days at room temperature. The colony-forming units were counted, multiplied by the dilution factor and the volume plated, and then normalized with the sample fresh weight. All colony forming units (CFU) data were transformed with log10 prior to statistical analysis and visualization.

Quantification of bacterial community size with qPCR

We quantified bacterial community size on samples of the same experiments, where one-half of the roots were freshly used for CFU plating and the other stored at -80 °C for culture-independent analysis. The roots were lyophilized, and DNA was extracted using the Nucleo-Spin Soil DNA extraction kit (Macherey-Nagel, Düren, Germany) following the manufacture's protocol.

Additionally, available DNA samples of the field experiments in Changins (CH, (Hu et al., 2018b)), Reckenholz (also CH) and Aurora (US, both (Cadot et al., 2021b)) were used for qPCR analysis. For all DNA samples, the concentration was measured using the AccuClear® Ultra High Sensitivity dsDNA Quantification Kit (Biotium, Fremont, United States) and samples were adjusted to 1 ng/µl. qPCR reactions were set up in a total volume of 20 µl containing HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia), 250 nM of each primer, 0.3% bovine serine albumin, and approximately 10 ng of root DNA. The size of the bacterial community was quantified on genomic DNA based on the bacterial 16S rRNA gene (primers 799F and 904R, sequences in Table S3; described in (Schlaeppi et al., 2014)) relative to the maize gene Actin (primers ZmActin1_F and ZmActin1_R1, sequences in Table S3; described in Erb et al., 2009. No template control reactions containing water were run in parallel as negative controls. The qPCR reactions were mixed (in triplicates for greenhouse experiment and in single reactions for samples from field experiment) using the Myra Liquid Handler (Bio Molecular Systems, Upper Coomera, Australia) and ran on a CFX96 Real Time System (Bio Rad, Hercules, California). The cycling program included an initial denaturation step at 95 °C for 15 min, followed by 80 cycles of 95 °C for 15 s, 63 °C for 40 s and 72 °C for 20 s, a hold phase at 72 °C for 10 min, followed by melting curve analysis (temperature incrementally increased by 0.5°C from 65 to 95 °C with steps held for 5 s). Raw data were exported directly from Bio-Rad CFX Manager 3.1 and imported into LinRegPCR version 2016.0 (Ruijter et al., 2009) to determine cycle threshold (Ct) and efficiency (E) using the default baseline limit option. The bacterial 16s rRNA gene signal was normalized to the plant signal using the following formula: 16S rRNA/plant gene = $EP_{\text{plant gene}}{}^{\text{Ct plant gene}}/E_{16S}{}^{\text{Ct 16S}}$, where Ct values of the individual reactions and mean E values over all reactions if a given primer pair and run were used for calculation (Bodenhausen et al., 2021).

Statistical analysis

Data analysis was performed in R version 4.0 (R core Team, 2016). We calculated the area under the bacterial growth curve using the function auc from the MESS package (Ekstrøm, 2016) and normalized with the growth of in control treatment (AUC norm). Raw bacterial growth data (AUC) were analysed by comparing the growth of the strain in the control treatment and the respective concentration of a compound using analysis of variance (one-sample t-tests). To compare the response of bacterial strains to compounds, a tolerance index was calculated from the area under the curve of the AUC norm values across all concentrations of the compounds tested. Since certain bacteria do not show growth inhibition at the highest concentration tested and we did not tested susceptibility, we decided to use tolerance indices instead of calculating an inhibitory concentration 50 (IC50). Tolerance indices were normalized using a maximal tolerance index of 1 representing no growth response to a compound. These TI norm values (for

simplicity referred as TI) were compared across strains using analysis of variance (ANOVA, TI norm ~ Strain). According to their TI norm value, strains were classified as tolerant (TI norm > 0.75), intermediate (TI norm 0.75 – 0.5) or susceptible (TI norm < 0.5). Using these tolerance groups, it was tested for a taxonomic signal in tolerance to the compounds using a Fisher's exact test (tolerance type (strain) ~ family). The correlation between tolerance indices of two different compounds was calculated using a Pearson's product-moment correlation test. As a measure for differential colonization on BX-producing plants, we calculated the log2fold change of the abundance of the corresponding OTU on WT and *bx1* roots and rhizosphere (log_2(abundance WT)-log_2(abundance *bx1*). Correlations between tolerance (TI norm) and BX colonization (log_2FC) were calculated using a Pearson's product-moment correlation test. Log transformed data for bacterial colonization on roots (CFU plating and qPCR) were checked for normality using Shapiro-Wilk-test and were analysed for variance using a t-test. Further packages used for the data analysis are the following: Tidyverse (Wickham et al., 2019), Broom (Robinson, 2014), DECIPHER (Wright, 2016) , DESeq2 (Love et al., 2014), emmeans (Lenth et al., 2019), ggthemes (Arnold, 2019), multcomp (Hothorn et al., 2008), phyloseq (McMurdie and Holmes, 2013), phytools (Revell, 2012), vegan (Oksanen et al., 2019) in combination with some custom functions.

Results

Maize root bacteria culture collection covers the abundant members of the maize root microbiome

We have built a culture collection of maize root bacteria (referred to as the 'MRB collection') isolated from B73 maize plants grown in pots filled with natural field soil from Changins. The MRB collection consists of 151 bacterial isolates representing 17 taxonomic families across the five major phyla Pseudomonadota (n = 69), Actinobacteriota (n = 56), Bacillota (n = 23), Bacteroidota (n = 2) and Deinococcota (n = 1; Fig. 1, Table S1). Among those, typical root colonizing families such as Pseudomonadaceae, Microbacteriaceae, and Rhizobiaceae are represented. We mapped the strains to microbiome profiles of maize roots from pot experiments where they were isolated from. The MRB collection accounted for 24 % of the root microbiome with 112 isolates mapping to abundant (>0.1% abundance) and 34 to low abundant (<0.1%) community members (ASVs, Fig. 1 inner ring, Fig 1b).

Next, we assessed whether the maize root bacteria isolates could be detected in root microbiomes of maize grown in the field and in other field soils. We mapped the MRB isolates to the taxonomic units (ASVs / OTUs) of following microbiome datasets: field-grown maize in Changins (CH, Hu et al. 2018), Reckenholz (also CH) and Aurora (US, both Cadot et al. 2021) and a pot experiment with field soil from Sheffield (UK, Cotton et al. 2019; Fig. 1, outer rings). Consistent with the pot experiments for isolation, the majority (139/151) of the MRB isolates also mapped to abundant (>0.1%) members of the root microbiomes of maize grown in the original field Changins (i.e., the field from which soil was used for the pot experiments, Fig. S1c). The majority of the MRB isolates were detected as abundant members in root microbiomes of maize grown in other field soils from Switzerland (117/151), the US (140/151), and the UK (84/151). Specifically isolates belonging to Pseudomonadaceae, Microbacteriaceae and Oxalobacteraceae were detected in all four soils as abundant members. Taken together, the MRB collection is taxonomically diverse, covers the major bacterial families, and represents largely the abundant members of maize root microbiomes in field soils around the globe.

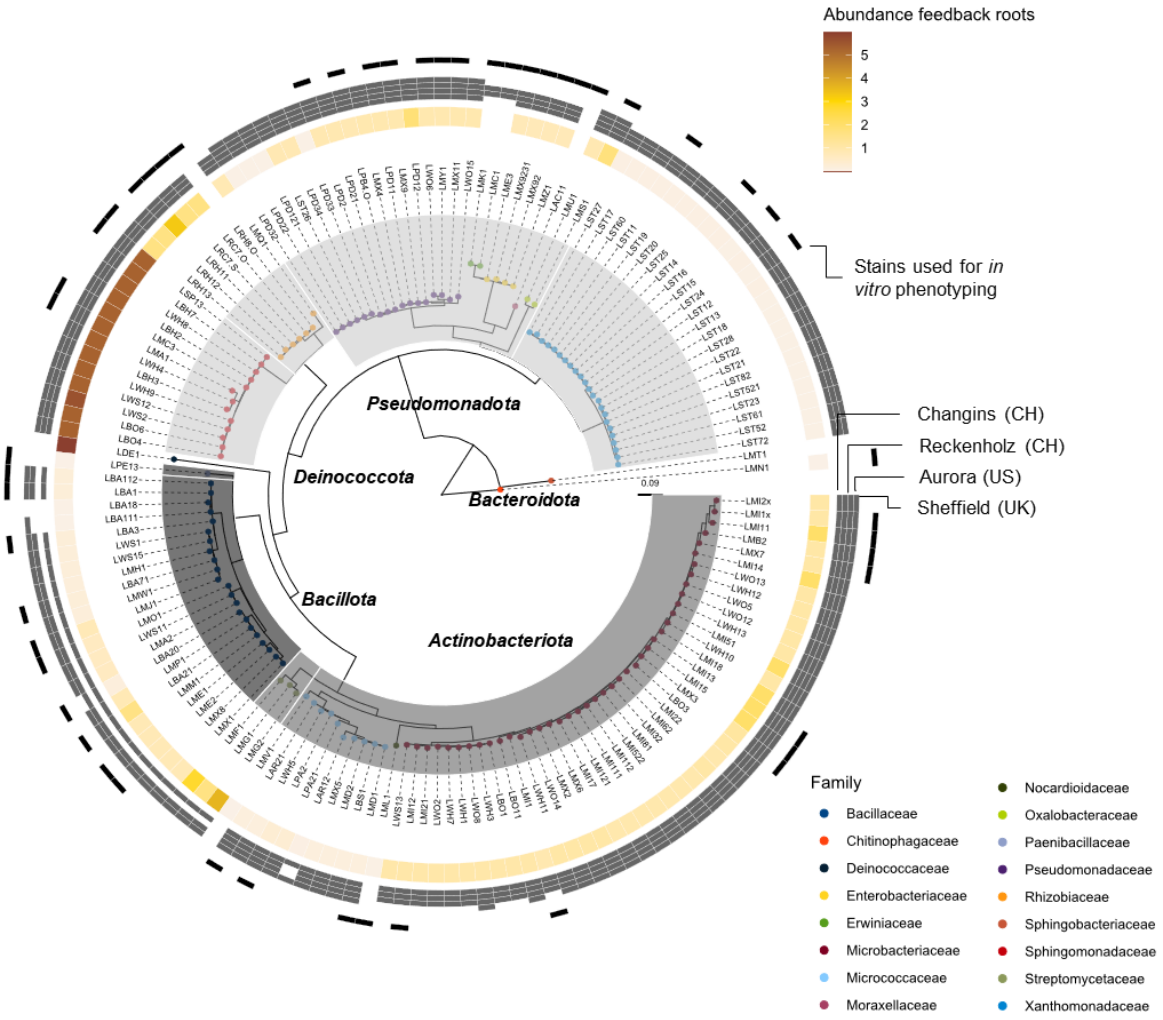


Figure 1. Phylogeny maize root bacteria strain collection. Maximum likelihood phylogeny, constructed from the alignment of 16s rRNA sequences. Leaf nodes are coloured by taxonomic affiliation (family level). The innermost ring shows the quantitative abundance (%) of the corresponding ASVs of the isolates on wild-type maize roots grown in field soil in the greenhouse (origin of isolation) and qualitative abundance (> 0.1 % abundance) on wild-type roots grown in field soil (Changins – CH, Reckenholz – CH, Aurora – US and Sheffield – UK). In the outermost black ring, the strains used for the growth assays are marked.

Benzoxazinoids and aminophenoxazinones selectively inhibit maize root bacteria *in vitro*

To investigate the tolerance of MRB to benzoxazinoids, we screened a representative set of 52 MRB isolates (Fig. 1, black outer ring) using *in vitro* growth assays with DIMBOA-Glc, the main compound exuded by maize roots, and MBOA and AMPO, the main degradation products accumulating in the rhizosphere (Hu et al., 2018b). We measured bacterial growth over time based on optical density in liquid cultures in 96-well plates using concentration ranges of 250 to 5000 µM for MBOA, up to 2500 µM for DIMBOA-Glc, and 10 to 50 µM for AMPO. We calculated the area under the bacterial growth curve (AUC), normalized it to the growth of the strain in the control treatment, and defined a tolerance index (TI) across the tested concentrations to compare and group the different strains according to their tolerance. We defined tolerance groups with TI values > 0.75 describing tolerant strains, values 0.5 – 0.75 referring to intermediately tolerant

strains, and values < 0.5 describing strains that are susceptible to the tested chemical (see Methods for details). Fig. 2a-c exemplifies the approach with the MBOA-tolerant *Pseudomonas* LPD2 (inhibited only at highest concentrations, TI = 0.88) and the MBOA susceptible *Rhizobium* LRC7.O (inhibited already at the lowest concentration, TI = 0.25). Fig. 3b-f reports the TI of all strains to all compounds and in the supplementary Fig. S3-S7, we document all the underlying BX-dependent growth responses.

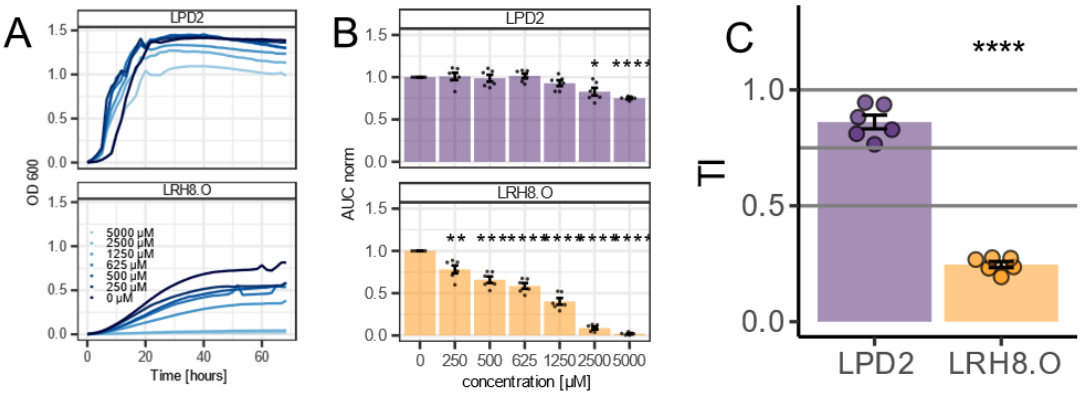


Figure 2. *In vitro* growth of maize root bacteria in MBOA. **A)** Bacterial growth curves (OD_{600}) of a representative tolerant strain of *Pseudomonadaceae* (LPD2) and a representative susceptible strain of *Rhizobiaceae* (LRH8.O) at different concentrations of MBOA over a time course of 68 hours. **B)** Area under the curve (AUC), normalized to the BX-free control treatment **C)** Tolerance index (TI). Means ± SE bar graphs and individual datapoints are shown (n = 6. Results of pairwise t-test is shown inside the panels, p-value < 0.05 = *.

For the glucoside of DIMBOA, DIMBOA-Glc we only tested two concentrations due to the availability of the compound and here we only focus on the effect of the high DIMBOA-Glc concentration tested. DIMBOA-Glc did not affect the growth of a majority of MRB strains (AUC at 2500 µM > 0.75, 43/52 strains) or even improved the growth of some strains (AUC at 2500 µM > 1, 21/52; Fig. 3b and S4). DIMBOA-Glc only inhibited the growth of six strains, predominantly belonging to the family of Bacillaceae. DIMBOA is the bioactive aglycon of DIMBOA-Glc and another major compound measured in root exudates of maize (Hu et al. 2018). However, because DIMBOA immediately converted to MBOA in our screening system, we did not screen the MRB isolates for tolerance to this compound.

In contrast to DIMBOA-Glc, MBOA, which is known for its bioactivity against microbes (Bravo et al., 1997; Machado et al., 2020; Neal et al., 2012), moderately affected a third of the MRB strains (18/52 strains) and strongly inhibited approximately a third of the strains (22/52 strains; Fig. 3c). The most susceptible strains belonged to the *Rhizobiaceae* and the *Moraxellaceae* families. Only 12 strains were tolerant to MBOA (TI > 0.75), belonging to *Pseudomonadaceae*, *Bacillaceae* and *Microbacteriaceae*. Strains belonging to the same family typically showed a similar tolerance level to MBOA (Fisher exact test: tolerance group ~ family: p = 0.00010). We

noticed that tolerance of the *Microbacteriaceae* ranged from the most tolerant strain LMI1x to the second most susceptible strain LMI1 with many strains of intermediate tolerance.

The aminophenoxazinone AMPO is a degradation product of MBOA. AMPO could not be tested in the same concentration range as DIMBOA-Glc and MBOA, because it precipitates at concentrations >50 µM. At the highest testable concentration, most of the tolerant strains began to show reduced growth (Fig. S5) suggesting that we covered with 0 – 50 µM the dynamic range of AMPO toxicity. The large majority of MRB strains were tolerant (43/52 strains) or only moderately affected (5/52 strains) and only 4 strains were susceptible (TI < 0.5) to AMPO (Fig. 2e). The affected strains belonged to the Bacillaceae and Micrococcaceae (Fisher exact test: tolerance group ~ family: p = 0.04263). A direct comparison of 50 µM MBOA and 50 µM AMPO revealed higher toxicity of the latter compound with only one strain was significantly inhibited by MBOA compared to the control (AUC, t.test < 0.05, AUC norm < 0.8; Fig. S6c). Twenty strains were inhibited by the same concentration of AMPO (t.test < 0.05) and with a stronger change in growth (AUC norm 0.03 – 0.97). In conclusion, MRB were largely unaffected by DIMBOA-Glc whereas they exhibited a broad range of tolerances to MBOA, the most abundant toxic compound in the rhizosphere. Although AMPO is more toxic than MBOA, only a small fraction of the MRB was clearly susceptible to this compound, which is generally at low concentrations in the rhizosphere.

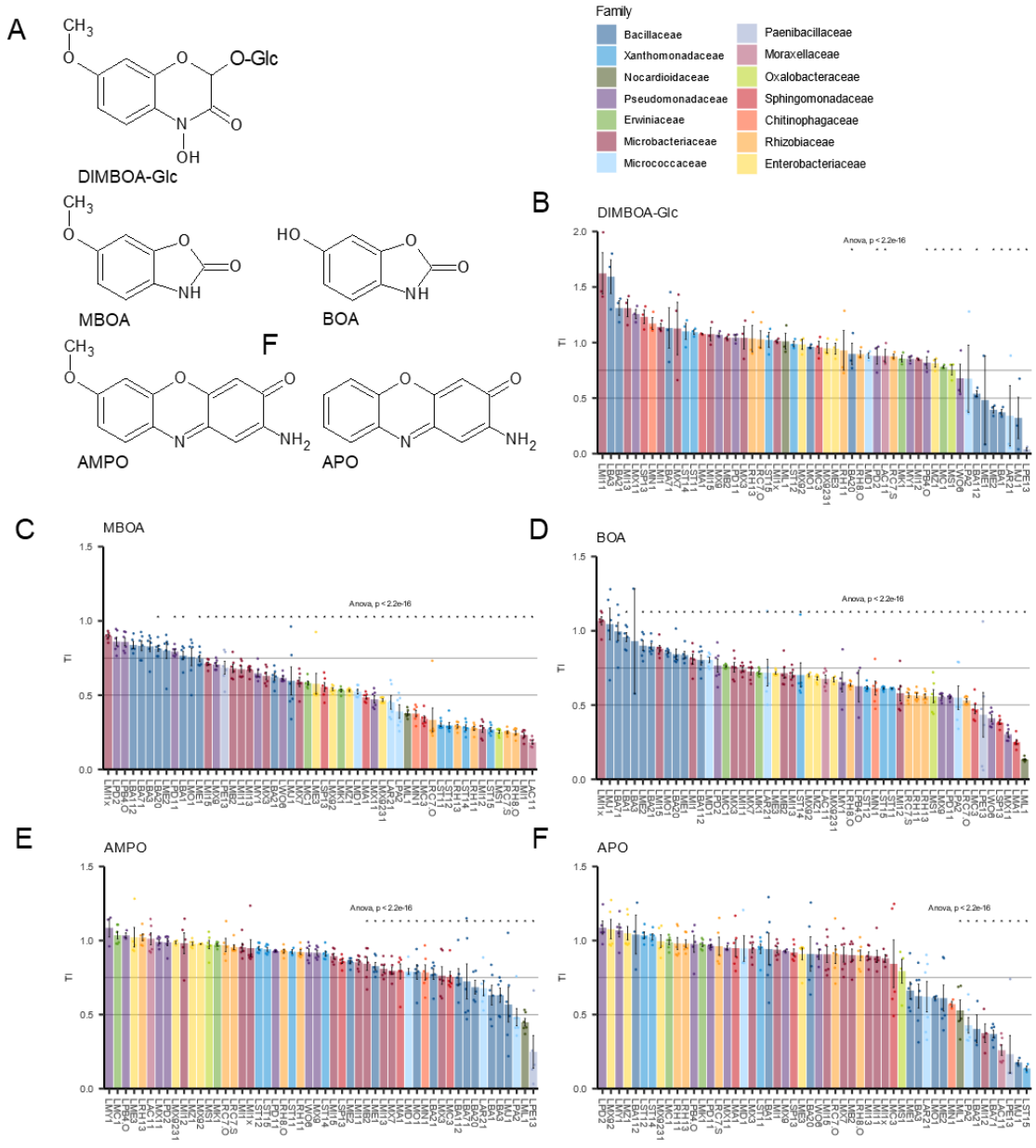


Figure 3. Taxa-specific and compound-specific growth response of maize root bacteria in benzoxazinoids and aminophenoxazinones. A) Chemical structures of the compounds tested. **B)** AUC value in 2500 µM DIMBOA-Glc. Tolerance index in **C)** MBOA, **D)** BOA, **E)** AMPO and **F)** APO. Means ± SE bar graphs and individual datapoints are shown (n = 6, for DIMBOA-Glc n = 3). Results of pairwise t-test and ANOVA are shown inside the panels. Results of pairwise t-test is shown inside the panels, p-value < 0.05 = *.

Tolerance to methoxylated and non-methoxylated benzoxazinoids and aminophenoxazinones correlates

For a broader investigation of bacterial tolerance to benzoxazinoids, we screened the MRB isolates also for their tolerance against BOA and APO, the 'non-methoxylated relatives' of MBOA and AMPO, respectively. Unlike MBOA, the majority of MRB strains were tolerant (16/52 strains) or only moderately affected (30/52 strains) by BOA while just 6 of the strains were notably susceptible (Fig. 3d). Toxicity of BOA was lower compared to MBOA as evidenced that

more strains could still tolerate BOA concentrations higher than 2500 (21/52) compared to 2500 µM MBOA (10/52; Fig. S2ab). Similar taxonomic groups were susceptible (*Rhizobiaceae*) or tolerant (*Bacillaceae*) to both BOA and MBOA, which was further supported by a significant positive correlation of TIs of all MRB isolates to these compounds (Fig. 3a). Also, the tolerance pattern to APO was comparable to AMPO with a large fraction of tolerant MRB strains (36/52 strains), few moderately affected (7/52 strains) and few susceptible isolates (9/52; Fig. 3f). Overall, 16/52 strains were intermediately tolerant to APO (TI norm < 0.75), whereas these were only 9/52 strains in AMPO (TI norm < 0.75) indicating higher toxicity of the non-methoxylated compound APO. Tolerance of MRB isolates to APO and AMPO correlated positively (Fig. 4a) revealing a similar tolerance profile to both compounds.

Finally, we tested whether bacterial tolerance to benzoxazinoid degradation compounds might be coupled with the tolerance to aminophenoxazinones, i.e. whether (M)BOA-tolerant bacteria were also A(M)PO tolerant. Correlation analysis with all strains revealed a significant, albeit weak negative correlation for tolerances to MBOA and AMPO (Fig. 4c) while no relationship was found for bacterial tolerances to BOA and APO (Fig. 4d). In summary, we find stronger antimicrobial effects for methoxylated than non-methoxylated benzoxazinoids, but not aminophenoxazinones, and that the tolerance profiles of the MRB strains were similar for methoxylated and non-methoxylated compounds. These findings suggest that the bacterial tolerance mechanism(s) may operate on the shared main chemical structure of the two benzoxazinoids, while the addition of a methoxy group enhances the level of toxicity.

Benzoxazinoid tolerance in maize root bacteria is related to cell wall properties

Besides testing whether taxonomically related strains exhibited similar tolerance levels to MBOA, we tested if benzoxazinoid tolerance depends on cell wall structure by comparing Gram-positive vs. Gram-negative bacterial isolates. Gram-positive bacteria are characterized by thick peptidoglycan layer in the bacterial cell wall while gram-negative bacteria have thin peptidoglycan layers which are located between an inner cell membrane and a bacterial outer membrane. We found that the tested Gram-positive isolates were overall more tolerant to MBOA, and BOA compared to the Gram-negative ones (Fig 3g, i). For AMPO and APO, as well as for DIMBOA-Glc (Fig 3f, h, j) we found the opposite with the Gram-negative bacteria being more tolerant. Together this indicates that features of the different cell wall structures may at least partially explain the tolerance patterns of the different bacteria to benzoxazinoids and aminophenoxazinones.

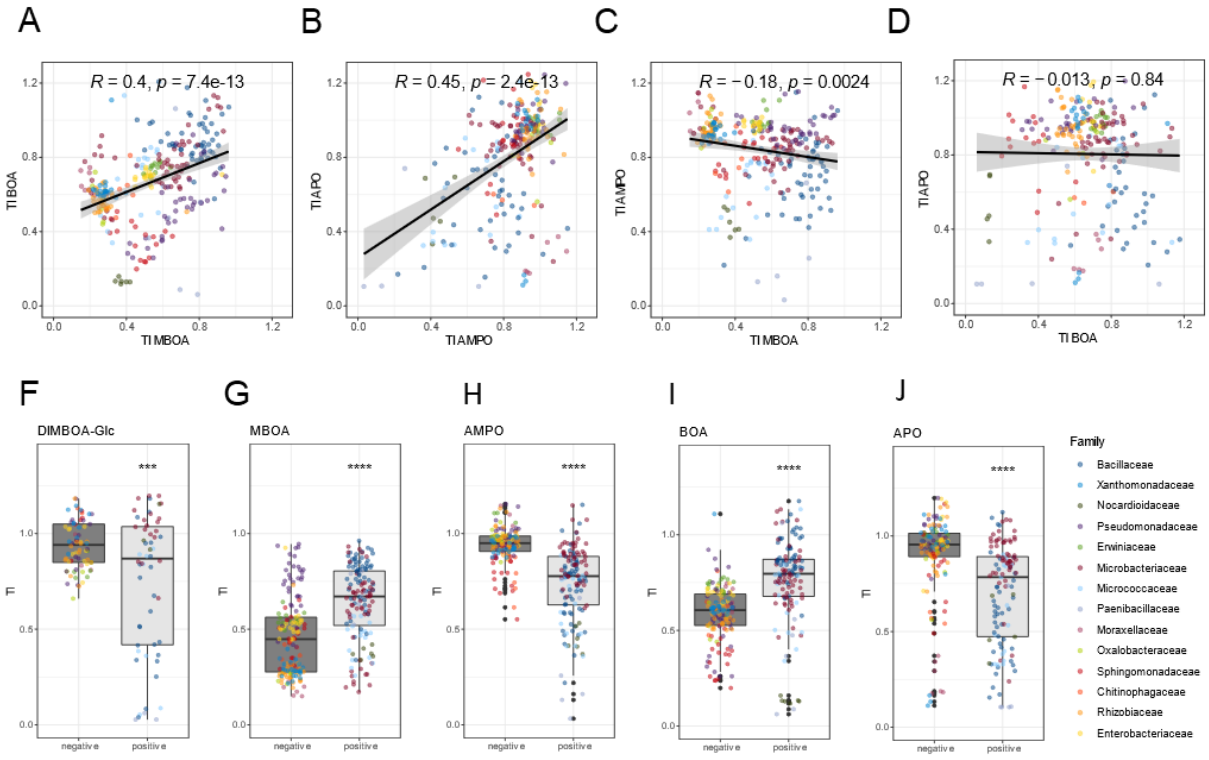


Figure 4. Mechanism of benzoxazinoid and aminophenoxazinone tolerance in maize root bacteria: Correlation of tolerance index for all strains between two compounds tested **A)** MBOA and BOA, **B)** AMPO and APO, **C)** MBOA and AMPO, **D)** BOA and APO. Pearson's product-moment correlation test was performed. cor, correlation coefficient; p, p-value. **E)** summarized TI in Gram-positive and Gram-negative strains in DIMBOA-Glc **F)** MBOA, **G)** MBOA, **H)** AMPO, **I)** BOA and **J)** APO. Results of pairwise t-test is shown inside the panels, p-value < 0.05 = *.

Bacterial tolerance to MBOA explains BX-dependent abundance on maize roots

Culture-independent analyses of maize root microbiomes revealed the enrichment of certain bacterial groups on roots of BX-producing plants (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019). The underlying mechanisms of enrichment and whether tolerance to benzoxazinoids could be involved remain unknown. Therefore, we inspected the root microbiota profiles of BX-producing (wild-type B73) and BX-deficient (*bx1* mutant) maize lines, which were grown in the same soil where the isolates originate from (Hu et al., 2018b). With the idea that an enrichment on BX-producing plants could point to BX-tolerant taxa, we tested for difference in mean abundance between WT and *bx1* roots and rhizospheres (Fig. S8). This cultivation-independent analysis revealed that members of the *Bacillaceae* and *Microbacteriaceae* were generally enriched on wild-type plants, while *Xanthomonadaceae* and *Rhizobiaceae* sequences were depleted. This finding, partially reminiscent of the *in-vitro* tests of the MRB strains (Fig. 3), suggested that the mentioned families rather contain BX-tolerant and non-tolerant bacteria, respectively (Fig. S8).

Therefore, we hypothesized that the MRB strain's tolerance to BX compounds explains differential abundance of their corresponding microbiota representative on BX-producing vs. BX-

deficient roots. We investigated whether community members that were enriched on WT roots were more tolerant to benzoxazinoids, whereas benzoxazinoids might be more toxic to members depleted on BX-producing roots. To test this hypothesis, we correlated the TIs of MRB with the changes in abundance on WT vs. *bx1* roots and rhizospheres. We were specifically interested, which of the BX compounds would best explain differential abundance of community members. Bacterial tolerance to DIMBOA-Glc correlated weakly negative, but still significantly, with BX-dependent abundance in the root and rhizosphere microbiotas (Fig. S10a). Bacterial tolerance to MBOA correlated strongly and significantly positively with BX-dependent abundance in the root (Fig. 5a) and rhizosphere microbiota (Fig. 5b). A similar positive albeit a bit weaker correlation was found for bacterial tolerance to BOA (Fig. 5c, Fig. S10c). In contrast, bacterial tolerance to aminophenoxazinones correlated negatively with BX-dependent abundance in the root and rhizosphere microbiotas, stronger for AMPO than for APO (Fig. 5c, Fig. S10b, d). It is noteworthy, that the strongest positive correlation was found for MBOA, which is both, the most abundant compound accumulating in the rhizosphere (Hu et al. 2018) and the compound to which we found the broadest range of bacterial tolerances. We conclude that bacterial tolerance to MBOA, at least partially, explains the differential abundance patterns of bacteria on BX-producing vs. deficient roots and that MBOA acts with its antibiotic activity as the driving factor to structure the maize root microbiome.

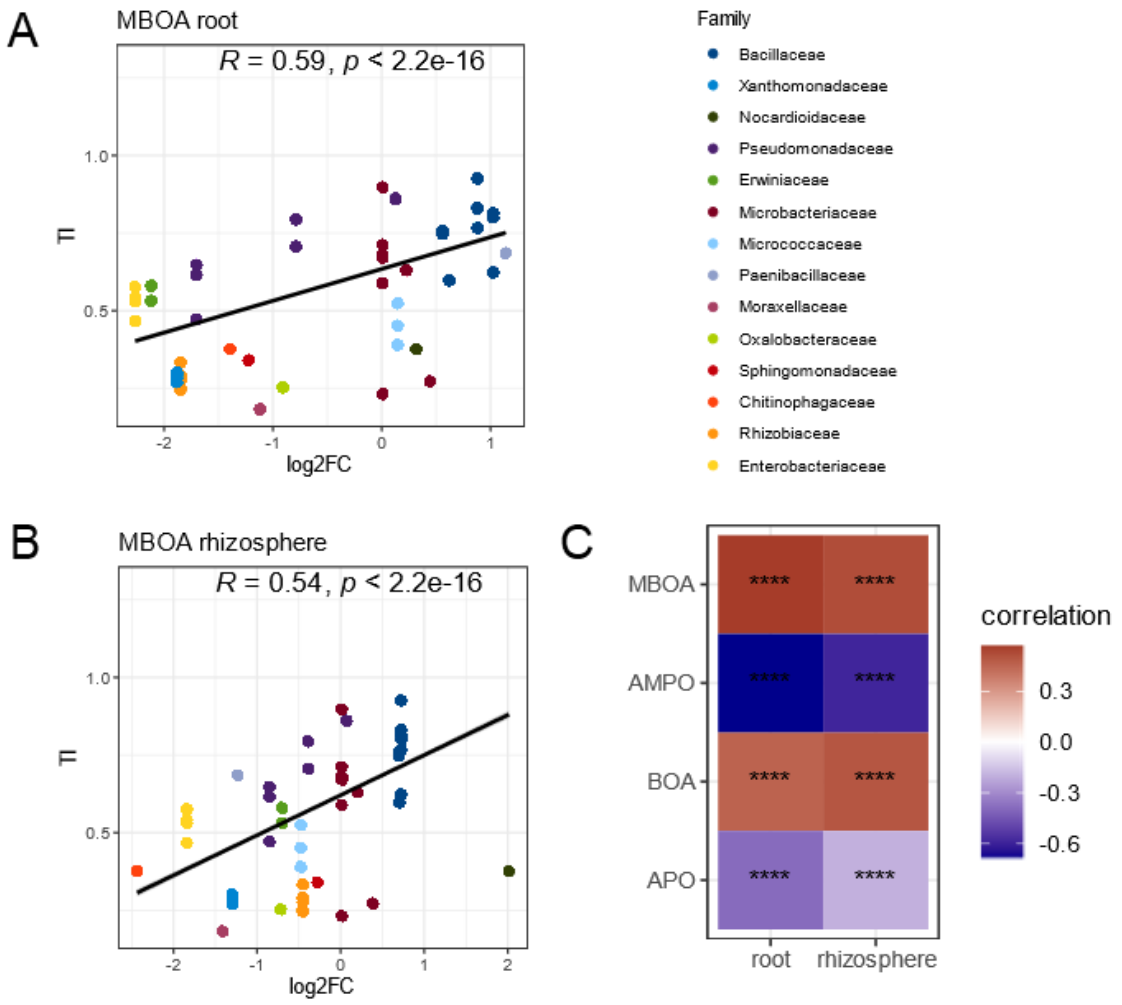


Figure 5: **Correlation of tolerance indices of MRB in MBOA to log2FC on WT roots and rhizospheres in the maize field: A)** Correlation on roots. **B)** correlation in rhizosphere. Pearson's product-moment correlation test was performed. cor, correlation coefficient; p, p-value. **C)** Heatmap of correlation values R between tolerance indices in benzoxazinoids and aminophenoxazinones. Asterixs indicate significant correlations (p. value < 0.05).

Benzoxazinoid exudation does not alter bacterial community size on maize roots

Microbiota analyses profile the relative abundances of community members in a semi-quantitative manner but without reference to absolute abundances of individual members or the overall microbiome (i.e., community size). After finding that benzoxazinoids can negatively affect the growth of MRB, we hypothesized that their antimicrobial activity may not only change community composition but may also reduce the overall community size. We tested this hypothesis by quantifying bacterial community size using a plating assay (colony-forming units of root extracts) and quantitative PCR (measuring bacterial DNA on roots). We set up two greenhouse experiments where we compared roots of wild-type BX-producing and BX-deficient *bx1* mutant plants grown in field soil. Additionally, we quantified bacterial community size using qPCR on root DNA extracts of earlier field experiments with WT and *bx1* plants (Cadot et al., 2021b). The cultivation-dependent quantifications of bacterial cell numbers did not reveal

differences in community size at the roots of the two genotypes (Fig. 6ab). Analogously, we did not find differences in a load of bacterial DNA on roots of both genotypes using cultivation-independent qPCR (Fig. 6cd). These findings from greenhouse experiments were supported by field samples, where we found the same bacterial DNA load comparing both genotypes (Fig. 6efg). Thus, benzoxazinoid exudation does not affect the community size of the bacterial maize root microbiome.

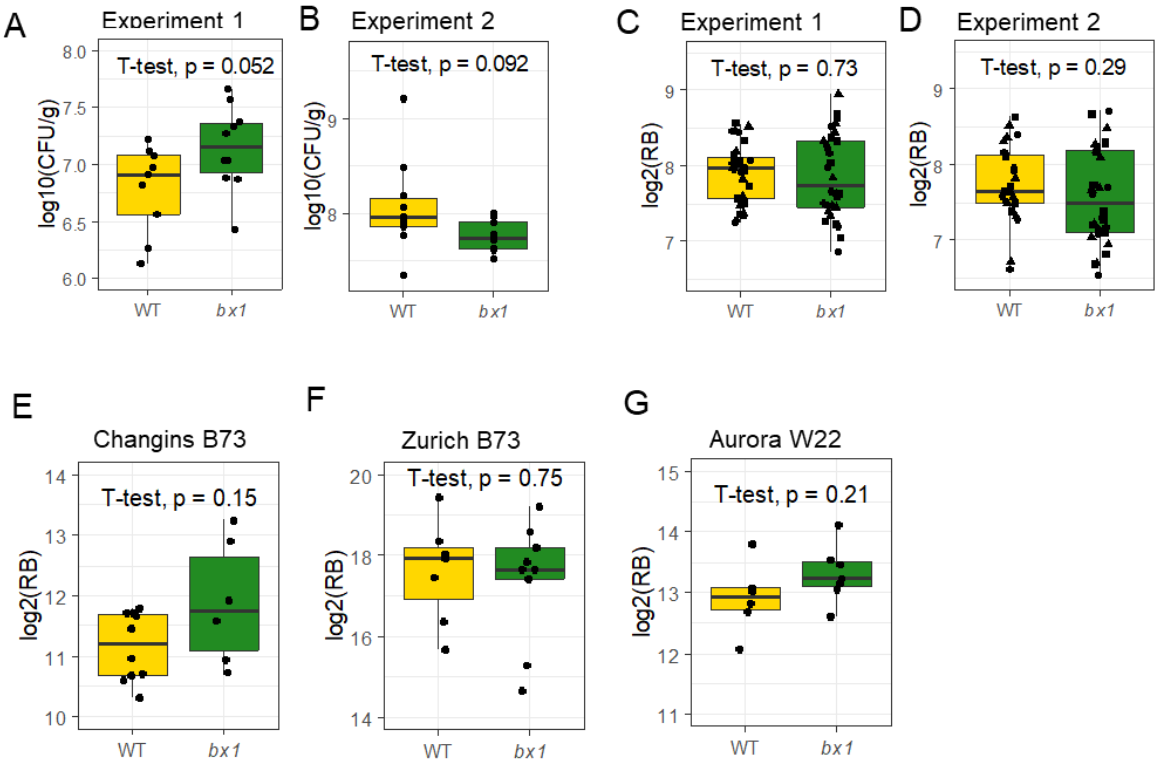


Figure 6. Size of bacterial community on maize roots. Microbiological and qPCR analyses of bacterial root colonization. **A-B)** Bacterial root colonization was assessed by plating colony forming units (CFU) on roots, $\log_{10}$ CFU counts plotted, and statistically significant differences between WT and *bx1* are indicated (t-test). **C-H)** DNA extracts from the same plants were used for qPCR analysis. The bacterial signal, derived from qPCR primers 799F and 904R, was normalized relative to the plant signal of the plant actin gene (ZmActin1). Statistically significant differences between WT and *bx1* are indicated (t-test).

Discussion

Maize root-associated microbial communities are shaped by root exudes benzoxazinoids (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019). Here we established a collection of maize root bacteria and tested their tolerance to benzoxazinoids and aminophenoxazinones. We find those compounds to inhibit the growth of MRB in an isolate-dependent and compound-dependent manner *in vitro*. The growth response to the main benzoxazinoid structuring the root microbiome, MBOA explains the BX-dependent colonization of the isolates on maize roots grown in the field. This finding demonstrates that benzoxazinoids act as antimicrobials to structure the maize root associated microbiomes.

MRB collection as a tool to study plant-microbe interactions in maize

To study the direct effect of benzoxazinoids on single maize root microbiome members, we established a maize root bacteria strain collection. It includes 151 isolates covering 5 major bacterial phyla and 17 bacterial families. Several of the culture representatives are abundant members of the maize root microbiome. For example, *Pseudomonadaceae*, *Microbacteriaceae*, and *Rhizobiaceae*. Other strain collections from maize roots exist (Niu et al., 2017, Beirinckx et al., 2020). These collections are taxonomically narrow (Niu et al. 2017) or originate from a different soil. The MRB collection originates from the soil where microbiota-structuring by benzoxazinoids occur (Hu et al. 2018, Cadot et al. 2021). Ensuring the same origin of the strain collection is important because microbes adapt specific to soil. Thus, the MRB collection is a tool to unravel the mechanistic of BX-dependent structuring of maize root microbiomes. Furthermore, the collection is a tool to decipher the BX-dependent feedbacks in maize. It can also be used to identify strains that promote plant growth or control diseases.

Benzoxazinoid derivates and aminophenoxazinones selectively inhibit maize root bacteria

Benzoxazinoids are exuded from maize roots to the surrounding soil as DIMBOA-Glc, then rapidly converted to more stable benzoxazolinones MBOA (Macías et al., 2004) and further to the aminophenoxazinone AMPO (Belz and Hurle, 2005; Kumar et al., 1993; Macías et al., 2004). We tested the growth of 52 MRB isolates in DIMBOA-Glc, MBOA and AMPO *in vitro*. We found that DIMBOA-Glc did not inhibit the growth of most of the isolates tested (Fig. 3b). A few isolates even grew better in DIMBOA-Glc, probably because they can use the attached glucose for growth. MBOA and AMPO selectively inhibit the growth of MRB (Fig. 3ce). In the rhizosphere, AMPO is only low abundant and has a low solubility. Although AMPO is more toxic than MBOA at the same concentration, only a small fraction of the MRB was susceptible. MBOA is the most abundant and stable benzoxazinoid in the rhizosphere (Hu et al., 2018b). Due to the solubility of the compound, we tested ten times higher concentrations. Among the compounds tested, we find MBOA as most

selective benzoxazinoid in the rhizosphere, because MRB exhibited the broadest range of tolerances to MBOA. MBOA tolerances negatively correlate with AMPO tolerances. This points to a specific adaption and an ecological trade-off of MRB to tolerate different benzoxazinoids in the soil.

To investigate the effect of the methoxy group for antimicrobial activity, we compared the growth response of MRB in methoxylated to non-methoxylated benzoxazinoids, namely MBOA with BOA and AMPO with APO. Among all strains tested, the inhibitory effect of MBOA is stronger than its non-methoxylated related compound BOA (Fig. 3d). Our observation is consistent with earlier work in yeast (*C. albicans*). The methoxy group makes MBOA more reactive and thus more toxic (de Bruijn et al., 2018). For the aminophenoxazinones it is opposite. Across the MRB collection APO is more toxic than the methoxylated AMPO. This may be due to the low solubility of AMPO which would lead to the exclusion of AMPO from cells and thus limiting toxicity. Another possibility is an altered accessibility of the molecule altering its toxicity as it was reported for toluene and benzene. In this case the benzene without any side groups is more toxic than the related compound toluene which has a methyl group and thus a limited accessibility to biological molecules (Dean, 1978).

For MBOA, MRB exhibit a broad range of tolerance levels indicating a strong selective function. Tolerance levels show a taxonomic signal but are within some families they differ strongly between strains. Isolates belonging to *Pseudomonadaceae* and the *Bacillaceae* were highly tolerant to MBOA. *Rhizobiaceae* and the *Xanthomonadaceae* are strongly inhibited by MBOA. We find that Gram-positive bacteria are more tolerant to the MBOA and BOA. For AMPO and APO, Gram-negative bacteria show higher tolerance (Fig. 4hj). These results show bacteria harbour the intrinsic resistance to benzoxazinoids. Gram-negative bacteria are often more tolerant to antibiotics. This might be due to the outer cell membrane protecting them and arises from selection pressure. Enhanced tolerance is possibly mediated by membrane architecture and permeability (Blair et al., 2015). The first indications of bacterial tolerance mechanisms to MBOA come from a recent study investigating *Photorhabdus*, bacterial symbionts of entomopathogenic nematodes (Machado et al., 2020). In an artificial evolution experiment, *Photorhabdus* strains were selected for high MBOA tolerance. MBOA-tolerant strains acquired mutations in the RNA polymerase and the aquaporin Z. Mutations in the aquaporin channel prevent the influx of the toxin into the cytoplasm (Blair et al., 2015; Nikaido, 1989). We speculate that similar mechanisms including cell membrane permeability and target modification may explain MBOA tolerance in MRB. Which exact mechanism causes benzoxazinoid tolerance in maize root bacteria remains to be investigated.

BX-tolerance correlates with BX-dependent colonization of maize root bacteria in the field

The broad range of tolerance of MRB to benzoxazinoids, prompted us to test if these may explain the abundance of MRB on BX-producing versus BX-deficient roots. We mapped our strains to the root microbiome of plants grown in the field (Hu et al. 2018). Indeed, bacterial tolerance to MBOA correlates with BX-dependent colonization of the corresponding OTUs. This shows that benzoxazinoids act as antibiotics to structure the microbiome community in maize roots. This correlation of *in vitro* tolerance with field abundances for works well for benzoxazinoids. This approach may also explain the structuring of microbiomes by other plant specialized metabolites (e.g. for *Arabidopsis* with coumarins or glucosinolates, or for maize with zealexins or flavonoids) (Harbort et al. 2020, Ding et al. 2020, Yu et al. 2021).

Observing the antimicrobial activity of benzoxazinoids led us to investigate the effect of benzoxazinoids on community size of the maize root microbiome. We measured the bacterial community size on maize roots by culture-dependent and culture-independent methods. We found no differences in community size. Thus, the antimicrobial effect of benzoxazinoids does not expand to the community to reduce its size on roots. This aligns with the study of Cadot et al 2021, which did not detect BX-dependent differences in alpha diversity. The unchanged community size in demonstrates that benzoxazinoids specifically inhibit certain microbes. More BX-tolerant bacteria will take over their niche space and dominate BX-producing roots. The antimicrobial effect of benzoxazinoids partially explains community composition on maize roots. Further chemical microbial mechanisms may influence a benzoxazinoid root microbiome. DIMBOA affects bacterial chemotaxis and attracts Pseudomonas putida (Neal et al., 2012). DIMBOA reduces biofilm formation and swarming motility of *Ralstonia solanacearum* (Guo et al., 2016). Certain bacteria can degrade benzoxazinoids (Schütz et al., 2019) and may thus change the chemical environment and affect microbe-microbe interactions (Niehaus et al., 2019).

The mechanistic of BX-dependent microbiome assembly of single microbes

A BX-tolerant microbiome may be beneficial for plant growth. We found that certain inhibited isolates belong to families with well-known plant pathogens (*Xanthomonas* and *Agrobacterium*) (Maresh et al., 2006). MBOA-tolerant isolates belong to families with well-known plant beneficial strains (*Pseudomonas* and *Bacillus*) (Neal et al., 2012; Neal and Ton, 2013; Santos et al., 2020). Thus, benzoxazinoids may select for a healthy maize root microbiome by inhibiting pathogenic microbes and favour beneficial microbes which evolved tolerance to benzoxazinoids. This explanation goes in line with the findings of BX-dependent soil feedback by Hu et al. 2018. BX-conditioned microbiomes improve the plant resistance to an aboveground insect pest of the

next generation of maize plants. The same happens when the soil was conditioned with a *bx1* plant supplemented with MBOA.

A similar mechanism occurs in the *Arabidopsis thaliana* rhizosphere where the dominant coumarin, scopoletin, inhibits soil-borne fungal pathogens. Growth-promoting rhizobacteria are coumarin tolerant, suggesting that plants assemble a health-promoting root microbiota by coumarin exudation (Stringlis et al., 2018). To test for such mechanisms, single strains or SynComs may be grown on WT and *bx1* plants in axenic microcosms (Niu et al., 2017; Wagner et al., 2021). Altering root exudation to favour tolerant, beneficial strains would ultimately improve plant health.

On the other end of the scale of microbial complexity, understanding mechanisms of bacterial benzoxazinoid tolerance may be used to improve the establishment of single biocontrol strains or consortia. Often, bacterial strains employed for biocontrol fail to establish due to unknown reasons. Tolerance to plant specialized metabolites thus may be a promising trait to engineer to improve colonization of biocontrol bacteria. Enhancing the tolerance of biocontrol strains towards host secondary metabolites may improve their establishment on host plants. Thus, uncovering BX-tolerance mechanisms would pave the way to improve biocontrol strains.

Conclusion

Benzoxazinoids selectively act as antibiotics on members of the maize root microbiome and their tolerance to the dominant benzoxazinoid MBOA explains their abundance on benzoxazinoid producing maize roots. These findings provide evidence that benzoxazinoids shape the root microbiomes of host plants, acknowledging plant specialized metabolites as important traits for the plant to select for their healthy microbiome.

Author statement

Experiments were planned and conducted by L.T. Co-authors contributed to the mapping of the bacterial 16s sequences to the microbiome datasets (Fig. 1), the calculation of the phylogenetic tree from the 16s sequences (Fig. 1) and the growing of the maize plants (Fig. 6). Co-authors performed the qPCR of the bacterial communities on maize roots (Fig. 6).

Acknowledgements

We thank Selma Cadot for the preparation of the root DNA samples used for qPCR, Niklas Schandry for developing and introduction to the high-throughput phenotyping pipeline, Sandro Rechsteiner for the isolation of the initial maize root bacteria strain collection and Florian Enz for the plant maintenance. This work was supported by the Interfaculty Research Collaboration "One Health" of the University of Bern.

Supplementary figures

Table S1. Phyla represented in MRB strain collection

Phylum	**Isolates**
Actinobacteriota	56
Bacillota	23
Bacteroidota	2
Deinococcota	1
Pseudomonadota	69

Table S2. Families represented in MRB strain collection

Family	**Isolates**
Bacillaceae	22
Chitinophagales	1
Deinococcaceae	1
Enterobacteriaceae	4
Erwiniaceae	2
Microbacteriaceae	42
Micrococcaceae	10
Nocardioidaceae	1
Oxalobacteraceae	2
Paenibacillaceae	1
Pseudomonadaceae	16
Moraxellaceae	1
Rhizobiaceae	7
Sphingobacteriaceae	1
Sphingomonadaceae	13
Streptomycetaceae	3
Xanthomonadaceae	24

Table S3. Tolerance groups and statistics of maize root bacteria in benzoxazinoids

group	MBOA	BOA	AMPO	APO	DIMBOA-Glc
tolerant	12	16	43	36	43
intermediate	18	30	5	7	4
susceptible	22	6	4	9	5
Fisher exact test: type ~ family	0.0001	2.59E-05	0.04263	0.00723	NA

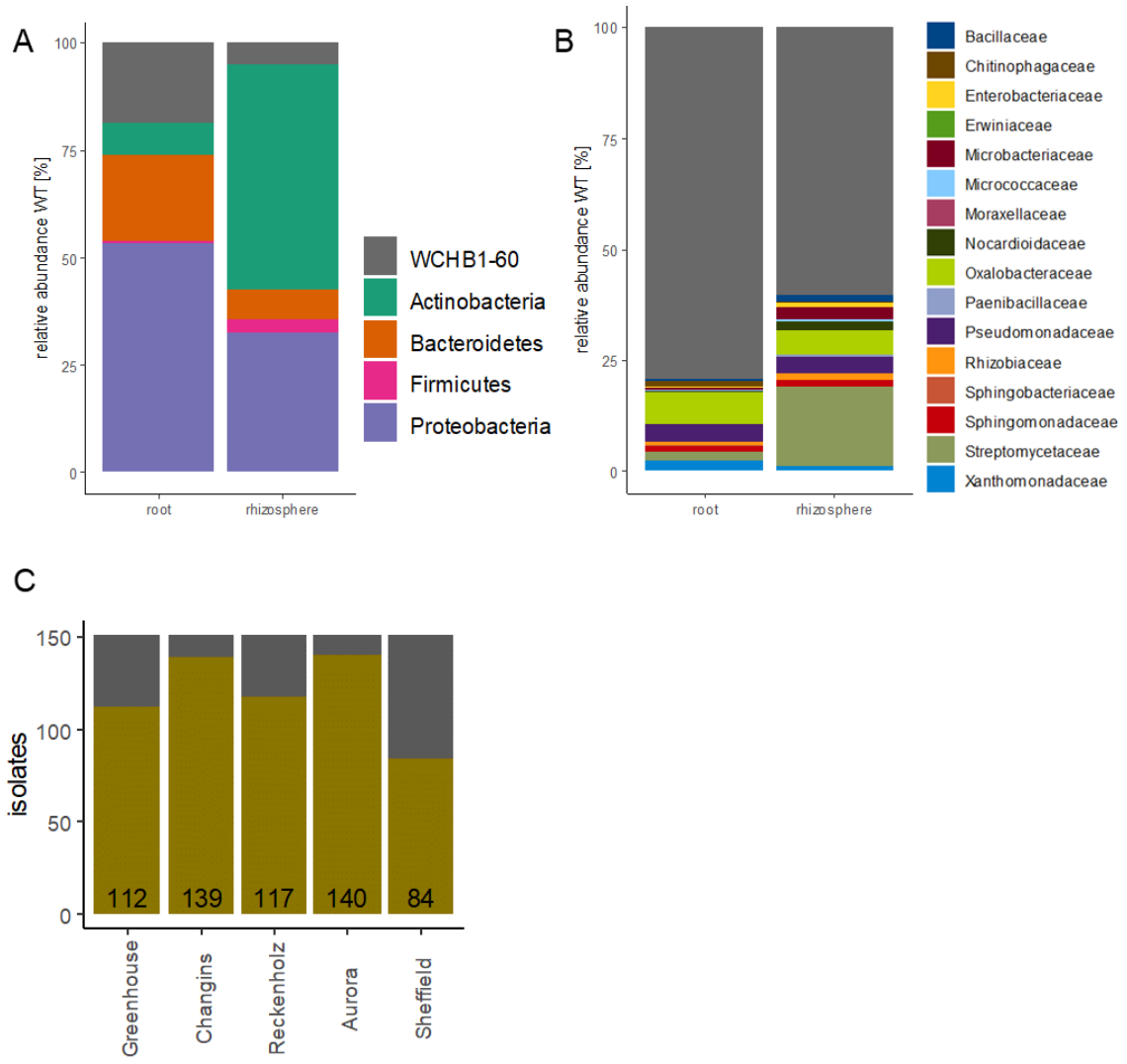


Figure S1. Abundance of strains form maize root bacteria collection in field root and rhizosphere microbiome OTU data Changins field **A)** Cumulative abundance in strain collection in the field maize root microbiome for phyla. **B)** for families. **C)** Isolates mapping to abundant members (> 0.1 % abundance) in the different datasets.

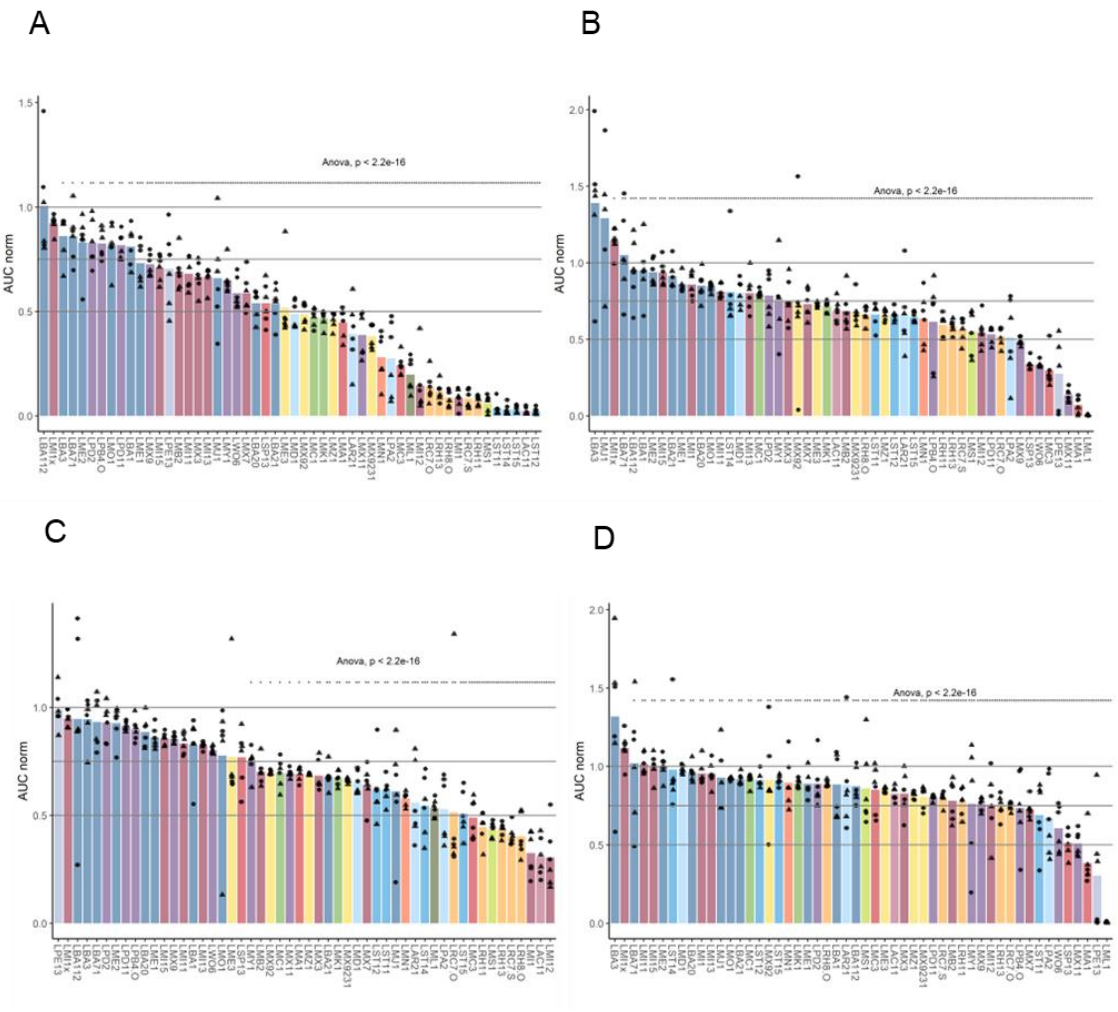


Figure S2. Absolute growth of maize root bacteria in defined concentrations of benzoxazinoids. Area under the curve normalized to the BX-free control treatment, AUC norm in **A)** MBOA 2500 µM **B)** BOA 2500 µM **C)** MBOA 1250 µM and **D)** BOA 1250 µM for all MRB strains (coloured according to family assignment). Overall significant differences calculated by ANOVA. Asterisks indicate significant differences to the most tolerant strain for each chemical (t. test).

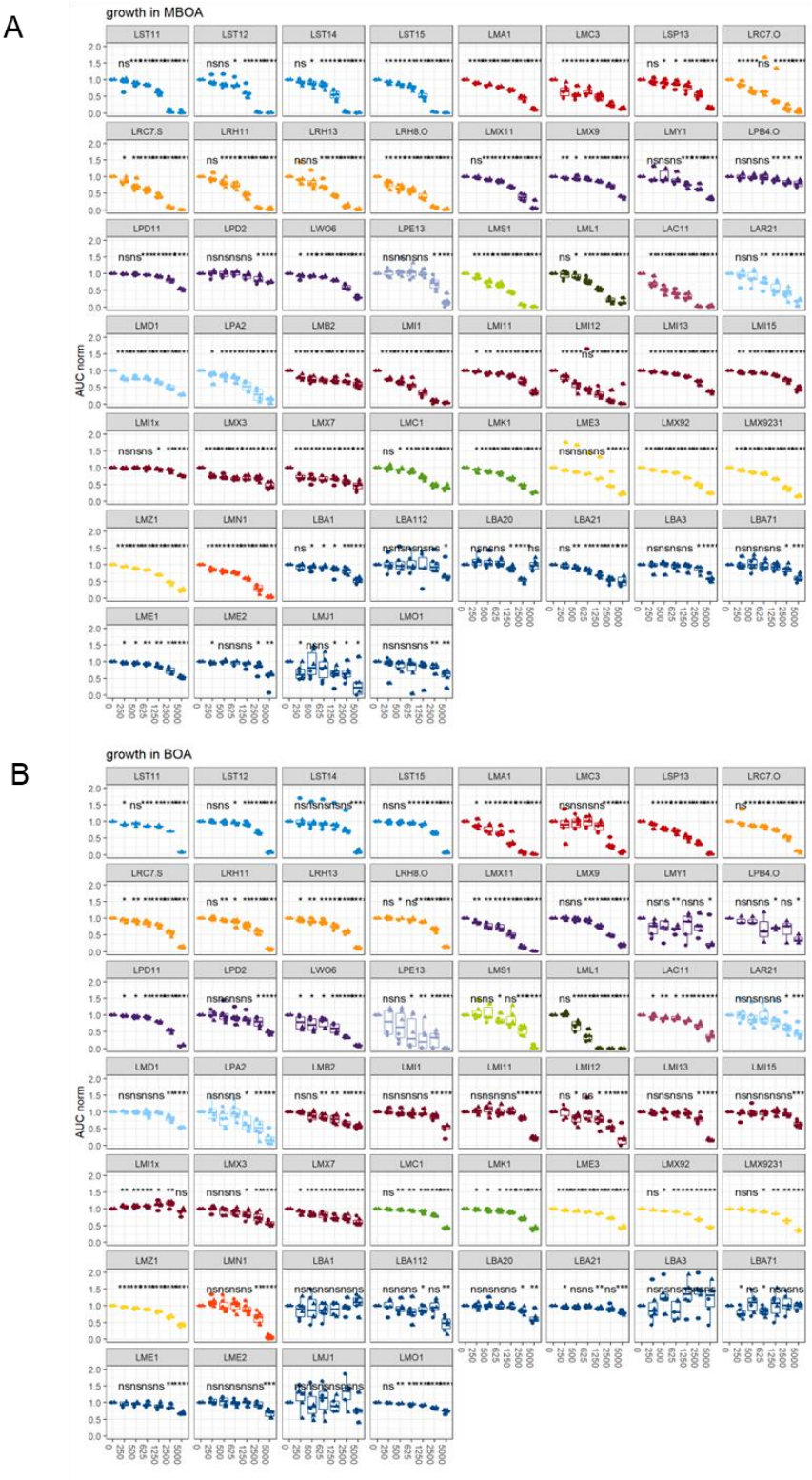


Figure S3. Absolute growth of maize root bacteria in all tested concentrations of benzoxazinoids Area under the curve normalized to the BX-free control treatment, AUC norm in all tested concentrations of **A)** MBOA (0-5000 µM) **B)** BOA (0-5000 µM). MRB strains (coloured according to family assignment). Asterisks indicate significant differences to the control (DMSO) (t.test).

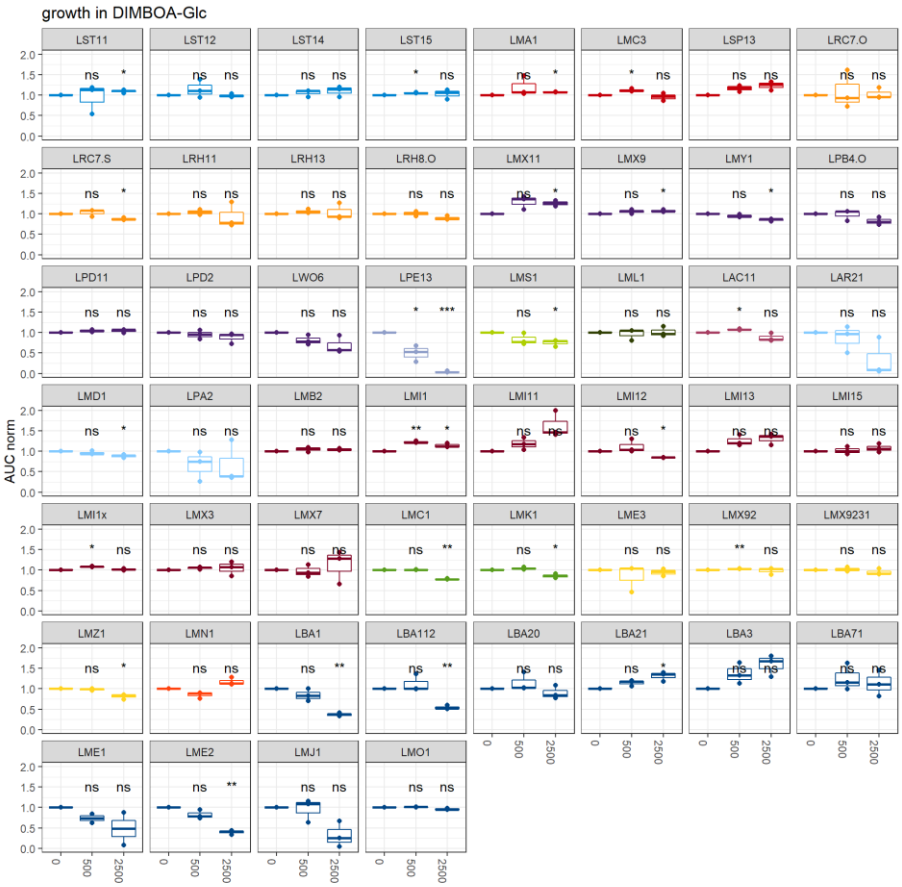


Figure S4. Absolute growth of maize root bacteria in all tested concentrations of DIMBOA-Glc Area under the curve normalized to the BX-free control treatment, AUC norm in all tested concentrations of DIMBOA Glc 500 µM and 2500 µM. MRB strains (coloured according to family assignment). Asterisks indicate significant differences to the control treatment (DMSO) (t.test).

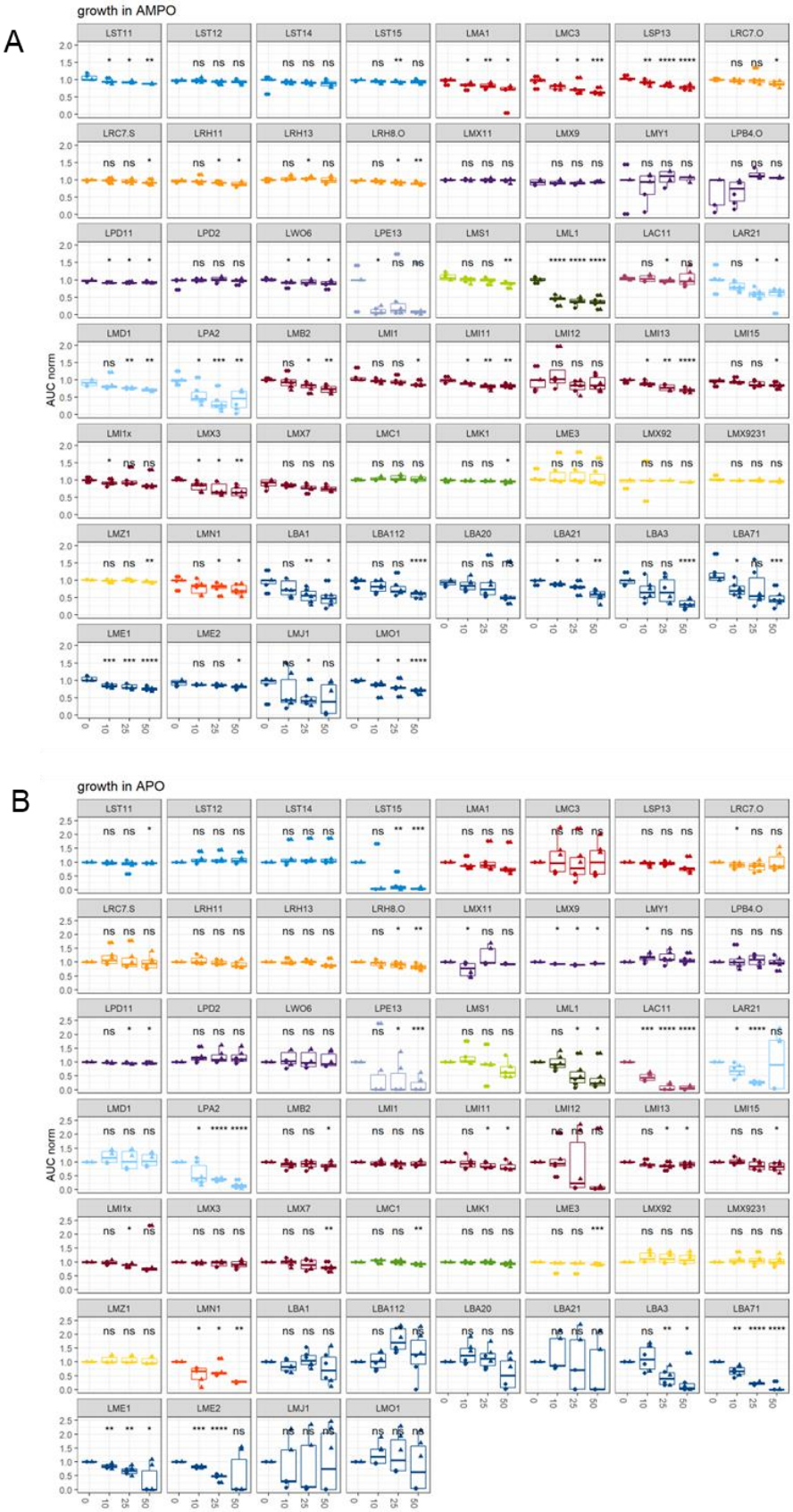


Figure S5. Absolute growth of maize root bacteria in all tested concentrations of aminophenoxazinones Area under the curve normalized to the BX-free control treatment, AUC norm in all tested concentrations of **A)** AMPO (0-50 µM) **B)** APO (0-50 µM). MRB strains (coloured according to family assignment). Asterisks indicate significant differences to the control (DMSO) (t.test).

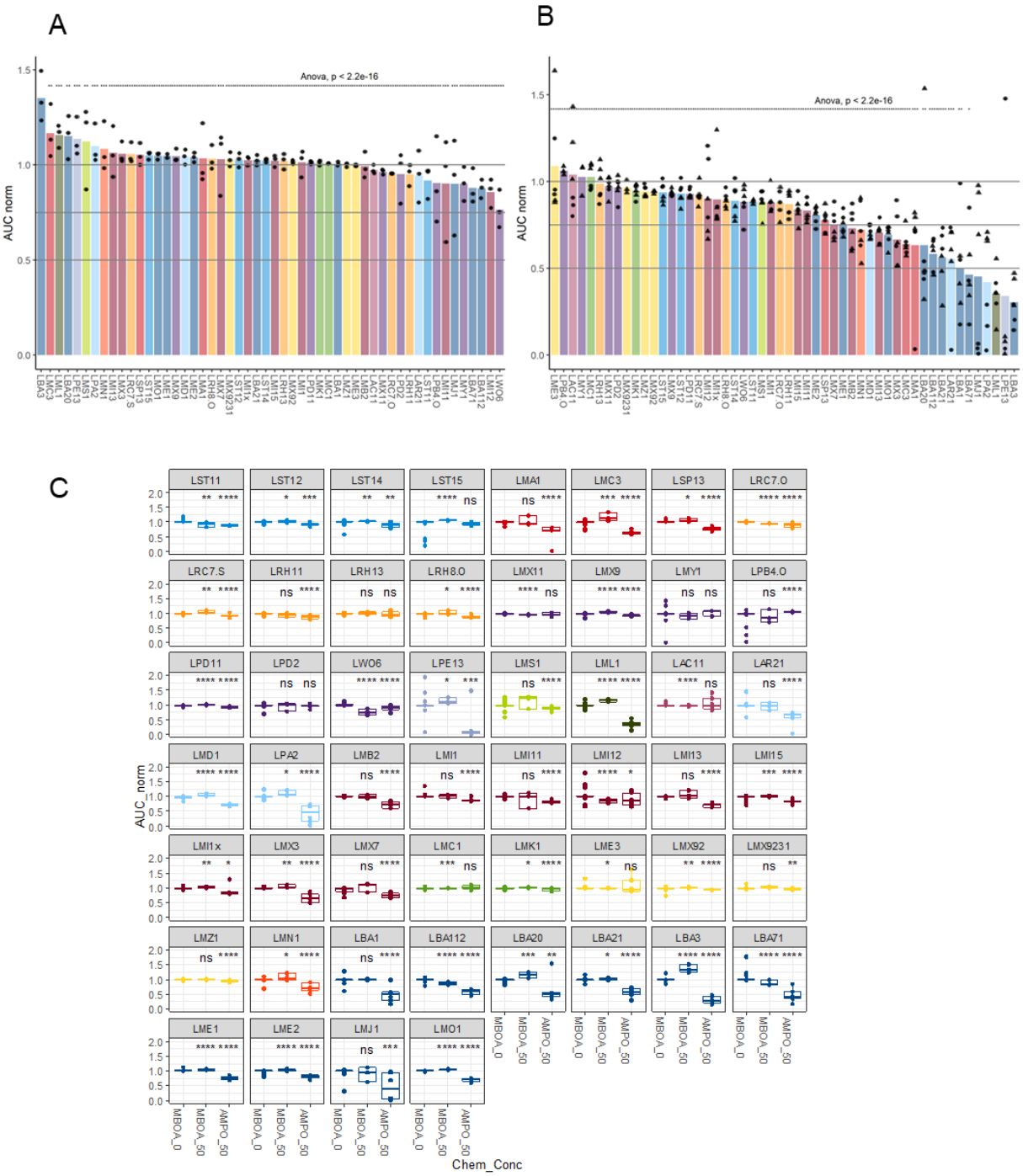
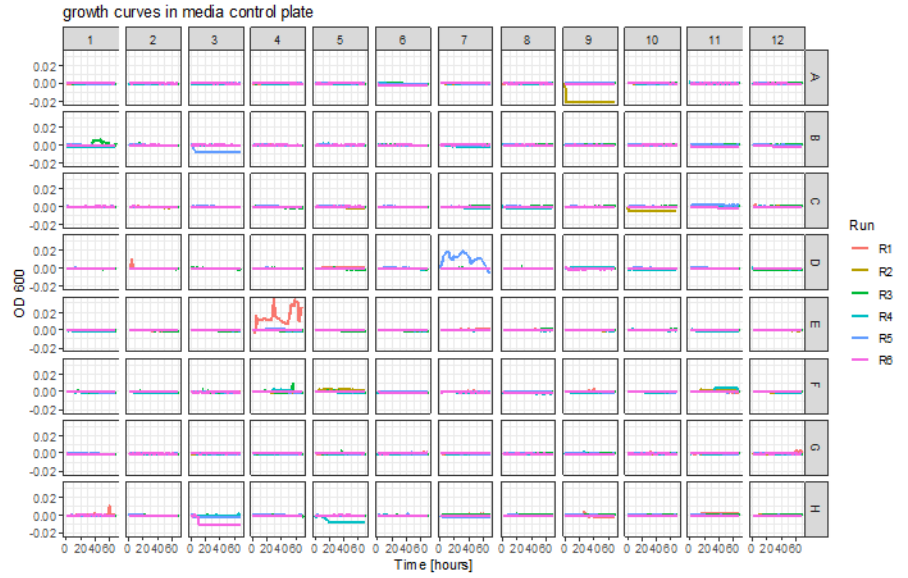


Figure S6: Absolute growth of maize root bacteria in defined concentrations of MBOA and AMPO. Area under the curve normalized to the BX-free control treatment, AUC norm in **A)** MBOA 50 µM and **B)** AMPO 50 µM for all MRB strains (coloured according to family assignment). Overall significant differences calculated by ANOVA. Asterisks indicate significant differences to the most tolerant strain for each chemical (t.test). **C)** Comparison of AUC norm in control treatment (DMSO) with AUC in MBOA 50 µM and AMPO 50 µM.

A



B

Plate A

	1	2	3	4	5	6	7	8	9	10	11	12
A	LBA71	LME2	LME1	LBA3	LBA71	LME2	LME1	LBA3	LBA71	LME2	LME1	LBA3
B	LSP13	LST11	LME3	LRH11	LSP13	LST11	LME3	LRH11	LSP13	LST11	LME3	LRH11
C	LPB4.R	LRC7.O	LMU1	LAR21	LPB4.R	LRC7.O	LMU1	LAR21	LPB4.R	LRC7.O	LMU1	LAR21
D	LMX8	LMN1	LMF1	LST17	LMX8	LMN1	LMF1	LST17	LMX8	LMN1	LMF1	LST17
E	LMK1	LMI13	LMX11	LPD1	LMK1	LMI13	LMX11	LPD1	LMK1	LMI13	LMX11	LPD1
F	LRH8.S	LMA1	LMB2	LMX3	LRH8.S	LMA1	LMB2	LMX3	LRH8.S	LMA1	LMB2	LMX3
G	LBA1	LMI11	LMR1	NBC	LBA1	LMI11	LMR1	NBC	LBA1	LMI11	LMR1	NBC
H	LMI1	LRH13	LAC11	LPA2	LMI1	LRH13	LAC11	LPA2	LMI1	LRH13	LAC11	LPA2

Plate B

	1	2	3	4	5	6	7	8	9	10	11	12
A	LMX7	LBA20	LST12	LRH8.O	LMX7	LBA20	LST12	LRH8.O	LMX7	LBA20	LST12	LRH8.O
B	LML1	LMI14	LMS1	LST15	LML1	LMI14	LMS1	LST15	LML1	LMI14	LMS1	LST15
C	LMX9	LPB4.O	LPE1.3.1	NBC	LMX9	LPB4.O	LPE1.3.1	NBC	LMX9	LPB4.O	LPE1.3.1	NBC
D	LMJ1	LPD2_E2	LMG1	LMC3	LMJ1	LPD2_E2	LMG1	LMC3	LMJ1	LPD2_E2	LMG1	LMC3
E	LMZ1	LMD1	LRC7.S	LST14	LMZ1	LMD1	LRC7.S	LST14	LMZ1	LMD1	LRC7.S	LST14
F	LMI1x	LMI12	LBA112	LMY1	LMI1x	LMI12	LBA112	LMY1	LMI1x	LMI12	LBA112	LMY1
G	LMI15	LMO1	LMC1	LMX92	LMI15	LMO1	LMC1	LMX92	LMI15	LMO1	LMC1	LMX92
H	LAR4	LBA21	LWO6	LMX9231	LAR4	LBA21	LWO6	LMX9231	LAR4	LBA21	LWO6	LMX9231

C

Nr	Treatment	Plate	Concenetration [uM]	Compound	Medium
1	Dummy_1	0	0	0	0
2	Ctrl_1_DMSO_TSB_A	A	Ctrl	DMSO	TSB
3	250_1_BOA_TSB_A	A	250	BOA	TSB
4	500_1_BOA_TSB_A	A	500	BOA	TSB
5	625_1_BOA_TSB_A	A	625	BOA	TSB
6	1250_1_BOA_TSB_A	A	1250	BOA	TSB
7	2500_1_BOA_TSB_A	A	2500	BOA	TSB
8	5000_1_BOA_TSB_A	A	5000	BOA	TSB
9	Ctrl_1_DMSO_TSB_B	B	Ctrl	DMSO	TSB
10	250_1_BOA_TSB_B	B	250	BOA	TSB
11	500_1_BOA_TSB_B	B	500	BOA	TSB
12	625_1_BOA_TSB_B	B	625	BOA	TSB
13	1250_1_BOA_TSB_B	B	1250	BOA	TSB
14	2500_1_BOA_TSB_B	B	2500	BOA	TSB
15	5000_1_BOA_TSB_B	B	5000	BOA	TSB
16	medium	0	0	no	TSB
17	2500_1_DMG_TSB_A	A	2500	DMG	TSB
18	2500_2_DMG_TSB_B	B	2500	DMG	TSB
19	2500_1_DMG_TSB_A	A	2500	DMG	TSB
20	2500_2_DMG_TSB_B	B	2500	DMG	TSB
21	500_1_DMG_TSB_A	A	500	DMG	TSB
22	500_1_DMG_TSB_B	B	500	DMG	TSB
23	500_1_DMG_TSB_A	A	500	DMG	TSB
24	500_1_DMG_TSB_B	B	500	DMG	TSB
25	Dummy_1	0	0	0	0

Figure S7. Bacterial growth in media control plate and setup. A) Optical density in plate with media alone, no bacteria inoculated as a sterile control **B)** Randomized plate layout strains. Each strain is tested in 3 replicates and the strains are split into two plates**. C)** Setup of the plates with different treatments in one run.

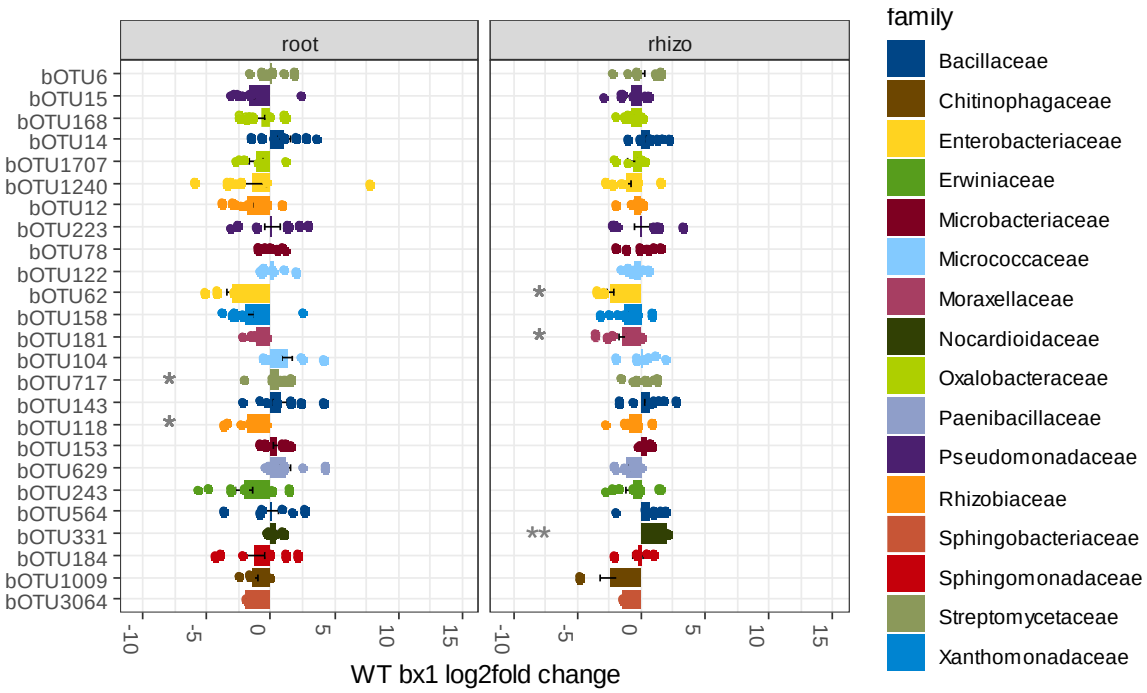
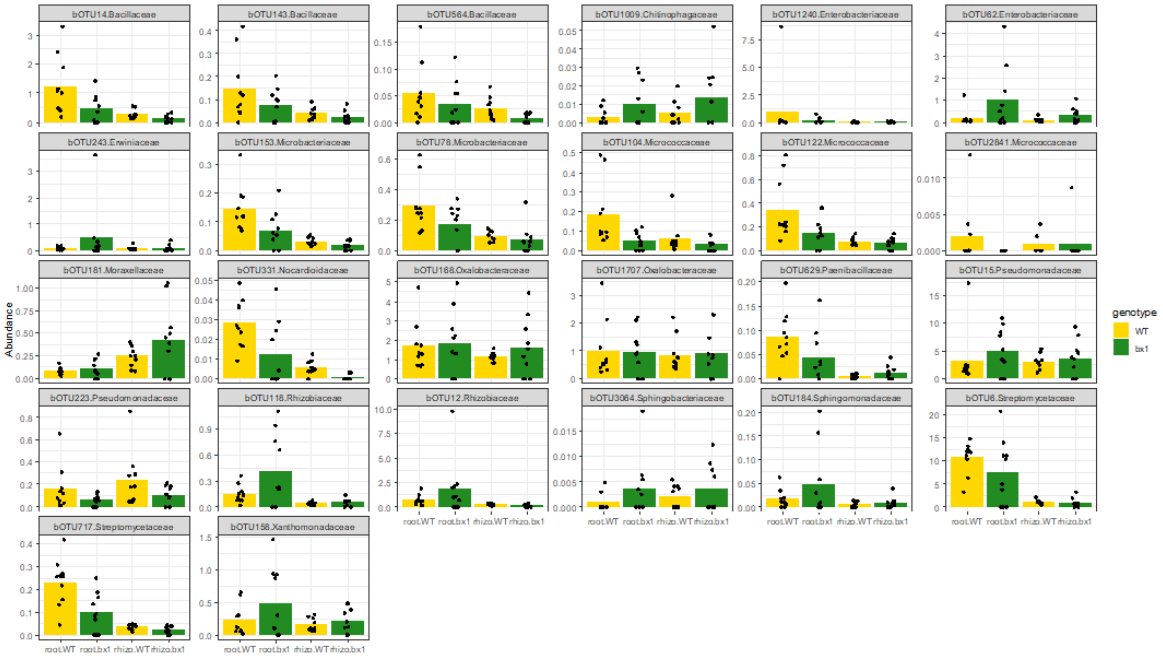


Figure S8: Differential abundance of isolate selected OTUs on roots and in rhizosphere of field grown maize. log2fold change values > 1 indicate OTUs enriched on WT while values < 1 indicate OTUs depleted on WT. Asterisks indicate significant differences of log2fold ratio (t.test).



genotype	OTU	family	p.value	p.value.signif
WT	bOTU14	Bacillaceae	0.01518	*
WT	bOTU143	Bacillaceae	0.039115	*
bx1	bOTU153	Microbacteriaceae	0.04049	*
bx1	bOTU181	Moraxellaceae	0.028461	*
bx1	bOTU118	Rhizobiaceae	0.022012	*
WT	bOTU12	Rhizobiaceae	0.012735	*
bx1	bOTU6	Streptomycetaceae	0.013531	*
bx1	bOTU717	Streptomycetaceae	0.019974	*
WT	bOTU153	Microbacteriaceae	0.00125	**
WT	bOTU78	Microbacteriaceae	0.003559	**
WT	bOTU122	Micrococcaceae	0.008515	**
WT	bOTU181	Moraxellaceae	0.001129	**
WT	bOTU629	Paenibacillaceae	0.001077	**
WT	bOTU118	Rhizobiaceae	0.009594	**
WT	bOTU331	Nocardioidaceae	0.000213	***
WT	bOTU717	Streptomycetaceae	0.000188	***
WT	bOTU6	Streptomycetaceae	7.99E-06	****

compartment	OTU	family	p.value	p.value.signif
rhizo	bOTU564	Bacillaceae	0.034755	*
root	bOTU153	Microbacteriaceae	0.031023	*
rhizo	bOTU153	Microbacteriaceae	0.048017	*
root	bOTU104	Micrococcaceae	0.028989	*
root	bOTU331	Nocardioidaceae	0.026529	*
rhizo	bOTU717	Streptomycetaceae	0.049187	*
root	bOTU717	Streptomycetaceae	0.005895	**
rhizo	bOTU331	Nocardioidaceae	0.000917	***

Figure S9: Strains mapping to greenhouse root and rhizosphere microbiome. OTUs representing mapping strains in **A)** root and rhizosphere microbiome profile of plants grown in the Changins field soil. Asterisks indicate significant differences to the most tolerant strain for each chemical (t.test). **B)** table with t.test for compartment enrichment per genotype and **C)** genotye enrichment per compartment

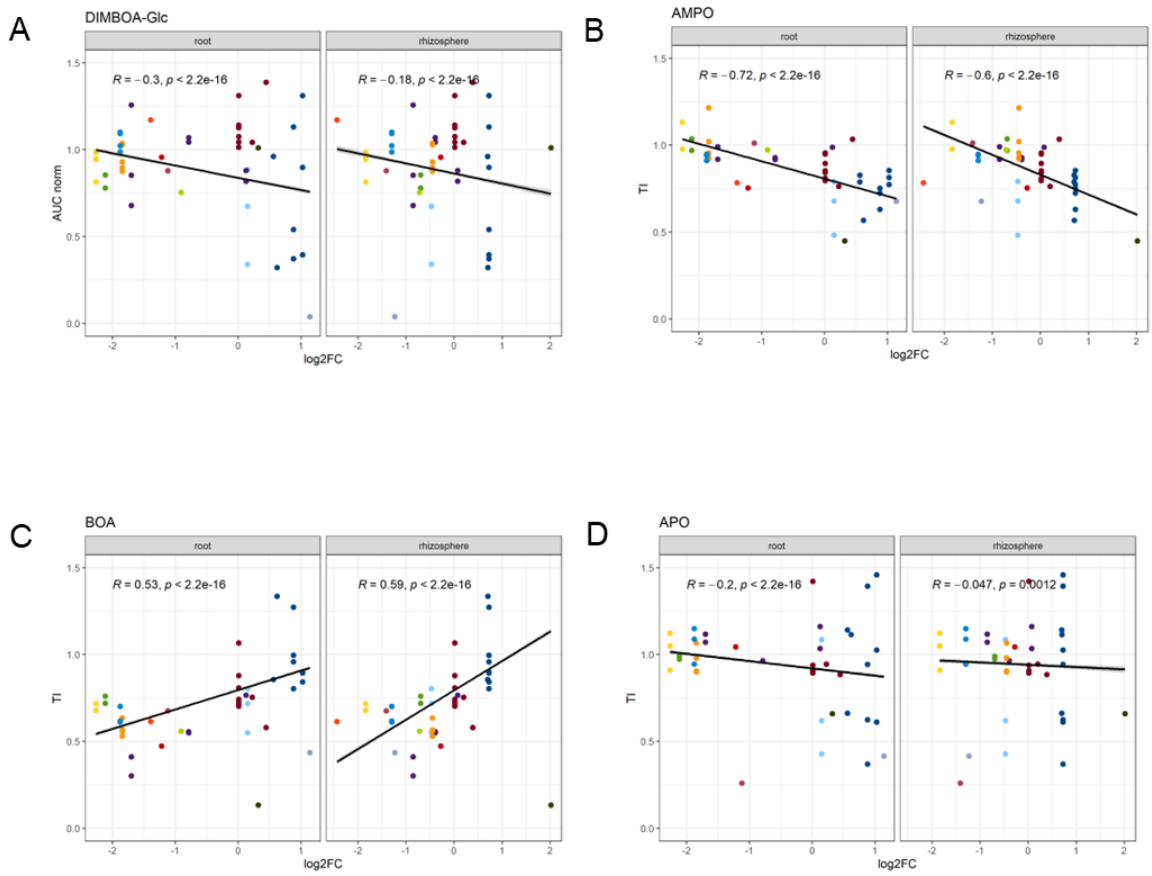


Figure S10. Correlation of tolerance indices of MRB in MBOA to log2FC on WT roots and rhizospheres in the maize field. Correlation on roots and rhizosphere **A)** for DIMBOA-Glc **B)** AMPO **C)** BOA **D)** APO. Pearson's product-moment correlation test was performed. cor, correlation coefficient; p, p-value.

Table S4: Plant growth conditions

Isolation event	Isolation event	Soil collection	Duration	Greenhouse settings	Fertilization	Isolation media	Comment
Isolation 1	Feedback BX+	Changins in 2014	10 weeks	1	unknown	Flour, Pseudomonas	Restreaked on LB
Isolation 2	MBOA	Changins in 2019 (Spring)	14 weeks	2	Weeks 1 - 14	10% TSB MBOA	Restreaked on TSB
Isolation 5	Fridge	Changins in 2019 (Spring)	12 weeks	2	Weeks 1 - 12	10% TSB MBOA	Restreaked on TSB
Isolation 3	Compartment	Changins in 2019 (Spring)	12 weeks	2	weeks 1 - 5	10% TSB MBOA	Restreaked on TSB
NA	Only for CFU count community size	Changins in 2019 (Summer, Valentin)	6 weeks	2	weeks 1 - 6	NA	Only for CFU count community size
Isolation 4	Species	Changins in 2019 (Winter)	7 weeks	2	weeks 1 - 7	10% TSB & 10% TSB MBOA	Restreaked on TSB

Greenhouse settings 1: 26 °C ± 2 °C, 55% relative humidity, 14:10 h light/dark, 50,000 lm m−2

Greenhouse settings 2: 16 h light (26 °C), 8 h dark (23 °C), 50 % relative humidity, ~550 µmol m-2s-1 light

Fertilization regime:

weeks 1 – 4: 100 ml; 0.2 % Plantactive Typ K [3], 0.0001 % Sequestrene Rapid [4]

weeks 5 – 12: 200 ml; 0.2 % Plantactive Typ K, 0.02 % Sequestrene Rapid

3) Hauert HBG Duenger AG, Grossaffoltern, Switzerland

4) Maag, Westland Schweiz GmbH, Dielsdorf, Switzerland

Table S5: Primer sequences

Name	**Target**	**Direction**	**Sequence 5'-3'**	**Reference**
27f	16s rRNA bacteria	forward	AGAGTTTGATCCTGGCTCAG	
1492r	16s rRNA bacteria	reverse	GGTTACCTTGTTACGACTT	Turner et al. 1999
ZmActin1_F	ZmActin1	forward	CCATGAGGCCACGTACAACT	Erb et al. 2009
ZmActin1_R1	ZmActin1	reverse	GGTAAAACCCCCACTGAGGA	
799F	16s rRNA bacteria	forward	*	Schlaeppi et al. 2014
904R	16s rRNA bacteria	reverse	*	Schlaeppi et al. 2014

Table S6: Stock solutions of compounds used for in vitro growth assays

Compound	mol_weight	stock conc [mM]	stock_mg.ml	solvent	work_conc [uM]	vol_stock_1ml [ul]
MBOA	1 maize root bacteria 5	606	100	DMSO	500	0.82
BOA	135.1	500	67.55	DMSO	500	1
DIMBOA-Glc	373.1	500	186.55	DMSO	500	1
AMPO	242.23	15	3.6	DMSO	30	1
APO	212.21	15	3.18	DMSO	30	1
Ctrl	0	0	0	DMSO	2.06 ml/ml	1

Chapter 2

The benzoxazinoid-metabolising N-acyl homoserine lactonase BxdA structures the maize rhizosphere microbiome

Unpublished work

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Abstract

Root-associated microbial communities are shaped by exuded plant specialized metabolites. Yet, little is known about the underlying molecular mechanisms. We investigated how benzoxazinoids released by maize structure rhizosphere microbiota on a phenotypical, genetic and biochemical level. We found that the rhizosphere of benzoxazinoid-producing maize genotypes is specifically enriched in bacterial taxa that metabolise MBOA, the major spontaneous benzoxazinoid breakdown product, to the aminophenoxazinone AMPO. At the same time, MBOA was found to serve as a carbon source for these bacteria. Comparative genomics and transcriptomics of 39 different *Microbacteria* revealed a single gene cluster that was strongly associated with AMPO biosynthesis. Through heterologous expression, we identified BxdA, a N-acyl homoserine lactonase that converts MBOA to AMPO. *BxdA* was strongly enriched in different genera of maize-root associated bacteria. These results demonstrate that plant-produced secondary metabolites structure the rhizosphere microbiome by selecting for metabolisation-competent bacterial strains. *BxdA* represents a novel benzoxazinoid metabolisation gene whose carriers successfully colonize the maize rhizosphere and thereby shape the plant's chemical environmental footprint.

Introduction

Plant microbiomes are important for plant and ecosystem health. Microorganisms form specialized relationships with their hosts, and the microbiome structure and function are tightly controlled by plant factors. Understanding the mechanisms shaping and stabilizing microbiomes are thus important to harness the functions of microbiomes to improve plant health in agricultural systems (French et al., 2021; Singh et al., 2020; Vandenkoornhuyse et al., 2015). Root-associated microbes promote plant growth, provide nutrients, and protect plants from pathogens, but can also act as pathogens themselves (Bulgarelli et al., 2013; Mendes et al., 2013).

A key mechanisms how plants shape root associated microbiomes is through the root exudates (Sasse et al., 2018). Plants secrete up to one-fifth of their assimilated carbon into soil (Nguyen, 2003). Root exudates contain primary metabolites such as sugars, amino acids, organic acids and fatty acids along with plant specialized metabolites. Primary metabolites that nourish the rhizosphere microbes (Canarini et al., 2019). Root microbes can metabolise root exudates and their substrate preference shapes microbial communities (Seitz et al., 2022; Zhalnina et al., 2018). For example the ability to catabolize carbohydrates is enriched in root associated microbes (Levy et al., 2018). On oat roots, synchronisation of microbial metabolism with root exudation is exemplified. The chemical composition of the root exudates changes with plant development and causes simultaneous shifts in the bacterial community, enriching bacteria which use the enriched substrates (Zhalnina et al., 2018). On tomato roots, enriched bacterial genes encode for the metabolism of plant polysaccharides, trehalose, and iron acquisition (Oyserman et al., 2022). Bacteria colonizing the rhizosphere of white lupins belonging to the genus *Burkholderia* metabolise citrate and oxalate (Weisskopf et al., 2006). A seven-member community can metabolise maize root exudates *in vitro*, specifically degrading primary metabolites, while also depleting compounds related to plant specialized metabolites such as benzoxazinoids and flavonoids (Krumbach et al., 2021). These findings support that root exudates are key drivers for community assembly and indicate how primary metabolites mechanistically structure root microbiomes.

Additionally, plants secrete diverse specialized plant metabolites that govern interactions of plants with the environment, protect the plant against insects and pathogens increase abiotic stress tolerance (Erb and Kliebenstein, 2020). Root exuded plant specialized metabolites shape plant species-specific root microbiomes (Jacoby et al., 2020b; Lareen et al., 2016; Pang et al., 2021). For example specialized metabolites produced by *Arabidopsis thaliana* such as glucosinolates (Kudjordjie et al., 2021), camalexins (Koprivova et al., 2019), triterpenes (Huang et al., 2019), and coumarins (Harbort et al., 2020; Stringlis et al., 2018; Voges et al., 2019) structure root microbiomes. The saponin tomatine structures the rhizosphere microbiome of

tomato (Nakayasu et al., 2021). The staple crop maize produces a variety of specialized metabolites, which structure root microbiomes, such as benzoxazinoids (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019; Schütz et al., 2021), diterpenoids (Murphy et al., 2021), zealexins (Ding et al., 2020) and flavonoids (Yu et al., 2021). Mechanistically plant specialized metabolites like benzoxazinoids, coumarins and flavonoids function as antimicrobials, reduce biofilm formation, inhibit quorum sensing, act as signalling molecules for chemotaxis or affect motility. Bacteria also metabolise plant specialized metabolites. Members of the *Arabidopsis* microbiome can use triterpenes as carbon source (Huang et al., 2019). *Sphingobium*, is enriched on tomato roots in presence of tomatine and can use tomatine as carbon source (Nakayasu et al., 2021). The soil bacterium *Pseudomonas mandelii* metabolises 7-hydroxycoumarin and the first step is catalyzed by an alcohol dehydrogenase which is a gene located in the hydroxycoumarin degradation gene cluster (Krikštaponis et al., 2021). Together these findings support the importance of metabolic adaptations for rhizosphere colonisation of bacteria, but it remains largely unknown how these mechanisms act on a community level to shape the rhizosphere microbiome.

Benzoxazinoids are indole-derived plant defence compounds produced by the *Poaceae* family, such as the crops wheat, maize and rye (Frey et al., 2009; Niemeyer, 2009; Wouters et al., 2016). Young seedlings produce high amounts of benzoxazinoids, but shoots and roots produce and exude them throughout plant growth (Dafoe et al., 2011; Zhou et al., 2018). In maize, the methoxylated benzoxazinoids dominate, while rye only produces non-methoxylated benzoxazinoids, and wheat forms a mixture of both (Belz and Hurle, 2005; Frey et al., 2009). DI(M)BOA-Glc is the main benzoxazinoid exuded by maize roots (chemical structures in Fig. S1, complete names in Table S1). (Hu et al., 2018b). Upon release, the glycosides get hydrolysed by glucosidases, for which it is unknown if they are microbe or plant-derived. DI(M)BOA aglucons degrade to the more stable (M)BOA, which is a spontaneous reaction (Macías et al., 2004). In sterile soils, MBOA is stable, but in the soil, microbes convert MBOA to reactive aminophenols (M)AP (Kumar et al., 1993; Niemeyer, 2009; Zikmundová et al., 2002). Aminophenols are further converted on three routes to three different metabolite classes. (I) Aminophenoxazinones A(M)PO may form from a spontaneous reaction in the presence of oxygen (Guo et al., 2022). This can be further acetylated to AA(M)PO. (II) Acetamides H(M)PAA (*MHPA) form by an acetylation reaction from aminophenol. They may be further nitrated. (III) Malonic acids H(M)PMA form by an acylation from aminophenol (Friebe et al., 1998; Nair et al., 1990; Schulz et al., 2013; Schütz et al., 2019; Understrup et al., 2005; Zikmundová et al., 2002). (M)BOA is stable in soil for a few days, while the corresponding 2-amino phenoxazine-3-ones are detectable for months (Macías et al., 2004). While chemical structures and class of benzoxazinoid breakdown have been analytically

well defined and it becomes increasingly clear that soil microbes present key organism in their metabolisation.

Benzoxazinoids have allelopathic properties to inhibit the growth and germination of neighbouring plants. Aminophenoxazinones are more potent allelopathic compounds than the original precursors (Fomsgaard et al., 2004). AMPO inhibits plant growth through inhibition of the histone deacetylase (Venturelli et al., 2015). Benzoxazinoids are multifunctional defensive compounds protecting the plant form pests and pathogens. They deter and toxify insects such as the agricultural pest *Spodoptera littoralis* (Dafoe et al., 2011; Zhou et al., 2018). Benzoxazinoids inhibit the growth of fungal pathogens (Couture et al., 1971; Niemeyer, 2009). The fungal endophyte in maize *seeds Fusarium verticillioides* overcomes benzoxazinoid toxicity by detoxifying BOA to HMPMA. Genes encoding this enzyme were horizontally transferred to other fungal species. This indicates that plant specialized metabolites exert evolutionary pressure on these fungi (Glenn et al., 2016). Exuded from maize roots, benzoxazinoids shape root-associated microbial communities (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2021). Benzoxazinoids inhibit the growth of root bacteria *in vitro* (Schandry et al., 2021). Apart from their antimicrobial activity, possibly due to their ability to intercalate to DNA (Hashimoto and Koichi, 1996), benzoxazinoids chemotactically attract the beneficial rhizobacterium *Pseudomonas putida* (Neal et al., 2012) and inhibit the virulence of *Agrobacterium tumefaciens* (Maresh et al., 2006). Yet, the biochemical mechanisms and the genetic basis of these microbial transformations largely remain to be discovered.

The following studies demonstrate the importance of soil microbes in benzoxazinoid metabolisation. In wheat-grown field soil, aminophenoxazinones are the most abundant benzoxazinoid microbial transformation products (Mwendwa et al., 2021). Limited knowledge exists on the mechanism forming aminophenoxazinones in soil except for the need for oxygen and microbial activity. The bacterial isolate *Acinetobacter* sp. was shown to convert BOA to APO *in vitro* (Chase et al., 1991). A more recent study identified the metal-dependent hydrolase for the degradation of Cl-BOA to Cl-APO in *Pigmentiphaga* (Dong et al., 2016). APO forms in a co-culture of the fungi *Fusarium verticillioides* with the bacterium *Bacillus mojavensis* grown on BOA (Bacon et al., 2007). The acetamide HMPAA forms from BOA in presence of the soil fungus *Fusarium sambucus* (Zikmundová et al., 2002). The malonamic acid HPMA forms from BOA by a metallo-β-lactamase from the specialized maize seed endophytic fungus *Fusarium verticillioides* (Glenn et al., 2016). Recently, bacterial metabolism of DI(M)BOA-Glc, DI(M)BOA and MBOA were studied, and degradation products formed in the expected sequence. Microbes converted DI(M)BOA-Glc to DI(M)BOA and further to (M)BOA and A(M)PO. Further they tested hydroxylated BOA-6-OH, which is the modification of BOA by plants. By a nitration reaction, NBOA-6-OH formed in the

cultures (Schütz et al., 2021). Supplementation of AP to *Pseudomonas* cultures yielded APO or catechol (Zhao et al., 2000). AAP supplementation yielded a coloured precipitate corresponding to the nitro aromatic compound N-(2-hydroxy-5-nitrophenyl)-acetamide (Schütz et al., 2019). Together these findings prove diverse routes for benzoxazinoid conversions by microbes. These studies were performed with soil bacteria isolated from different environments, but bacteria isolated from benzoxazinoid producing roots were not yet investigated for their ability to metabolise benzoxazinoids.

Microbial benzoxazinoid degradation products are more potent allelopathic compounds than their precursors (Fomsgaard et al., 2004). Among those, aminophenoxazinones are the most abundant group in soil (Mwendwa et al., 2021). Thus, it may be beneficial for a plant to associate with microbes converting exuded benzoxazinoids. We hypothesized that BX-producing plants would recruit AMPO-forming bacteria forming potent allelopathic compounds. Further, we sought to identify the specific taxa being capable of the conversion of MBOA to AMPO. To characterize the mechanism, we aimed to identify the bacterial gene responsible this reaction. There are already a few isolates capable of metabolising BOA and related compounds and a few genes known. To expand on this knowledge, we aimed for a more microbiome-based view compared to previous studies. Three main interests guided our research: (I) Where are MBOA-metabolising bacteria most abundant? Do they associate with roots, rhizosphere, or soil compartment? Are they recruited to BX-producing roots? (II) Which bacterial taxa metabolise MBOA? Is this trait taxonomically widespread or restricted to certain taxa? (II) How do maize root bacteria metabolise MBOA? Which are the chemical mechanisms? How is MBOA metabolisation genetically encoded? Thus, we started our investigations by plating maize root extracts on agar plates supplemented with MBOA. Bacteria that form AMPO are abundant community members on maize roots. They are depleted on roots of a BX-deficient maize mutant. We found a subset of maize root bacteria that convert MBOA to AMPO. Those bacteria belong to few and taxonomically distinct lineages. Using comparative genomics, we identified a candidate gene cluster for benzoxazinoid metabolisation. This is different from genes reported in the literature.

Materials and Methods

Plating experiment

To assess the number of AMPO-producing colonies on roots, we grew wild-type maize plants and BX-deficient *bx1*(B73) maize, wheat (CH Claro), *Medicago sativa* (Sativa, Rheinau, Switzerland), *Brassica napus* (Botanik Saemereien AG, Pfaeffikon, Switzerland) and *Arabidopsis thaliana* (Col-0). We grew the plants in soil collected from the field in Changins (Hu et al., 2018b) or 'Q-Matte' (1health soil, Wasmiuddin unpublished, Frauenkappelen, Switzerland) in the greenhouse for 6-7 weeks (Table S2). We harvested the roots and washed 10 cm fragments twice in the sterile distilled water by shaking the tubes 30 times. With a sterile scalpel, we chopped the root fragments into small pieces. Then we transferred them into a 50 ml Falcon tube containing 10 ml sterile magnesium chloride buffer supplemented with Tween20 (MgCl2Tween, 10 mM MgCl2 + 0.05 % Tween, both Sigma-Aldrich, St. Louis, USA). We homogenized the roots with a laboratory blender for 1 minute at 20'000 rpm and vortexed them for 15 s. We resuspended the pellet from the washing step in 5 ml MgCl2Tween. This fraction represents the rhizosphere compartment. For the soil extracts, we mixed 5 g of soil from the pot with 5 ml MgCl2Tween and vortexed it for 15 s.

To quantify bacterial community size, we plated of root, rhizosphere, and soil extracts. We serially diluted the extracts and plated 20 µl on 10 % TSB agar (Sigma-Aldrich, St. Louis, USA) plates (12 x 12 cm, Greiner bio-one, Kremsmünster, Austria) containing cycloheximide (10 mg/l, Sigma-Aldrich, St. Louis, USA) and DMSO (2 ml/l, Sigma-Aldrich, St. Louis, USA). To spread the drops for counting we tilted the plates and incubated them for 6 days at room temperature. We counted colony-forming units (CFU), multiplied them by the dilution factor and normalized them with the sample's fresh weight. Before statistical analysis, we transformed CFU counts by log10.

To count the number of AMPO-forming colonies in the extracts, we spread one dilution on a square agar plate containing MBOA. Depending on the plant species and the compartment, we selected a dilution between 1:10-1 and 1:10-4 to reach a colony density which is countable. We spread the 50 µl of the sample with a delta cell spreader on square agar plates 10% TSB supplemented with cycloheximide and MBOA (200 mg/l, Sigma-Aldrich, St. Louis, USA). For 10 days we incubated the plates at room temperature (21 - 25 °C). We photographed the plates and counted the red colonies on the pictures. To get the proportion of AMPO-forming colonies per sample, we divided the count of AMPO-forming colonies by the total CFU.

Bacterial strains and cultures

Maize root bacteria (chapter 1, this thesis; Thoenen et al. unpublished) and *Arabidopsis* bacteria (AtSPHERE, Bai et al. 2015) were grown on tryptic soy broth plates (TSB, Sigma-Aldrich,

St. Louis, USA) at 25 °C – 28°C or TSB liquid medium. To screen for AMPO-formation of single isolates, we plated a loop of pure bacterial cultures on TSB agar plates supplemented with MBOA (200 mg / l) or DMSO (2 ml / l) as control. We incubated the plates for 10 days at room temperature, assessed the phenotype by eye and photographed the plates

In vitro growth & metabolisation assays

Prior to the setup of the metabolisation assays, we prepared liquid pre-cultures in a 96-well format from fresh plates. Pre-cultures from plates were prepared with freshly picked isolates and inoculated with an inoculation needle (Greiner bio-one, Kremsmünster, Austria) to 1 ml of 50 % liquid TSB in 2 ml 96-well deep-well plates (Semadeni, Ostermundigen, Switzerland), covered with a Breathe-Easy membrane (Diversified Biotech, Dedham, USA) and grown until stationary phase for 4 days at 28°C and 180 rpm.

Before the setup of the metabolisation assays, we prepared liquid pre-cultures in a 96-well format from fresh plates. We filled 1 ml of 50 % liquid TSB in 2 ml 96-well deep-well plates (Semadeni, Ostermundigen, Switzerland). We inoculated colonies from fresh plates (4-7 days old) using an inoculation needle (Greiner bio-one, Kremsmünster, Austria) [KS2]. With a Breathe-Easy membrane (Diversified Biotech, Dedham, USA) we covered the plates. We grew the cultures to the stationary phase for 4 days at 28°C and 180 rpm. To set up the metabolisation assays we inoculated bacterial cultures into the medium with benzoxazinoid compounds. For the stock solutions, we dissolved pure DIMBOA-Glc, DIMBOA, MBOA or BOA (Sigma-Aldrich, St. Louis, USA) in DMSO. Pure DIMBOA-Glc and DIMBOA were isolated from natural source in our laboratory. Stock concentrations differed depending on their solubility (Table S3). For the treatment solutions, we added stock solutions to 50 % liquid TSB. The concentration for all compounds is 500 µM and the DMSO concentration is constant. We filled the treatment solutions to 200 µl 96-well microtiter plates (Corning, Corning, USA) using an 8-channel pipette or a liquid handling system (Mettler Toledo, Liquidator 96™, Columbus, USA). Shortly before the start of the assay, we inoculated 4 µl of the pre-cultures into the plates. We piled the plates with a lid and inserted them into a stacker (BioStack 4, Agilent Technologies, Santa Clara, United States). The plate reader (Synergy H1, Agilent Technologies, Santa Clara, United States) connected to the stacker measures optical density (absorbance at 600 nm) every 100 min over 68 hours. Before each measurement, the reader shook the plates for 120 s. To check for contamination, we included "no bacteria controls" on each plate. Additionally, we included a plate containing only medium. To measure MBOA metabolisation over time, we removed plates from the stack after 16 h, 24 h, 44 h, 68 h and 96 h. This procedure applies to the time-series experiment, the *Microbacteria* screen, the carbon source and BOA assays. For the initial metabolite screen of all maize root bacteria and the transcriptome experiment, we incubated the plates on a laboratory shaker at 28 °C. To avoid

evaporation, we sealed the plates a stripe of Breathe-Easy membrane. We recorded optical density of the cultures at the end of the experiment in a plate reader (Tecan Infinite M200 multimode microplate reader equipped with monochromator optics, Tecan Group Ltd., Männedorf, Switzerland). The initial metabolite screen ended after 68 h and the transcriptome experiment after 16 h. We exported bacterial growth data from the software (Gen 5, Agilent Technologies, Santa Clara, United States) to excel. We used R studio statistical software to calculate the area under the growth curve (x-axis for time and y-axis for OD600).

Assessing MBOA metabolisation in anaerobic conditions

We performed a metabolisation experiment in anaerobic conditions to test the need for oxygen for AMPO formation. As described above, we prepared treatment solutions with 500 µM or 2500 µM MBOA in 15 ml Falcon tubes. Prior to the experiment, we pre-incubated the treatments over three days in a sealed jar under anaerobic environment to remove oxygen from the medium. To start the experiment, we inoculated a loop of bacteria from fresh plates. An anaerobic environment was created for half of the samples with an environment generator according to the manufacture instruction (TRILAB, Jenny Science, Rain, Switzerland). We grew the cultures either under anaerobic or aerobic conditions at in an incubator at 28 °C (Memmert, Schwabach, Germany). After 68 hours of growth, we measured the optical density of the cultures.

Metabolite extraction from bacterial cultures

At the end of the experiment, we examined colour changes in the cultures by eye. To fix bacterial cultures, we added 150 µl bacterial cultures to 350 µl of the extraction buffer (100 % Methanol + 0.14 % formic acid) in non-sterile round bottom 96-well plates (Thermo Fisher Scientific, Waltham, USA). We stored the fixed samples with a final concentration of 70 % methanol and 0.1 % formic acid at -80 °C. To reduce the number of samples, we pooled three replicates of the same culture. For the transcriptome experiment (n =5) and the anaerobic experiment (n =3) we did not pool samples. We diluted the pooled sample by mixing 50 µl to 700 µl MeOH 70% + 0.1 % FA. We filtered the cultures through regenerated cellulose membrane filters (CHROMAFIL RC, 0,2 µm, Macherey-Nagel, Düren, Germany) by centrifugation (6200 rpm for 2 min) to remove bacterial debris. To avoid any residual particles, we centrifuged the cultures at 13'000 rpm for 10 min at 4 °C. We aliquoted the supernatants in glass vials (VWR, Dietikon, Switzerland) and stored the samples for a few days at 20°C until analysis.

Profiling benzoxazinoid degradation products in bacterial cultures

Using an Acquity I-Class UHPLC system (Waters, Milford, US) coupled to a Xevo G2-XS QTOF mass spectrometer (Waters, Milford, US) equipped with a LockSpray dual electrospray ion source (Waters, Milford, US) we quantified benzoxazinoids in samples of filtered bacterial

cultures. Gradient elution was performed on an Acquity BEH C18 column (2.1 x 100 mm i.d., 1.7 mm particle size (Waters, Milford, US) at 98–50% A over 6 min, 50-100% B over 2 min, holding at 100% B for 2 min, re-equilibrating at 98% A for 2 min, where A = water + 0.1% formic acid and B = acetonitrile + 0.1% formic acid. The flow rate was 0.4 ml/min. The temperature of the column was maintained at 40°C, and the injection volume was 1 µl. The QTOF MS was operated in sensitivity mode with a positive polarity. The data were acquired over an m/z range of 50–1200 with scans of 0.1 s at collision energy of 6 V (low energy) and a collision energy ramp from 10 to 30 V (high energy). The capillary and cone voltages were set to 2 kV and 20 V, respectively. The source temperature was maintained at 140°C, the desolvation temperature was 400°C at 1000 L/hr and cone gas flows was 100 L/hr. Accurate mass measurements (<2 ppm) were obtained by infusing a solution of leucin encephalin at 200 ng/mL at a flow rate of 10 µl/min through the Lockspray probe (Waters, Milford, US). For each expected benzoxazinoid compound, four standards with concentrations of 10 ng/ml, 50 ng/ml, 200 ng/ml and 400 ng/ml were run together with the samples (DIMBOA-Glc, DIMBOA, HMBOA, MBOA-Glc, MBOA, BOA, AMPO, APO, AAMPO, AAPO, HMPMA) or 40 ng/ml, 200 ng/ml, 1 µg/ml and 10 µg/ml for MHPA and MAPH)

NMR identification of AMPO

To confirm the presence of AMPO in the bacterial cultures from LMB2 and LSP13, we isolated the red precipitate observed. By ^{1}H NMR spectroscopy (Bruker Advance 300, 1H: 300.18 MHz, Bruker Corp., Billerica, MA, USA) we analysed the sample. Briefly, supernatants of cultures from LMB2 and LSP13 were extracted twice with Et_2O, dried with Na_2SO_4 and filtered. The red precipitate left was collected with acetone. These two organic phases were combined, concentrated under reduced pressure, and dried over P_2O_5. The ^{1}H NMR spectrum of the red residue obtained was recorded in DMSO-d_6 and compared to an authentic AMPO sample, therefore confirming its presence in our bacterial cultures.

DNA extraction, library preparation and genome sequencing

For total DNA was isolated extracted using a DNeasy UltraClean Microbial Kit (Qiagen, 12224-50) according to the protocol provided with the kit. For all *Microbacteria* except LMI1, LMI11, LMI12, LMI13, LMI1x, LMB2, LM3X and LMX7 strains DNA was extracted without kit following the GES method of DNA extraction (Pitcher et al., 1989). Briefly, 2-4 mL of each bacterial strain was grown in TSB at 28 °C overnight, centrifuged for 10 min at 12'000 rpm at RT, the media was removed, and the bacterial pellet was re-suspended in 200 µL TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0). Thereafter 500 µL of GES solution was added to each bacterial suspension and incubated for 10 min at RT, before the addition of 250 µL of 7.5 M ammonium acetate. The mixture was gently mixed and incubated for 10 min on ice. Thereafter, 500 µL phenol chloroform isoamyl alcohol mixture, 25:24:1 (Sigma-Aldrich, St. Louis, USA) was added, vigorously mixed and

centrifuged for 15 min at 12'000 rpm at 4 °C. The upper aqueous layer was transferred to a fresh tube and 500 µL of chloroform isoamyl alcohol mixture 24:1 (Sigma-Aldrich, St. Louis, USA) was added, vigorously mixed, and centrifuged for 15 min at 12'000 rpm at 4 °C. Once again, the upper layer of fluid was transferred to a new tube and mixed with 0.7 vol. 100 % isopropanol, mixed well, and stored at -20 °C overnight. Precipitated DNA was recovered by centrifugation at 12'000 rpm for 15 min at 4 °C. The DNA pellet was washed once with 80 % ethanol and twice with 70 % ethanol. The pellet was dissolved slowly in 80 µL water with the aid of heating at 55 °C for 1 h. The quantity, purity and length of the total genomic DNA was assessed using a Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA), a DS-11 FX spectrophotometer (DeNovix, Wilmington, US) and an FEMTO Pulse System with a Genomic DNA 165 kb Kit (Agilent, Basel, Switzerland), respectively. Sequencing libraries were made using an Illumina DNA Prep Library Kit (Illumina, San Diego, US) in combination with IDT for Illumina DNA/RNA UD Indexes Set C, Tagmentation (Illumina, San Diego, US) according to the Illumina DNA Prep Reference Guide (Illumina, San Diego, US). The input DNA was set at 200 ng and 5 PCR cycles were employed to amplify the fragmented DNA. Pooled DNA libraries were sequenced paired end on a NovaSeq 6000 SP Reagent Kit v1 (300 cycles; Illumina, San Diego, US) on an Illumina NovaSeq 6000 instrument. The quality of the sequencing run was assessed using Illumina Sequencing Analysis Viewer (Illumina version 2.4.7) and all base call files were demultiplexed and converted into FASTQ files using Illumina bcl2fastq conversion software v2.20. All steps from gDNA extraction to sequencing data generation were performed at the Next Generation Sequencing Platform, University of Bern, Switzerland.

PacBio sequencing

The AMPO-forming *Microbacteria* were additionally subjected to PacBio sequencing for high-resolution genomes. DNA was extracted using the GES method using fresh agar plate cultures instead of a pellet grown in liquid culture (Pitcher et al., 1989). Prior to SMRTbell library preparation, bacterial genomic DNA was assessed for quantity, quality and purity using a Qubit 4.0 flurometer (Qubit dsDNA HS Assay kit; Thermo Fisher Scientific), an Advanced Analytical FEMTO Pulse instrument (Genomic DNA 165 kb Kit; Agilent, Basel, Switzerland) and a Denovix DS-11 UV-Vis spectrophotometer, respectively. Multiplexed SMRTbell libraries were prepared for sequencing on the Sequel exactly according to the PacBio guideline entitled: “Procedure & Checklist – Preparing Multiplexed Microbial Libraries Using SMRTbell® Express Template Prep Kit 2.0" - Part Number 101-696-100 Version 08 (November 2021). Concisely, 1 µg of gDNA in 100 µL was used to shear the gDNA using a Covaris g-TUBE (Covaris, Wolburn, US). Subsequently, the sheared gDNA was concentrated and cleaned using AMPure PB beads. The samples were then quantified and qualified to be in the range of 12-15 Kb using a Qubit 4.0 flurometer (Qubit dsDNA

HS Assay kit, Thermo Fisher Scientific, Waltham, USA) and an Advanced Analytical FEMTO Pulse instrument (Genomic DNA 165 kb Kit, Agilent, Basel, Switzerland), respectively. The rest of the procedure as referenced above was followed including removal of single strand overhangs, DNA damage repair, end-repair & A-tailing, ligation of barcoded overhang adapters and then purification of the library using AMPure PB beads. The libraries were quality controlled using the steps described above and then were pooled using the PacBio microbial multiplexing calculator. Prior to and after size selection, the library pool was purified using AMPure PB beads. Size selection was performed a BluePippin instrument (Sage Science, Beverly, US) using BluePippin with dye free, 0.75% Agarose Cassettes and S1 Marker (Sage Science, Beverly, US) wherein the selection cut-off was set at 6000 bp. Library pool concentration and size was again assessed using a Thermo Fisher Scientific Qubit 4.0 flurometer and an Advanced Analytical FEMTO Pulse instrument (as described above), respectively. PacBio Sequencing primer v4 and Sequel DNA Polymerase 3.0 were annealed and bound, respectively, to the DNA template libraries. The polymerase binding time was 1 h and the complex was cleaned using 1.2 X AMPure PB beads. The libraries were loaded at an on-plate concentration of 150pM using adaptive loading, along with the use of Spike-In internal control. SMRT sequencing was performed in CLR mode on the Sequel IIe with Sequel Sequencing kit 3.0, SMRT Cells 8M, a 2h pre-extension followed by a 15 h movie time and via PacBio SMRT Link v10.1. Thereafter, the CCS generation and barcode demultiplexing workflow was run in SMRT Link v10.1. All steps from gDNA extraction to sequencing data generation were performed at the Next Generation Sequencing Platform, University of Bern, Switzerland.

Genome assembly

The raw, paired end fastq sequences generated on Illumina sequencers were trimmed using fastp v. 0.20.1 (S. Chen et al., 2018) with default options. The read quality after trimming was assessed with fastQC v. 0.11.7 (Andrews, 2010). The genomes were assembled using SPAdes assembler v. 3.14.0 (Bankevich et al., 2012) with the options `--isolate –k 21,33,55,77,99,127 --cov-cutoff 'auto'`. The assemblies for samples that were sequenced on a PacBio sequencer were conducted using the continuous long reads (CLRs). The `fasta` sequences of the CLRs were extracted from the BAM files using samtools v. 1.10 (Li et al., 2009). The assembly was then conducted with Flye v. 2.9 (Kolmogorov et al., 2019). Since the same samples were also sequenced on Illumina sequencers, the CLR assembly was corrected with Illumina reads. The reads were first mapped to the assembly using the Burrows-Wheeler Aligner BWA, v 0.7.8 (Li and Durbin, 2009). The resulting SAM file was then sorted and indexed using samtools v. 1.10 (Li et al., 2009) before using Pilon v. 1.24 (Walker et al., 2014) to correct the assemblies.

The quality of the resulting assemblies was assessed with Quast v. 4.6.0 (Gurevich et al., 2013), BUSCO v. 5.1.3 (Seppey et al., 2019) and checked for contamination with ConFindr v. 0.7.2 (Low et al., 2019). The genomes were then annotated with the NCBI procaryotic genome assembly pipeline PGAP, v. 2022-04-14 (Tatusova et al., 2016). The annotated genomes were functionally annotated with EggNog v. 5.0.1 (Huerta-Cepas et al., 2019) and orthologues genes were determined using OrthoFinder v. 2.3.8 (Emms and Kelly, 2019). The annotated assemblies were then integrated into an instance of OpenGenomeBrowser (Roder et al., 2022) hosted at the Interfaculity Bioniformatics Unit (University of Bern).

Comparative genomics

To find genes that are involved in the transformation of MBOA to AMPO, the 39 strains of *Microbacteria* were phenotypically divided into AMPO-positive (n=16) and negative (n=23) groups. Two approaches were investigated independently. The first consisted of grouping the genes into orthogroups with OrthoFinder v. 2.3.8 (Emms and Kelly, 2019) and estimating significant associations between the phenotype and orthogroups applying Fisher's Exact Test using the gene trait matching tool in OpenGenomeBrowser (Roder et al., 2022). In the second approach, a kmer-similarity search strategy was conducted. The scaffolds of the assemblies were first divided into unique kmers of size 21 base pairs and counted using the tool K-Mer Counter v. 3.1.1 (Kokot et al., 2017). The resulting kmer libraries per sample were then merged into a single matrix using custom python scripts. In the next step, the kmers were scored based on their occurrence in AMPO-positive or negative strains. Specifically, the score of a kmer was increased by 1, if the kmer is present in a sample with AMPO-producing phenotype and was decreased by 1 if the kmer is present in a sample with AMPO-negative phenotype. This score can thus be seen as a correlation between genetic sequence and phenotype. The highest scoring kmers were then used to filter genes containing those kmers using custom python scripts. Since this approach relies on exact matches of kmers, the gene sequences containing high-scoring kmers were clustered with a 70% similarity cut-off using vsearch v. 2.17.1 (Rognes et al., 2016). The obtained centroid sequences were then searched with BLAST v. 2.10.0 (Altschul et al., 1990) against a database of all genes from all *Microbacteria* strains using 'blastn'. The BLAST output was filtered for matches with an e-value < 1e50 which resulted in a list of genes for each centroid sequence. These gene lists were then statistically assessed for their association with the phenotype using Fisher's Exact Test in R (v. 4.2.1). The p-values were corrected using the Benjamini-Hochberg method.

Transcriptome analysis

For the transcriptome experiment, bacterial cultures which were grown for 16 h in six individual wells were pooled, immediately stabilized by the addition of RNAprotect Bacteria

Reagent (Qiagen, Hilden, Germany). Bacterial cells were lysed by enzymatic lysis and proteinase K treatment and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with subsequent DNAse treatment using the RapidOut DNA removal kit (Thermo Fisher Scientific, Waltham, USA) following manufactures instructions.

The quantity and quality of the purified total RNA was assessed using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Waltham, USA) and an Advanced Analytical Fragment Analyzer System using a Fragment Analyzer RNA Kit (Agilent, Basel, Switzerland), respectively. One hundred ng of input RNA was first depleted of ribosomal RNA using an Illumina Ribo-Zero plus rRNA Depletion Kit (Illumina, San Diego, US) following Illumina 's guidelines. Thereafter cDNA libraries were made using an Illumina TruSeq Stranded total Library Prep Kit (Illumina, San Diego, US) in combination with TruSeq RNA UD Indexes (Illumina, San Diego, US) according to Illumina ’s reference guide documentation. Pooled cDNA libraries were sequenced paired end using an Illumina NovaSeq 6000 SP Reagent Kit v1.5 (100 cycles Illumina, San Diego, US) on an Illumina NovaSeq 6000 instrument. The run produced, on average, 14 million reads/sample. The quality of the sequencing run was assessed using Illumina Sequencing Analysis Viewer (Illumina version 2.4.7) and all base call files were demultiplexed and converted into FASTQ files using Illumina bcl2fastq conversion software v2.20. The quality control assessments, generation of libraries and sequencing was conducted by the Next Generation Sequencing Platform, University of Bern.

The quality of the RNA-Seq data was assessed using fastQC v. 0.11.7 (Andrews, 2010) and RSeQC v. 4.0.0 2 (Wang et al., 2012). The reads were mapped to the reference genome using HiSat2 v. 2.2.13 (Kim et al., 2019). The reference genome of strain LMB2 was prepared before the mapping step as follows: The General Features Format (GFF) file obtained from the assembly was transformed to the Gene Transfer Format (GTF) using AGAT v0.8.0 (Dainat, 2022) and subsequently transformed to Browser Extensible Data (BED) format using BEDOPS v. 2.4.39 (Neph et al., 2012). The HiSat2 index from the reference fasta file was created using the `hisat2-build` command. FeatureCounts v. 2.0.1 4 (Liao et al., 2013) was used to count the number of reads overlapping with each gene as specified in the genome annotation. The Bioconductor package (DESeq2 v1.32.0 5) (Love et al., 2014) was used to test for differential gene expression between the experimental groups. To annotate the genes with Gene Ontology (GO) terms, the genes from the reference assembly were translated to amino acid sequences using the `esl-translate` command in HMMER3 v. 3.3.2 (Mistry et al., 2013). Pfam domains were then searched using `hmmscan`. GO terms were then mapped to genes and their pfam domains using the pfam2go mapping file (http://current.geneontology.org/ontology/external2go/pfam2go). GO

term analysis was performed using the R Bioconductor package TopGO (Alexa and Rahnenfuhrer, 2022).

Phylogenetic tree construction

The species tree estimation for *Microbacteria* was obtained from OrthoFinder v. 2.3.8 (Emms and Kelly, 2019). The 16s trees were reconstructed as follows: First, the 16S sequences were concatenated and then aligned using MAFFT v. 7.475 (Katoh et al., 2002) with default options. The aligned sequences were then used as input to RAxML v. 8.2.12 (Stamatakis, 2014). The multi-threaded version ` raxmlHPC-PTHREADS` was used with the options ` -f a -p 12345 -x 12345 -T 23 -m GTRCAT` with 1000 bootstrap replicates. The phylogenetic tree was visualized and annotated in R using the package ggtree (Yu et al., 2017).

Confirmation of candidate genes

Plasmids for expression of *bxdA*, *bxdC*, *bxdF* and *bxdM* were ordered from Twist Bioscience. The DNA sequences of the genes were used to generate codon-optimized nucleotide sequences for expression in *E. coli*, applying default settings of Twist. Sequences were introduced to expression plasmid pET28a(+) with BamHI and HindIII restriction sites (Twist Bioscience HQ, San Francisco, US). All genes were amplified with Platinum Superfi polymerase II (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions by using the primers reported (Table S4) and cloned in pOPINF (N-ter His tag) digested with HindIII-HF and KpnI-HF. Cloning was performed with In-Fusion (Takara Bio, Shiga, Japan) according to manufacturer protocol and transformed in chemically competent *E. coli* Top10 (NEB, Ipswich, US) and plated on LB plates supplemented with carbenicillin 100 µg/ml (Sigma-Aldrich, St. Louis, USA). Plasmids were isolated from recombinant colonies and the identity of the inserted sequences confirmed by Sanger sequencing. Next, the constructs were used to transform chemically competent BL21 (DE3) (NEB, Ipswich, US). Correct uptake of the plasmids was verified trough colony PCR with vector specific primers (Table S4). Positive colonies were inoculated in 5 ml LB with carbenicillin 100 µg/ml and grown overnight at 37°C, 220 rpm. 100 µl of the preculture were inoculated in 100 ml 2xYT media with carbenicillin 100 µg/ml and incubated at 37°C, 220 rpm until they reached OD600 = 0.5-0.6. At this point cultures were incubated 15 min at 18°C, 220 rpm and then induced with IPTG 0.5 mM and incubated at 18°C, 220 rpm for 16 h. For purification, the cultures were harvested by centrifugation at 3200g, 10 min and resuspended in 10 mL of buffer A1 (50 mM tris-HCl pH 8, 50 mM glycine, 500 mM sodium chloride, 20 mM imidazole, 5% v/v glycerol, pH 8) supplemented with 0.2 mg/mL Lysozyme and EDTA free protease inhibitor cocktail (cOmplete, Roche, Basel, Switzerland) and incubated for 30 min on ice. Cells were disrupted by sonication using a Sonics Vibra Cell at 40% amplitude, 3s ON, 2s OFF, 2.5 min total time. The crude lysates were centrifuged at 35,000g for 30 min and the cleared lysates incubated with 200 µl Ni-NTA

agarose beads (Takara Bio, Shiga, Japan) for 1h at 4 °C. The beads were then sedimented by centrifugation at 1000g for 1 min and washed 4 times with buffer A1 before eluting the proteins with buffer B1 (50 mM tris-HCl pH 8, 50 mM glycine, 500 mM Sodium Chloride, 500 mM imidazole, 5% v/v glycerol, pH 8). Dialysis and buffer exchange were performed using buffer A4 (20 mM HEPES pH 7.5; 150 mM NaCl) in centrifugal concentrators with size exclusion appropriate for the protein size. Proteins were aliquoted in 50 µl and stored at -20°C. Protein concentration was determined spectrophotometrically at 280nm on an NanoPhotometer N60 (Implen, Munich, Germany) considering molecular weight and extinction coefficient. Protein purity and size was checked trough SDS-Page on Novex WedgeWell 12% Tris-Glycine Gel (Invitrogen, Waltham, US). Protein ladder used was Color Protein Standard Broad Range (NEB, Ipswich, US).

AMPO 0a, C, F, K and M were tested. All reactions were performed in a total volume of 100µl, in 25mM potassium phosphate buffer, pH=7.5 with 5µg protein. AMPO biosynthetic activity was tested by supplementing the enzyme with 1mM MBOA (30mM stock in MeOH, Sigma-Aldrich, St. Louis, USA). In addition, AMPO-C was supplemented with NADP+ and AMPO-M with NADP+ and NADPH. Reactions were initiated by protein addition and incubated at 30°C, 300rpm for 2h in the dark. Reactions were quenched by addition of 100µl MeOH, incubated on ice 15 minutes and then centrifuged at 15.000g for 15min. Then transferred to LC-MS glass vials.

LC-MS analysis were performed on a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Waltham, USA) equipped with Phenomenex Kinetex XB-C18 column (100 x 2.1mm, 2.6 µm, 100 Å, column temperature 40°C) coupled to a Bruker EVOQ Elite electrospray ionization. Analytical conditions consisted of A: H_2O + 0.1% FA and B: ACN, 0.6ml/min flow with the following gradient: 0-1 min, 15% B, 1-6 min, 15-35% B, 6.1-7.5 min, 100% B, 7.6-10 min, 15% B. Mass spectrometry data were acquired through ESI with capillary voltage of 3500 V and end plate offset of 500 V, nebulizer pressure of 2.5 bar with a drying gas flow of 11.0 l/min and a drying temperature of 250°C. Acquisition was performed at 12 Hz with a mass scan range from 80 to 1000 m/z. For tandem mass-spectrometry (Ms^2) collision energy, the stepping option model (from 20 to 50 eV) was used.

Statistical analysis

We used R version 4.0 (R core Team, 2016) for statistical analysis and visualization of the data. For the analysis of bacterial colonization, we used log transformed data. We checked for normality using Shapiro-Wilk-test. Using t-test or ANOVA we tested for variance. For the calculation of bacterial growth curves, we used the auc function from the MESS package (Ekstrøm, 2016). We calculated the relative growth by normalizing it with the growth in the control treatment (AUC norm). Raw chromatogram data were peak integrated using MassLynx 4.1 (Waters, Milford, US), using defined properties for the reference compounds in the standards. We

used the following packages for data analysis and visualizations: Tidyverse (Wickham et al., 2019), Broom (Robinson, 2014), DECIPHER (Wright, 2016), DESeq2 (Love et al., 2014), emmeans (Lenth et al., 2019), ggthemes (Arnold, 2019), pheatmap (Kolde, 2019), multcomp (Hothorn et al., 2008), phyloseq (McMurdie and Holmes, 2013), phytools (Revell, 2012), vegan (Oksanen et al., 2019) in combination with some custom functions.

Results

AMPO-forming colonies are abundant microbiome members on benzoxazinoid-producing maize roots

During preliminary experiments with maize root bacteria, we observed that some strains form a red precipitate when exposed to MBOA. Chemical analysis by UPLC-MS and revealed that these bacteria degrade MBOA and form the red precipitating degradation product AMPO, thus explaining the observed phenotype (Fig. S1d). To confirm that the precipitate mainly consists of AMPO, we subjected it to NMR analysis. The ^{1}H NMR spectrum of the red residue obtained was similar to an authentic AMPO sample, confirming that the red precipitate is AMPO (Fig. S7).

To investigate the prevalence of MBOA-to-AMPO metabolisation in bacterial communities, we plated maize root, rhizosphere and soil extracts of benzoxazinoid producing wild type maize plants and benzoxazinoid-deficient *bx1* mutant plants on MBOA-containing agar plates and counted red colonies (Fig 1a). In extracts from wild type plants ~7.7 % of the root bacteria, ~5.8% of the rhizosphere bacteria and ~11.4% of the soil bacteria formed AMPO (Fig. 1b). In extracts from *bx1* mutants, the proportion of bacteria forming AMPO decreased by more than 50% ($p < 0.014$, Fig 1b). In a second experiment, we plated extracts of wild type plants grown in two different soils and found AMPO-forming colonies in both soils (Fig. S2a).

To further test the specificity of enrichment of AMPO-forming bacteria to roots of BX-producing plants, we compared root extracts from maize with wheat (*Triticum aestivum*), which produces different types of benzoxazinoids (Corcuera et al., 1982; Niemeyer, 2009; Quader et al., 2001) and with lucerne (*Medicago sativa*), oilseed rape (*Brassica napus*) and *Arabidopsis* (Fig. 1c), all not producing benzoxazinoids. We found the highest proportion of AMPO-forming colonies on WT maize roots (7.7 %), followed by *Brassica* (1 %), *Triticum* (0.5 %), *Medicago* (0.07 %) and *Arabidopsis* (0.002 %). These findings highlight that AMPO-forming bacteria are enriched in proximity of BX-producing maize roots.

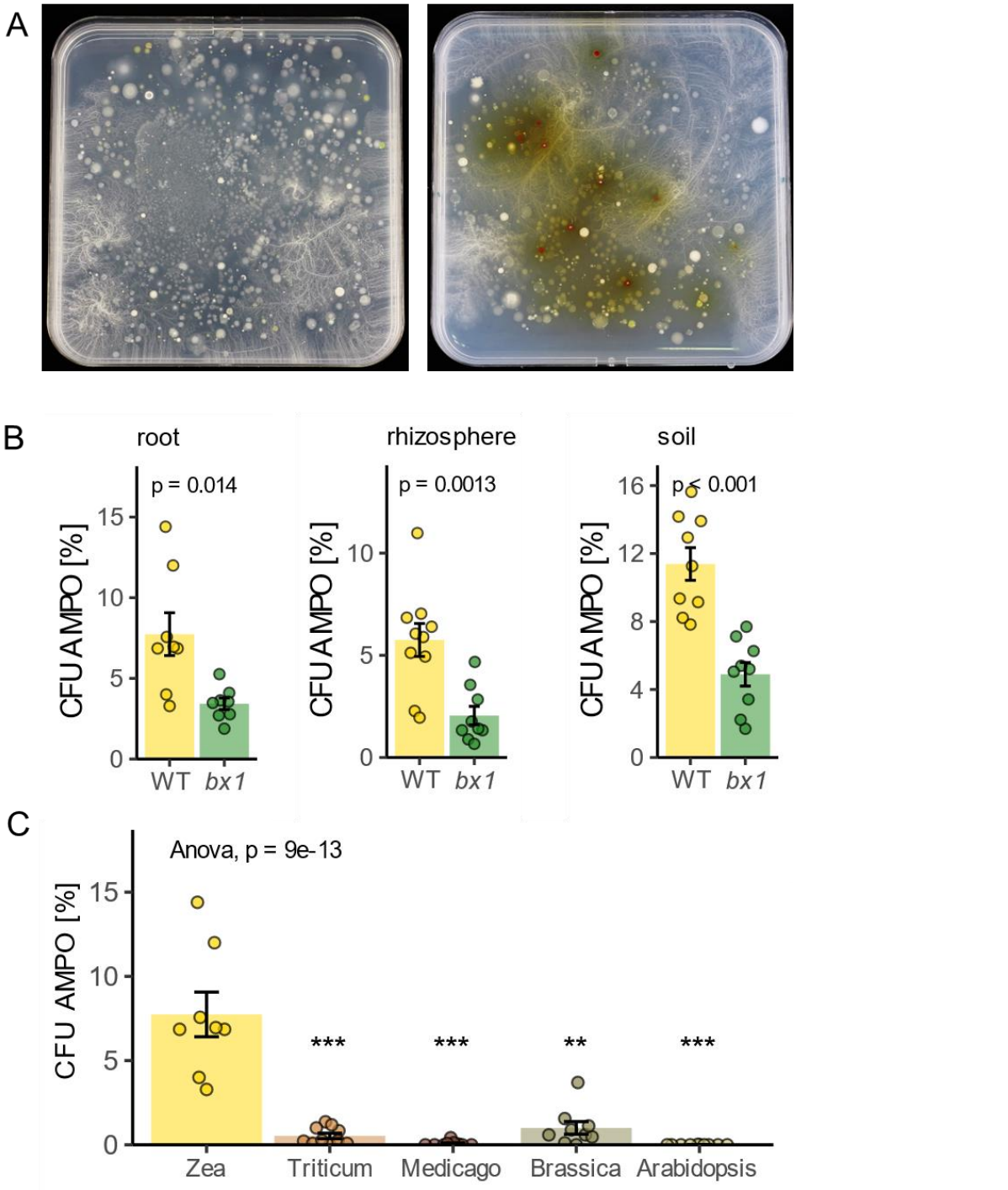


Figure 1. AMPO-forming colonies are abundant microbiome members on BX-producing maize roots. A) Maize root extracts plated on bacterial growth medium supplemented with DMSO (left) and MBOA (right) grown for 10 days. AMPO-forming colonies appear red on the MBOA-supplemented medium. **B)** Percentage of colony forming units (CFU) of AMPO-forming colonies on wild-type (WT) or benzoxazinoid-deficient *bx1* mutant maize roots, in rhizosphere and soil. **C)** AMPO-forming colonies on BX producing plants (*Zea, Triticum*) and non-BX-producing plants (*Medicago, Brassica, Arabidopsis*). Means ± SE bargraphs and individual datapoints are shown (n = 10, except WT n = 8, *bx1* n = 9). Results of pairwise t-test or ANOVA are shown inside the panels.

AMPO-formation is a taxonomically widespread in maize-associated bacteria

To find the taxonomic groups of bacteria capable of AMPO formation, we screened 151 strains of maize root bacteria using MBOA-containing agar plates (Fig. 2a). We identified 38 strains belonging to six different genera from two different phyla which produced AMPO when grown on MBOA-containing agar plates (Fig. 2c). *Microbacteria* (17) and *Pseudoarthrobacter* (3) of the phylum Actinobacteriota and *Sphingobium* (Pseudomonadota) (6) showed a strong dark red colour on plates, while *Enterobacter* (4), *Rhizobium* (6) and *Acinetobacter* (1) belonging to Pseudomonadota did colour the medium weaker red (Fig. S3). Chemical screening by UPLC-MS confirmed that all these strains form AMPO in the presence of MBOA in liquid culture (Fig. 3a). Thus, AMPO-formation is a taxonomically widespread trait in maize root bacteria.

To test whether MBOA-degrading bacteria can use MBOA as a carbon source, we tested a subset of strains in a minimal medium with MBOA as a sole carbon source (Fig. S4). Most of the strains could not grow in minimal medium supplemented with MBOA. These strains were previously identified to not metabolise MBOA (Fig. 2c). Certain strains were able to grow in minimal medium supplemented with MBOA, indicating that they can use MBOA as a carbon source. These strains correspond to the strains which previously identified to metabolise MBOA (Fig. 2c). At the same time, we also observed the red precipitate in the cultures, indicating that they form AMPO as a side product when metabolising MBOA. Together this points to a potential benefit of MBOA degradation for bacterial fitness in the rhizosphere.

As a comparison, we also screened a collection of *Arabidopsis* root bacteria (AtSphere, Bai et al. 2015) using the same plating assay. We only found a weak color change for 23 of the 200 strains (Fig. 2d). A subset of strains with no (10 strains) or slight color change (10) were tested for their ability to degrade MBOA and form AMPO in liquid cultures. As positive controls, AMPO-forming strains from the maize root bacteria collection were tested (LAC11, LMB2 and LSP13). None of the *Arabidopsis* strains metabolised significant amounts of MBOA (Fig. S5). Two strains (Root1280 and Root423) formed small amounts of AMPO. These findings confirm that AMPO-formation is largely specific for bacteria isolated from maize roots.

To confirm the prevalence of AMPO-forming bacteria in the maize rhizosphere using a cultivation-independent approach, we leveraged root microbiome data from different greenhouse and field experiments (Cadot et al. 2021). We mapped the 16S Sanger sequences of the AMPO-forming strains (Fig. 2c) to the microbiome data of maize grown in the greenhouse, in the fields of Changins (CH), Reckenholz (CH) and Aurora (US) (Fig. S2b). We found that community members corresponding to AMPO forming strains (*Sphingobium, Enterobacter, Rhizobia, Acinetobacter, Microbacterium* and *Pseudoarthrobacter*) accounted for 9% on maize roots grown in Changins field soil in the greenhouse, 2.9% on roots from the Changins field, 14.9%

on roots from Reckenholz, and 6.7% from Aurora. These results show that taxonomic units that map to AMPO-forming bacteria are widespread on maize roots growing in a variety of environments.

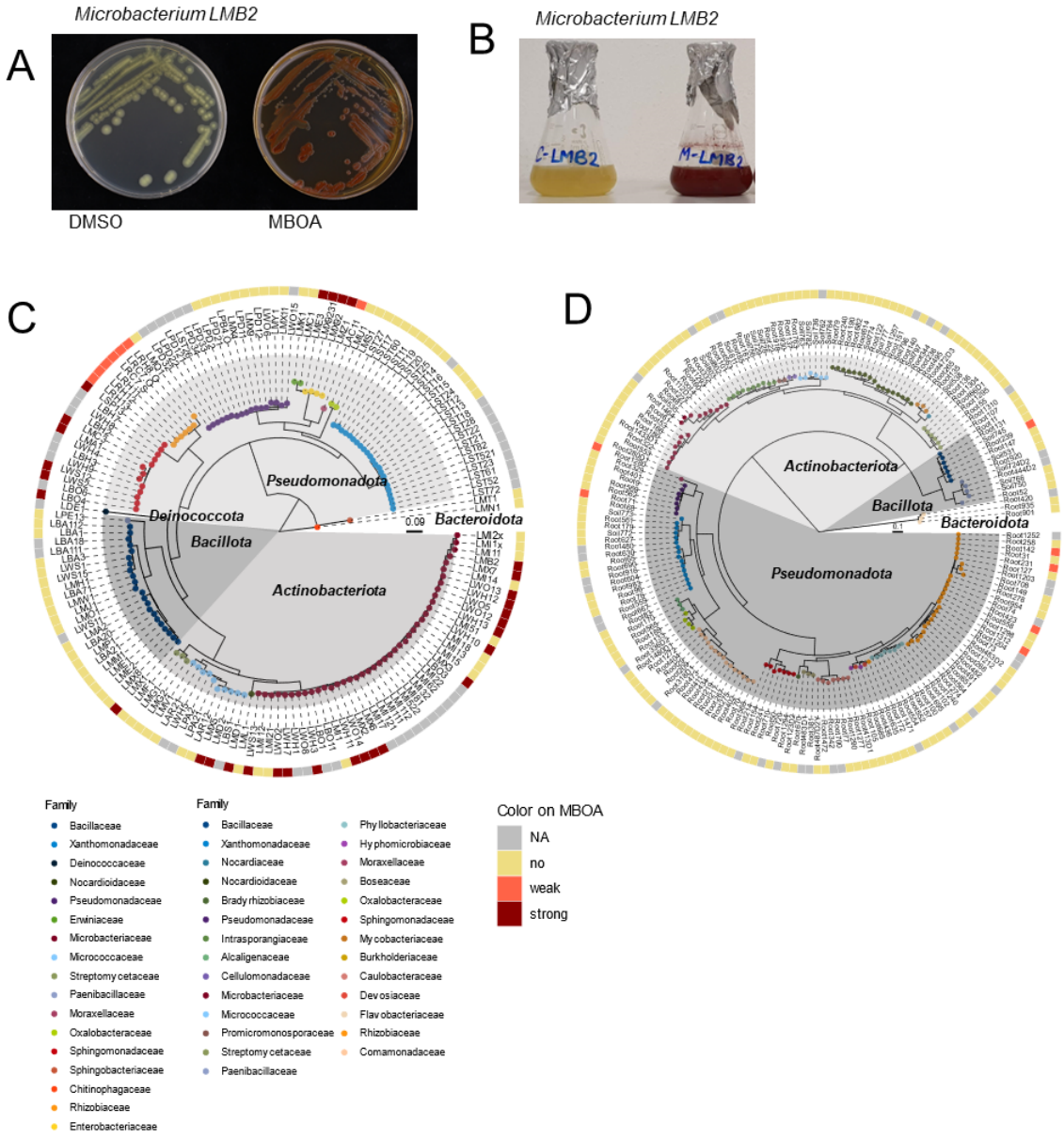


Figure 2. Benzoxazinoid degradation is a taxonomically widespread trait specific to maize root bacteria. A) Pure culture of AMPO-producing strain *Microbacterium* LMB2 on agar plates supplemented with DMSO (left) of MBOA (right) **B)** same in liquid medium. **C)** Phylogenetic tree of maize root bacteria and **D)** *Arabidopsis* root bacteria based on 16s rRNA sequences. Nodes represents isolates, node colour represents family assignment, shading indicates phylum assignment. Color of box indicates AMPO formation of the strain.

Detailed chemical characterization of benzoxazinoid metabolisation reveals multiple pathways

To obtain a quantitative chemical characterization of MBOA metabolisation, we exposed a set of 50 strains of maize root bacteria to 500 µM MBOA in liquid cultures and used targeted UPLC-MS analyses to quantify MBOA metabolisation. We identified 37 strains that did not

metabolise MBOA (Fig. 3a), while 13 other strains did degrade significant amounts of MBOA (< 350 µM MBOA compared to the control, Fig. 3a) and formed AMPO (> 0.13 µM). Three strains, LMB2, LMX7 and LME3 also formed AAMPO, a metabolisation product of AMPO. To investigate the specificity for MBOA degradation of maize root bacteria, we exposed a subset of strains to the non-methoxylated compound BOA. We found that all MBOA degrading strains also degraded BOA and two strains formed small amounts of APO (LMB2 and LMD1) (Fig. S6d). Thus, the ability to metabolise MBOA does not require the methoxy group.

To test if AMPO-forming bacteria can metabolise precursors of MBOA, we tested DIMBOA-Glc as a substrate. This compound is directly exuded by maize roots, is deglycosylated to DIMBOA on the root surface, with the latter then forming MBOA (Fig. S1a). As DIMBOA forms MBOA spontaneously (Fig. S6c), we did not include it in the experiment. For 25 strains of maize root bacteria, we did not find any evidence for DIMBOA-Glc metabolisation. For the other half of the strains, we found DIMBOA metabolisation (< 170 µM DIMBOA-Glc compared to control). For 16 strains, we detected MBOA (> 14 µM) and of those, 9 strains could form AMPO (> 1 µM) and of those, 3 strains could form AAMPO (> 1 µM) (Fig. 3b). 6 strains completely degraded DIMBOA-Glc, without any detectable metabolites forming (Fig 3c). Thus, additional metabolisation pathways apart from MBOA-AMPO conversion are present in maize root bacteria.

To characterize the temporal dynamics of MBOA degradation, we performed a time-series experiment with six selected strains where we detected AMPO formation in previous experiments (*Sphingobium* LSP13, *Microbacterium* LMB2, *Pseudoarthrobacter* LMD1, *Enterobacter* LME3, *Acinetobacter* LAC11 and *Rhizobium* LRC7.O) as well as some strains where we detected color changes on agar plates (*Pseudomonas* LMX9) or did not detect any degradation (*Bacillus* LBA112 and *Microbacterium* LMI1x) as negative controls. We measured benzoxazinoid metabolite profiles after 16h, 24h, 44h, 68h, and 96h and we confirmed strong AMPO formation for *Sphingobium* LSP13, *Microbacterium* LMB2, and *Pseudoarthrobacter* LMD1 (Fig. 3d). We detected almost a molar conversion of MBOA to AMPO by *Microbacterium* LMB2. In contrast, the formation of AMPO only partially explained the strong MBOA degradation by *Sphingobium* LSP13 and *Pseudoarthrobacter* LMD1, indicating potential alternative degradation routes of MBOA or further degradation of AMPO. For *Enterobacter* LME3 and *Acinetobacter* LAC11, we only detected small amounts of AMPO at the last time point, while we did not detect AMPO in cultures of *Rhizobium* LRC7.O as well as in the negative strains *Pseudomonas* LPD2, *Bacillus* LBA112 and *Microbacterium* LMI1x. These findings further indicate that different types of MBOA metabolisation mechanisms operate across maize root bacteria.

Literature suggests that MBOA is metabolised to the reactive intermediate MAP. Two MAP molecules would spontaneously react to form AMPO in presence of oxygen (Guo et al., 2022). To

confirm the requirement for oxygen in AMPO formation, we grew bacterial cultures in MBOA in anaerobic conditions. We found that AMPO formation was inhibited in anaerobic conditions (Fig. S9a). However, this result must be interpreted with caution, as bacterial growth was also strongly reduced in anaerobic conditions (Fig. S9b).

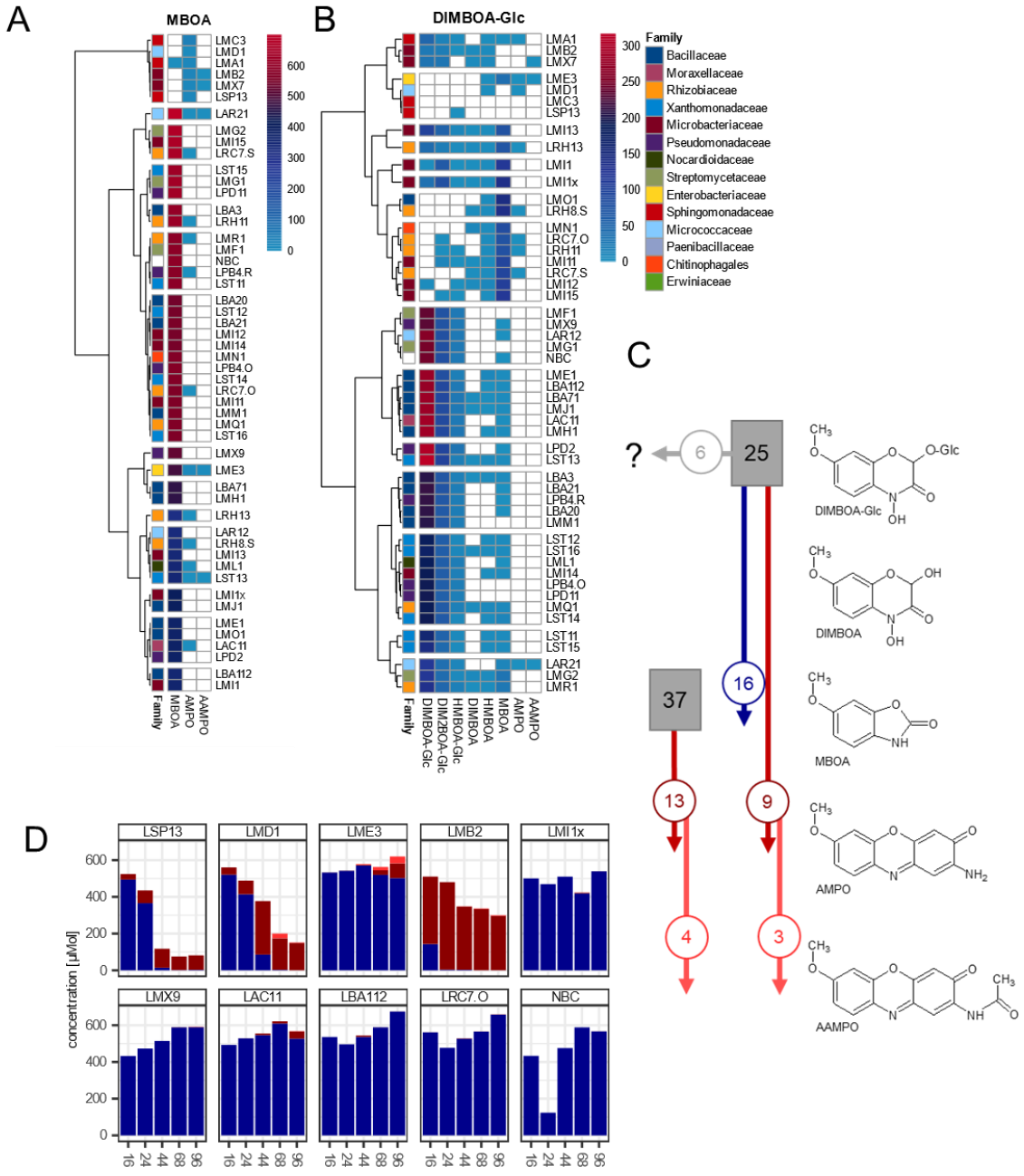


Figure 3. Complete degradation of benzoxazinoids by maize root bacteria. A) Hierarchically clustered heatmap displaying concentrations of MBOA and its metabolisation products in uMol from MBOA supplementation experiment. **B)** Same heatmap displaying DIMBOA-Glc and its metabolisation product from DIMBOA-Glc supplementation experiment. **C)** Overall metabolisation potential of DIMBOA-Glc and MBOA by MRB (50). **D)** Metabolisation products of MBOA over a time series (16h, 24h, 44h, 68h, 96h) for single strains. All measurements were made from three independently grown samples which were pooled in equal ratios prior to metabolite analysis.

AMPO formation in *Microbacteria* is associated with a specific gene cluster

To analyse the genetic basis for AMPO formation, we took advantage of the phenotypic heterogeneity in AMPO formation within the *Microbacteria* (Fig. 2c). We used 39 *Microbacteria* strains from maize (n=21) and *Arabidopsis* (n=18), with 16 strains capable to form AMPO. We first confirmed the AMPO formation capabilities on plates with metabolite analyses of all strains in liquid cultures (Fig. 4). All the 16 strains forming a red precipitate on MBOA-containing agar plates, also degraded MBOA and formed AMPO in liquid cultures (Fig. 4, Fig. S11a). Additionally, the metabolite analyses uncovered three strains (LTA6, LWH12, LWO13) capable of full MBOA degradation. For these strains, we detected AMPO degradation when pure AMPO was supplemented to the cultures (Fig. S11c). Thus, these strains were classified as MBOA-AMPO metabolisers for the analysis. All AMPO-positive *Microbacteria* were also able to use MBOA as a carbon source (Fig. S10). For comparative genomics, we included the genomes of these 39 *Microbacteria*.

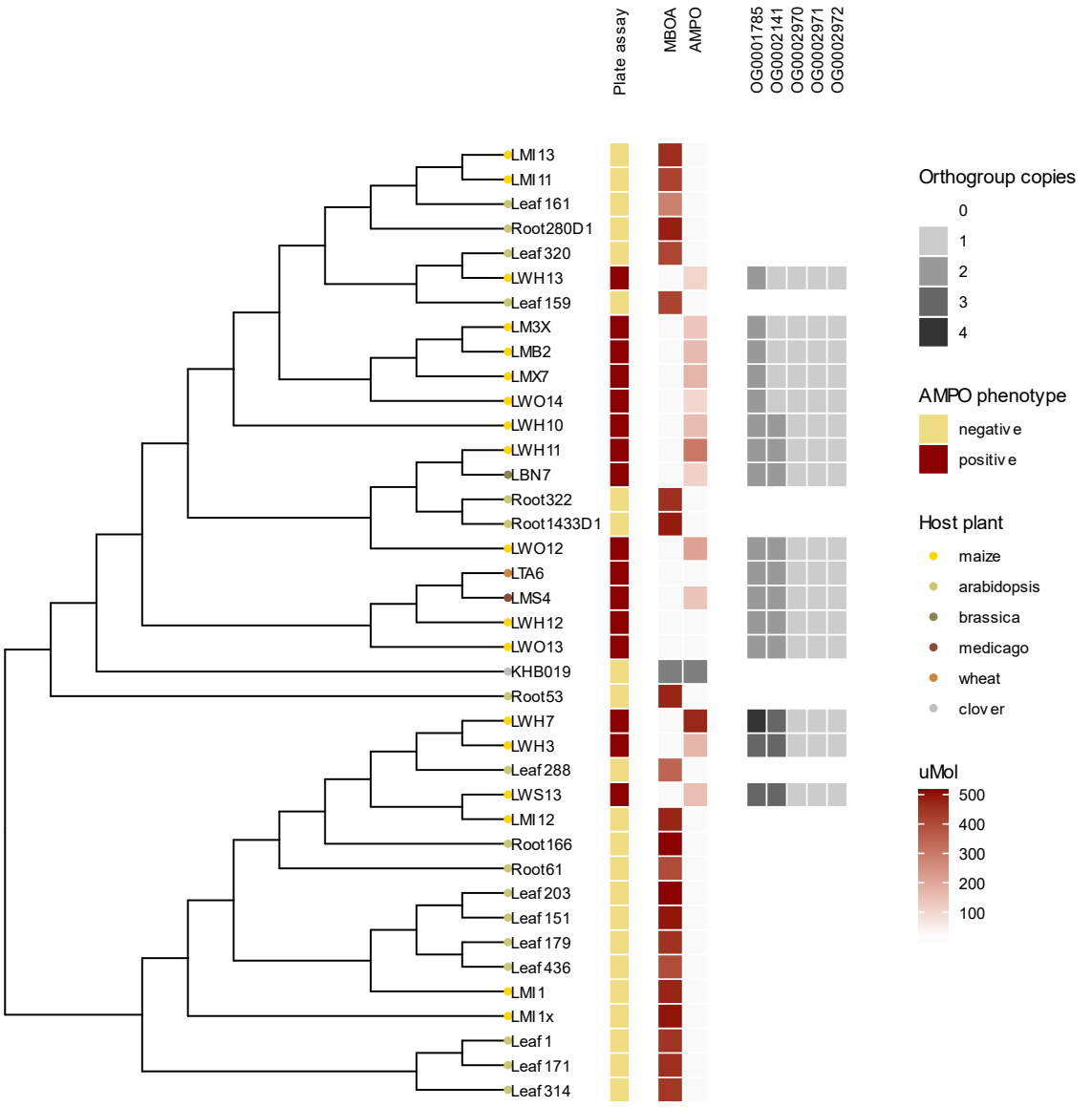


Figure 4. MBOA metabolisation of *Microbacteria*. Phylogenetically clustered heatmap displaying the phenotypes of the strains on the plate assay, along concentrations of MBOA and the metabolisation products AMPO in µMol from MBOA supplementation experiment. Strain KHB019 was not tested in metabolisation experiment. The heatmap in the third row displays the presence of the 5 selected orthogroups in *Microbacteria* across strains. Node colour indicates AMPO phenotype, node shape represents the host plant the strains were isolated from. All measurements were made from three independently grown samples which were pooled in equal ratios prior to metabolite analysis.

First, we used the genomes to search for orthologous gene groups associated with AMPO-positive strains using OrthoFinder (Emms and Kelly, 2019). Orthogroups are groups of related genes thought to originate from a single gene in the last common ancestor of a clade of species. We found five orthogroups which uniquely occurred in AMPO-positive strains (Table S6). Orthogroups OG0002970, OG0002971, and OG0002972 were present as single copies (Fig. 4). OG0001785 is present in two copies in all strains, except for three strains where it was present in three copies (LWS13 and LWH3) or even four (LWH7). OG0002141 was present in two copies,

but in three copies in three strains (LWS13, LWH3, LWH7). These orthogroups map to five different genes from the AMPO-positive strains.

Complementary to the orthogroup approach, we screened the genomes for short sequence strings that were associated with AMPO-positive strains using a custom k-mer approach. We identified a total of 377 k-mers with a score >=7 which were found in 1482 genes among all samples. Clustering these genes with a 70% identity threshold resulted in a total of 145 centroid sequences which were then searched using BLAST against all genes. The Fisher's exact test resulted in 17 gene clusters with significant associations with the phenotype (Fig. 5a, Table S7).

To further confirm putative candidate genes, we performed a transcriptome experiment. We grew the AMPO-positive *Microbacterium* LMB2 for 16 h in MBOA and in DMSO as a control and we measured growth, metabolite profiles, and total gene expression. In this short growth period, essential transcripts for AMPO formation should stay active. We found similar cell numbers of LMB2 in both DMSO and MBOA (Fig. S12a) and found a complete metabolisation of MBOA and high concentrations of AMPO formed during this short growth period (Fig. S12b). The transcript analysis revealed 2.8 % of differentially regulated genes (108 genes) with 14 being downregulated and 94 upregulated (Fig. 5a, Table S8).

Finally, the results from the orthogroups, the k-mer apprach and the transcriptome analysis were combined. In the case of LMB2, for which the results of all three methods were available, we found 3 genes that were present in all three approaches (Fig. 5a). Mapping the candidate genes with support by at least two of the three methods to the genomes of AMPO-positive strains revealed that 15 of them were located closely to each other, collectively pointing to a gene cluster for AMPO formation in *Microbacteria* (Fig. 5b). This gene cluster contained five orthogroups present in AMPO-positive and absent in AMPO negative *Microbacteria* (Fig. 5c) and eight genes that were detected with the k-mer approach. Transcripts of the entire gene cluster were significantly upregulated in presence of MBOA, corroborating an active role of this gene cluster in AMPO-formation.

We termed the identified cluster of 15 genes as *b*enzo*x*azinoid *d*egradation cluster naming the genes in sequence *bxdA* – *bxdO* (Fig. 5b). These genes encode 13 enzymes and two transcriptional regulators. The gene cluster is organized in two operons: *bxdD, bxdE*, *bxdF* build operon 1, *bxdG*, *bxdH bxdI*, *bxdJ* and *bxdK, bxdM* and *bxdN* are organized as operon 2 (Fig. S5c).

Table 1: List of genes present in *bxd* gene cluster

Gene	Annotation	Type
bxdA	N-acyl homoserine lactonase family protein	Enzyme
bxdB	RidA family protein	Enzyme
bxdC	acyl-CoA dehydrogenase family protein	Enzyme
bxdD	aldehyde dehydrogenase family protein	Enzyme
bxdE	thiamine pyrophosphate-dependent enzyme	Enzyme
bxdF	2-oxo acid dehydrogenase subunit E2	Enzyme
bxdG	VOC family protein	Enzyme
bxdH	GntR family transcriptional regulator	Transcriptional regulator
bxdI	acyl-CoA dehydrogenase family protein	Enzyme
bxdJ	flavin reductase	Enzyme
bxdK	RidA family protein	Enzyme
bxdL	M24 family metallopeptidase	Enzyme
bxdM	LacI family DNA-binding transcriptional regulator	Transcriptional regulator
bxdN	NAD(P)-dependent oxidoreductase	Enzyme
bxdO	NADPH-dependent F420 reductase	Enzyme

For in-depth analysis of the gene cluster across *Microbacteria*, we performed high-resolution sequencing of the AMPO-positive strains using PacBio. Aligning the *bxd* gene cluster across all strains revealed four types of cluster architectures (Fig. 5d). Interestingly, the types of cluster architectures agreed with the observed MBOA metabolisation phenotypes (Fig. 5d, S10a). Gene cluster type I is present in five strains (LMX3, LMB2, LMX7, LWO14 LWH13) and is characteristic of partial MBOA metabolisation and pure AMPO accumulation (strains form AMPO as the only degradation product). Gene cluster type II contains five additional genes and is found in four strains (LWH10, LWH11, LBN7, and LWO12) which form besides AMPO also AAMPO, and HMPAA. A third gene cluster, present in four strains (LMS4, LTA6, LWH12, and LWO13), corresponded to bacteria that metabolised MBOA without accumulation of AMPO. Finally, the fourth gene cluster type, containing many duplications of orthogroups, was found in three strains (LWH7, LWH3, and LWS13) which completely metabolised MBOA and formed a lot of AMPO (like group 1). All the AMPO-forming *Microbacteria* strains also degraded DIMBOA-Glc to a certain extent (Fig. S11b). This fine-grained analysis revealed multiple variants of the *bxd* gene cluster possibly representing multiple metabolic pathways of benzoxazinoid degradation in *Microbacteria*.

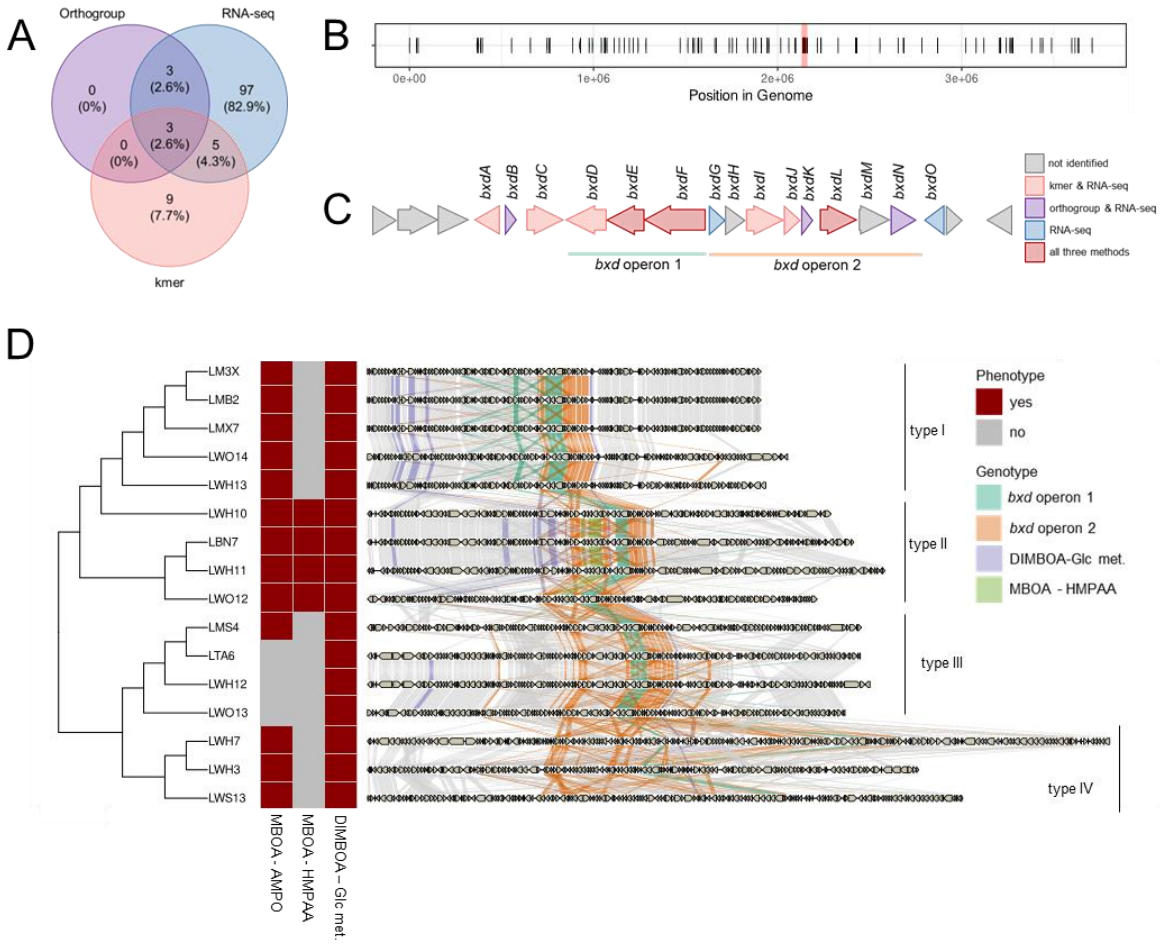


Figure 5. *bxd* gene cluster in *Microbacteria* A) Venn diagram visualizing the overlap of candidate genes identified with the 3 approaches: orthogroups, kmers and in RNAseq **B)** Position of *bxd* gene cluster in the genome of *Microbacterium* LMB2 **C)** Architecture of *bxd* gene cluster in *Microbacterium* LMB2. It includes 15 genes, named *bxdA* - *bxdO*. **D)** *bxd* gene cluster across all *Microbacteria* is present in four types (type I, type II, type III and type IV) corresponding to their phenotype.

A lactonase converts MBOA to AMPO

To determine which genes in the *bxd* cluster may be responsible for MBOA metabolisation to AMPO, we heterologously expressed four candidate genes of the cluster in *E. coli* and purified their enzymes for *in vitro* assays with MBOA. The candidate genes were selected according to their probable biochemical functions, making them likely candidates for MBOA metabolisation. The purified extracts from *E. coli* transformed with the empty vector as well as with the genes *bxdC*, *bxdF* and *bxdM* were unable to degrade MBOA (Fig. S12a). The protein extract enriched for *bxdA* (Fig. S12b) degraded MBOA and led to the accumulation of AMPO in these reactions (Fig. 6a). *bxdA* is about 34 kDa and is encoded by the first gene in the cluster. The annotation as a N-acyl homoserine lactonase family protein is in accordance with the presumed first reaction to degrade MBOA- the breaking of the lactone-like ring, possibly at the NH position in the MBOA molecule (Fig. 6b). Here we demonstrate that the N-acyl homoserine lactonase from *Microbacteria* is sufficient to convert MBOA to AMPO.

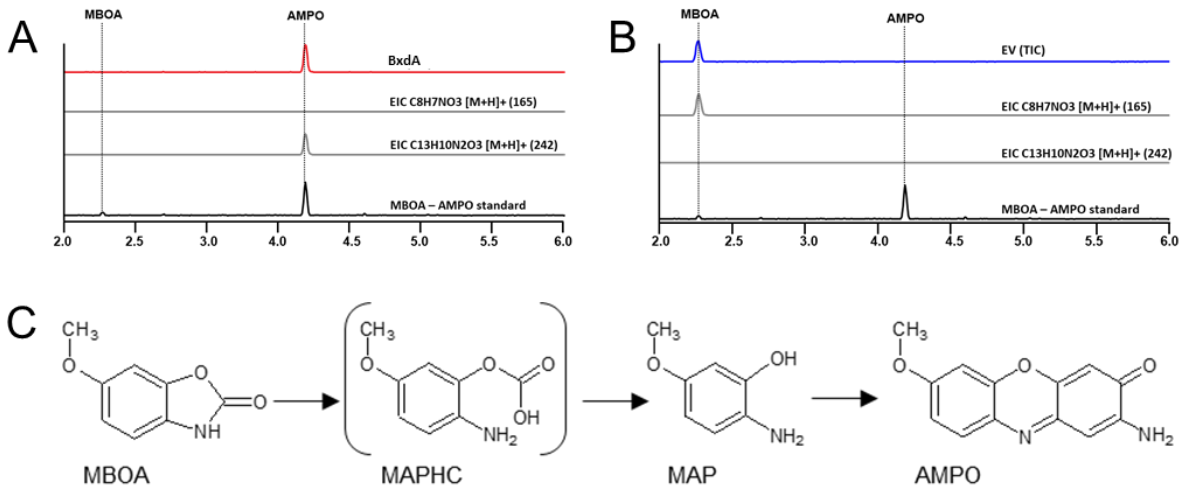


Figure 6: Heterologous expression of N-acyl homoserine lactonase bxdA in *E. coli* converts MBOA to AMPO. A) High pressure liquid chromatography-mass spectrometry (HPLC-MS) full scan (positive mode) of the purified enzyme *bxdA* in MBOA and **B)** for the empty vector (EV). **C)** Schematic illustration proposed for conversion of MBOA to AMPO by *bxdA*. Potential intermediate MAPHC is proposed but was not confirmed. MAPHC = (2-Amino-5-methoxyphenyl) hydrogen carbonate.

Lactonase *bxdA* is specific to maize root bacteria

Having identified an enzyme capable of MBOA degradation in *Microbacterium* LMB2, we wondered how widespread the corresponding gene is across AMPO-forming *Microbacteria* and in other bacterial lineages. We found that all AMPO-positive *Microbacteria* possess a homologous lactonase with high sequence similarity (76.25 – 100 %. Fig. S13a). A homologue of the N-acyl homoserine lactonase from *Microbacterium* LMB2 was also found to be present in the AMPO-forming *Pseudoarthrobacter* strains (LMD1) and *Sphingobium* strains (LSP13, LMA1 and LMC3) (Fig S13b). The gene variants in *Sphingobium* and *Pseudoarthrobacter* showed high similarity in amino acid sequences (58.9 and 78.93%, respectively). In *Pseudoarthrobacter* we found all the genes identified as *bxd* gene cluster type I in LMB2, except the aldehyde dehydrogenase family protein. This aligned with the chemical profile of *Pseudoarthrobacter* producing just AMPO (Table S5 and S8). In *Sphingobium* we found three copies of *bxdA*. In proximity of the lactonase, we identified several genes present in the *bxd* gene cluster of LMB2, including the aminopeptidase P family protein, NAD(P)-dependent oxidoreductase, VOC family protein and two copies of the MFS transporter (Table S5 and S8). The similarity of the genetic architecture in these three strains indicates the importance of the *bxd* gene cluster for benzoxazinoid metabolisation.

Beyond our collection of maize root bacteria, we blasted the *bxdA* gene against the NCBI data base, where we identified similar genes in other bacterial taxa (Sayers et al., 2021). The most similar *bxdA*-like genes were identified in the *Micrococcaceae* family annotated as N-acyl homoserine lactonase family proteins, specifically in *Arthrobacter* sp. isolated from rice (77.89 % identity) and followed by *Leucobacter* sp. (76.17 %) (Fig. S14c, Table S9). We also identified hits for *bxdA*-like genes in more distantly related bacteria, specifically in members of the

Pseudomonodata specifically in the family of *Burkholderiaceae* like *Paraburkholderia* sp. (63.82%) or in the family of *Pseudomonadaceae*, namely in *Pseudomonas poae* (59.67%) isolated from sugar beet.

Interestingly, the identified lactonase *bxdA* shared some sequence similarity (<44%), with phenazine synthesis genes (*phzA*) of several *Pseudomonas* and *Streptomyces* spp. reported to have similar functions (Fig. S14d). With the metal-dependent hydrolase which converts the BOA derivate CDHB to the aminophenoxazinone derivate CAPO, *bxdA* shares 42.58% identity. Compared to the metallo-ß-lactamase in *Fusarium pseudograminearum* converting BOA to AP, *bxdA* is 30.11% identical. Overall, the BLAST analyses suggest that the lactonase *bxdA*, a single enzyme responsible for the conversion of MBOA to AMPO, is specific for maize root bacteria and is rarely present in other bacterial genomes.

Discussion

Plants recruit distinct root microbial communities from soil by exuding primary and specialized metabolites (Jacoby et al., 2020a). The latter are important for the species-specific microbial communities forming on the root surface (Hu et al., 2018b), but the mechanisms underlying this phenomenon are not well understood. Here, we demonstrate that maize benzoxazinoids structure the maize root microbiome by favouring bacteria that can metabolise MBOA into AMPO. We identify a gene cluster containing an N-acyl homoserine lactonase that converts MBOA to AMPO (BxdA). We show that BxdA-carrying bacteria of different taxa are specifically enriched on maize roots, thus establishing BxdA as a likely molecular driver of root microbiome structuring. Here, we discuss the mechanisms and biological context of these findings.

Metabolic adaptation of microbiome members to host secondary metabolites shapes the root microbiome

Root associated microbes are known to metabolise secondary metabolites from their host plants. For instance, root-associated microbes from *Arabidopsis* metabolise host synthesized triterpenes (Huang et al., 2019). Several fungi and bacteria are also known to metabolise benzoxazinoids (Bacon et al., 2007; Chase et al., 1991; Glenn et al., 2016; Schulz et al., 2018; Schütz et al., 2019; Zikmundová et al., 2002). An example is the fungal endophyte colonizing maize seeds which can detoxify BOA to HMPMA. An associated set of genes was horizontally transferred to other fungi living in association with BX-producing sweetgrasses (Glenn et al., 2016). However, what is unknown so far is whether the capacity to metabolise host-plant specific compounds is a trait that structures microbial communities. Here, we demonstrate that maize root bacteria are efficient at degrading benzoxazinoids via a variety of routes. A major pathway is the production of AMPO from MBOA, which is present in two different phyla representing five different genera. Importantly, the capacity to metabolise MBOA to AMPO is strongly enriched in bacteria growing on the maize root surface compared to root surfaces of other plants. Enrichment of these bacteria is also indicated in field samples of various origins. Inhibiting benzoxazinoid biosynthesis in maize is sufficient to reduce AMPO producing bacteria by 50% demonstrating a direct link between benzoxazinoid production and MBOA metabolisation. Thus, the capacity to metabolise secondary metabolites directly structures the root microbiome. Given the high degree of microbiome specificity and the widespread nature of plant specialized metabolites, we propose that this mechanism may structure root microbiomes across the plant kingdom.

Mechanism of MBOA metabolisation and AMPO formation in the maize root microbiome

Several molecular and biochemical mechanisms are known by which single bacteria can metabolise plant specialized metabolites, including *Pseudomonas* which can degrade 7-

hydroxycoumarin catalyzed by an alcohol dehydrogenase (Krikštaponis et al., 2021) or *Herbaspirillum* which can catabolize the flavonoid naringenin through a monooxygenase (Marin et al., 2016). However, the molecular mechanisms that allow root bacteria to cope with exuded benzoxazinoids remain largely unknown (Schütz et al., 2019). Here, we found that maize root bacteria can metabolise MBOA as well as DIMBOA-Glc. Some form MBOA from DIMBOA-Glc, and a few can form AMPO, others degrade it to unidentified compounds. Strains degrading MBOA can also degrade the non-methoxylated relative BOA. This implies the involvement of an enzyme acting on the 5-ring structure of the benzoxazinoid molecule independent of the methoxy group. To date, only a few examples of microbes metabolising BOA to APO exist. These include an *Acinetobacter* strain and a co-culture of *Bacillus* with the fungus *Fusarium* (Bacon et al., 2007). However, the genetic basis of MBOA metabolisation is unclear. Combining multiple genomic and transcriptomic methods, we identified a gene cluster only present in AMPO-forming bacteria. Among the 15 genes in the cluster, there are 13 enzymes and two transcriptional regulators. We found the first enzyme in the gene cluster *bxdA* to metabolise MBOA to AMPO. This gene is annotated as N-acyl homoserine lactonase which is a metalloenzyme known to hydrolyse the ester bond of the homoserine lactone ring (Hopwood, 1997). In line, the identified lactonase cleaves the ester bond of the lactone ring in MBOA, yielding a reactive intermediate which would dimerize to form AMPO in presence of oxygen.

We find gene homologs of the lactonase of *Microbacterium* in the AMPO-forming *Pseudoarthrobacter* (78.93 % similar on amino acid sequence) and *Sphingobium* (58.86 %) strains. In *Sphingobium* LSP13 this gene is also upregulated when in a culture exposed to MBOA. This suggest that the same enzyme as in *Microbacteria* is also responsible for AMPO formation in other genera. To fully confirm the involvement of those genes, they need to be tested by heterologous expression separately. The N-acyl homoserine lactonase is known to degrade the N-acyl homoserine lactones produced by gram-negative bacteria for quorum sensing (Dong et al., 2001). They occur in various taxa (Kusada et al., 2019). The amino acid sequence of the tested N-acyl homoserine lactonase from *Microbacterium* LMB2 only shares 42.58 % identity with the metal-dependent hydrolase (CbaA) handling this the conversion of CBOA to CAPO in *Pigmentiphaga* (Dong et al., 2016). The metallo-β-lactamase from *Fusarium pseudograminearum* (Kettle et al., 2015) is 30.11 % similar. The most similar protein sequence is found in a *Leucobacter sp.* (family *Microbacteriaceae*) sharing 76.17 % sequence identity according to NCBI database (Sayers et al., 2021) and in *Burkholderia pyrrocinia* sharing 65.1 % sequence similarity according to GMGC database (Coelho et al., 2021). Thus, the lactonase gene in *Microbacteria* represents a new bacterial gene for benzoxazinoid degradation. Further the fact that there are only a few similar enzymes found, points to a highly specific genetic adaption to benzoxazinoids

restricted to root microbiomes associated with BX-producing plants. It would be interesting to find whether a similar coevolution also occurred for other plant specialized metabolites.

Within the AMPO-forming *Microbacteria* we found different types of the BXD gene cluster. The types of clusters match the strains' ability metabolising benzoxazinoids like DIMBOA-Glc and formation of other degradation products (HMPAA). This finding opens the door to further investigate the enzymes responsible for further benzoxazinoid conversions. Further these additional genes may encode for enzymes responsible for further degradation of MBOA to be used in the carbon cycle, which is supported by the finding that all AMPO forming *Microbacteria* can use MBOA as a sole carbon source for growth (Fig. S10). A similar gene cluster has been identified in Pigmentiphaga which can degrade completely 6-Chloro-2-benzoxazolinone (CDHB) and use it as a carbon source for growth (Dong et al., 2016). This hypothesis is supported by the fact that MBOA degrading maize root bacteria can use MBOA as a carbon source for bacterial growth. At the same time, they still form AMPO, which raises the possibility that they may primarily degrade MBOA as a carbon source and AMPO is formed as a side product. Knocking out the lactonase in *Microbacteria* and make non-functional mutants may be a good way to disentangle the functions of MBOA degradation for bacterial physiology. However, *Microbacteria* are an understudied genus and no protocols for genetic manipulation exist yet.

Biological relevance of AMPO formation

The metabolisation of secondary metabolites is likely to have multiple functions and biological consequences. Bacteria are generally thought to process secondary metabolites for detoxification, suppression of other microbes or use as carbon source (Blair et al., 2015; Cycoń et al., 2019). We find that AMPO-forming bacteria can use MBOA as a carbon source, conceivably by partially syphoning the intermediate MAP into their own metabolism. Thus, the advantage of being able to process is, in this case, not necessarily related to AMPO formation, but to the effective degradation of MBOA. Nevertheless, the production of AMPO may confer advantages, as it may allow the bacteria to expand their niche by suppression other microbes. Aminophenoxazinones act against both Gram-positive and Gram-negative bacteria (Kozlovskiĭ et al., 2004). The maize endophytic fungus *Fusarium verticillioides* metabolises BOA to a less toxic form (HMPMA). Co-culturing *F. verticillioides* with the bacterium *Bacillus mojavensis* causes APO formation. Since the fungus is susceptible APO, the bacterial APO formation inhibits fungal growth (Bacon et al., 2007). The identification of the lactonase BxdA as a mechanism of MBOA metabolisation and AMPO formation opens opportunities for manipulative approaches that test the benefits of this traits for maize root bacteria.

Plants themselves may benefit from recruiting bacteria that are able to process their secondary metabolites into other bioactive compounds. Aminophenoxazinones such as AMPO are

well-studied allelopathic compounds that can inhibit neighboring plants (Venturelli et al., 2015). This improves fitness of the host plant. Maize plants may thus recruit AMPO producing bacteria to suppress weeds. AMPO may control pathogens. Aminophenoxazinones inhibit agricultural pathogens, such as *Mucor miehei*, *Fusarium verticillioides*, and *Sclerotium rolfsii* (Bacon et al., 2007; Maskey et al., 2003). Thus, bacterial aminophenoxazinone formation may protect the maize rhizosphere from fungal pathogens. Thus, we suggest that the enrichment of AMPO producing bacteria could benefit both the plant and the bacteria carrying these traits, leading to a more stable and beneficial microbiome.

Conclusion

Plant-microbe interactions play an important role in shaping ecological communities and agricultural productivity (French et al., 2021). Understanding the role of plant specialized metabolites in shaping plant microbiomes is thus important for both fields. Our work shows that maize benzoxazinoids structure the maize root microbiome by favouring bacteria that can metabolise MBOA into AMPO. In general, plant specialized metabolites and root microbiomes interact to determine plant growth and defense (Hu et al. 2018). Through altering chemical profiles in the soil, benzoxazinoid metabolising maize root bacteria may suppress weeds and protect maize plants from fungal pathogens in the rhizosphere. From an agricultural point of view, the uncovered mechanisms may expand the use of benzoxazinoids to structure maize root microbiomes and may be employed to create more stable and healthier microbiomes, helping the plants to supress weeds and certain microbes, ultimately enhancing crop productivity.

Author statement

Experiments were planned and conducted by L.T. Co-authors contributed to the development of the analytical protocol and the running of the metabolite samples on the HPLC, the screening of the Arabidopsis strains for AMPO formation (Fig. 2d), the bioinformatic analysis for comparative genomics (Fig. 4 and Fig. 5), the transcriptome analysis (Fig. 5) and the anaerobic experiment (Fig. S9). Co-authors performed the NMR analysis (Fig. S7) and the experiments for the heterologous expression of the candidate genes in *E. coli* (Fig. 6).

Acknowledgements

We thank the Prof. Dr. Julia Vorholt and Prof. Dr. Paul Schulze-Lefert for sharing the *Microbacteria* strains from the AtSphere collection. Further we thank Thomas Roder for the development and support with the open genome browser and Dr. Pamela Nicholson from the Next-generation sequencing platform in Bern for technical support in with sequencing. This work was supported by the Interfaculty Research Collaboration "One Health" of the University of Bern.

Supplementary figures

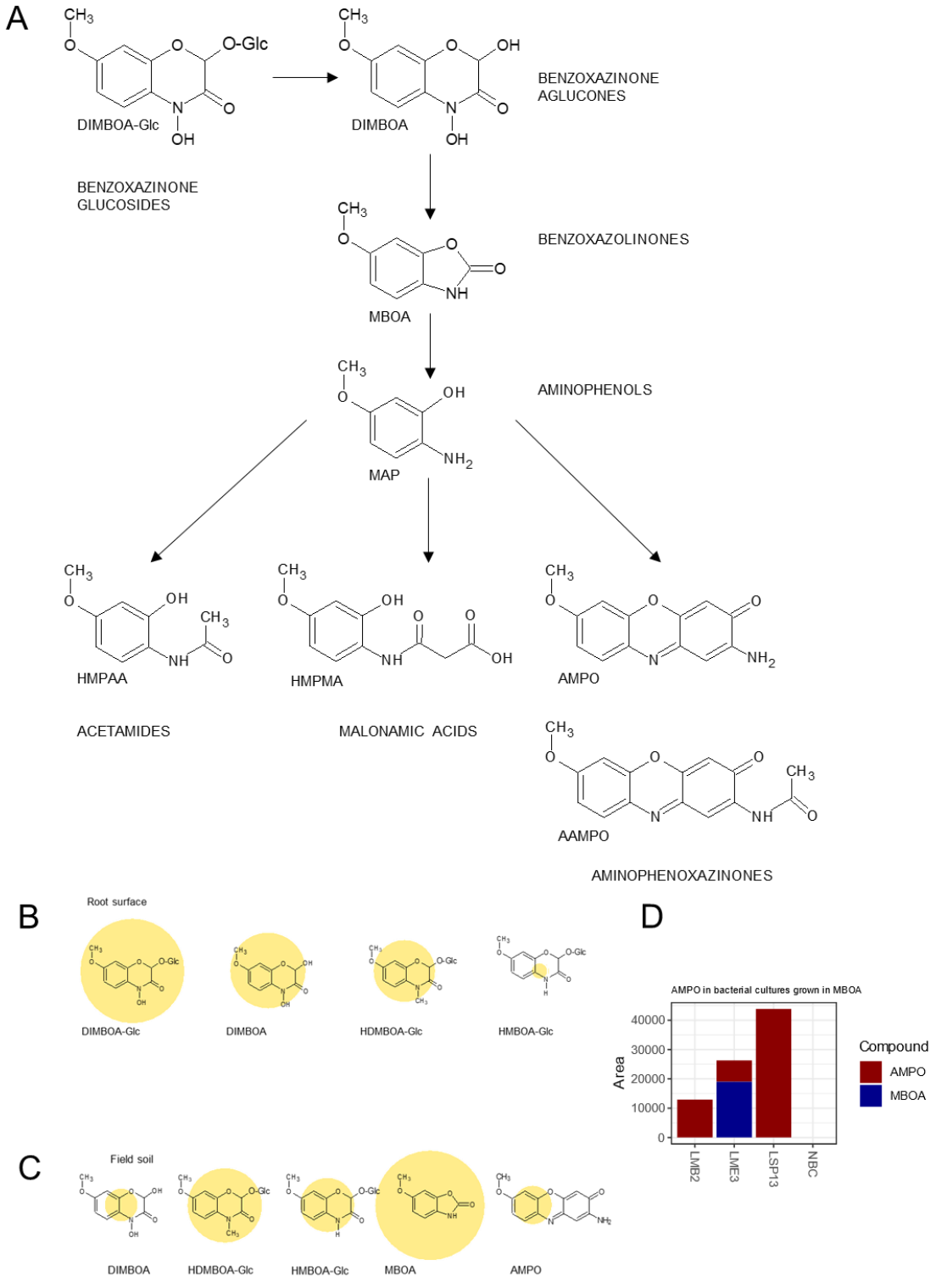


Figure S1: Benzoxazinoid metabolites in the rhizosphere. A) Benzoxazinoid degradation pathways in soil reported in literature. **B)** Exudation profiles of maize roots and **C)** in field soil. Size of bubble represents total amount of specific benzoxazinoid compounds detected. Data from Hu et al. 2018 presented. **D)** MBOA to AMPO conversion in three pure bacterial cultures.

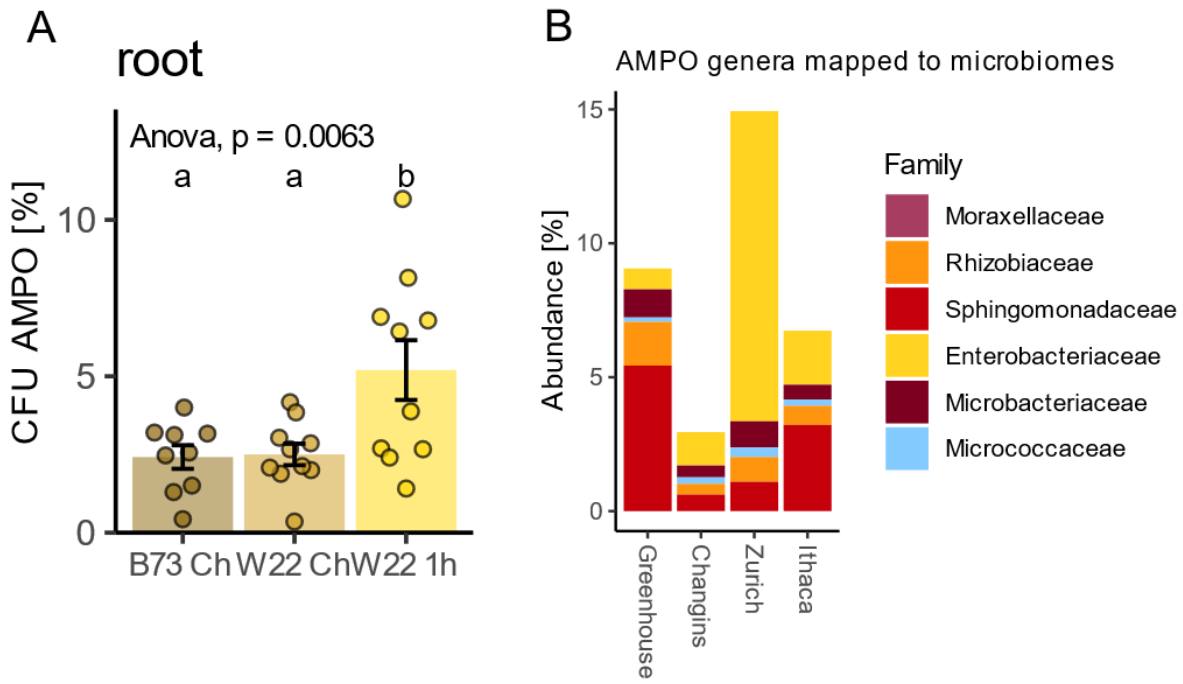


Figure S2: AMPO-forming colonies are abundant microbiome members on BX-producing maize roots. A) Percentage of colony forming units (CFU) of AMPO-forming colonies on WT maize roots W22 background and B73 background grown in 1health and Changins soil. Means ± SE boxplots and individual datapoints are shown (n = 10, except B73 in Ch soil n = 9). Results of ANOVA are shown inside the panel. Different letters indicate significant differences among treatments using least-square means (emmeans). **B)** Cumulative relative abundance of taxonomic units in field soil represented by AMPO-forming isolates. Datasets from greenhouse experiment with field soil and fields in Switzerland (Changins and Zurich) and the US (Ithaca), Hu et al. 2018 and Cadot et al. 2021 were used for this analysis.

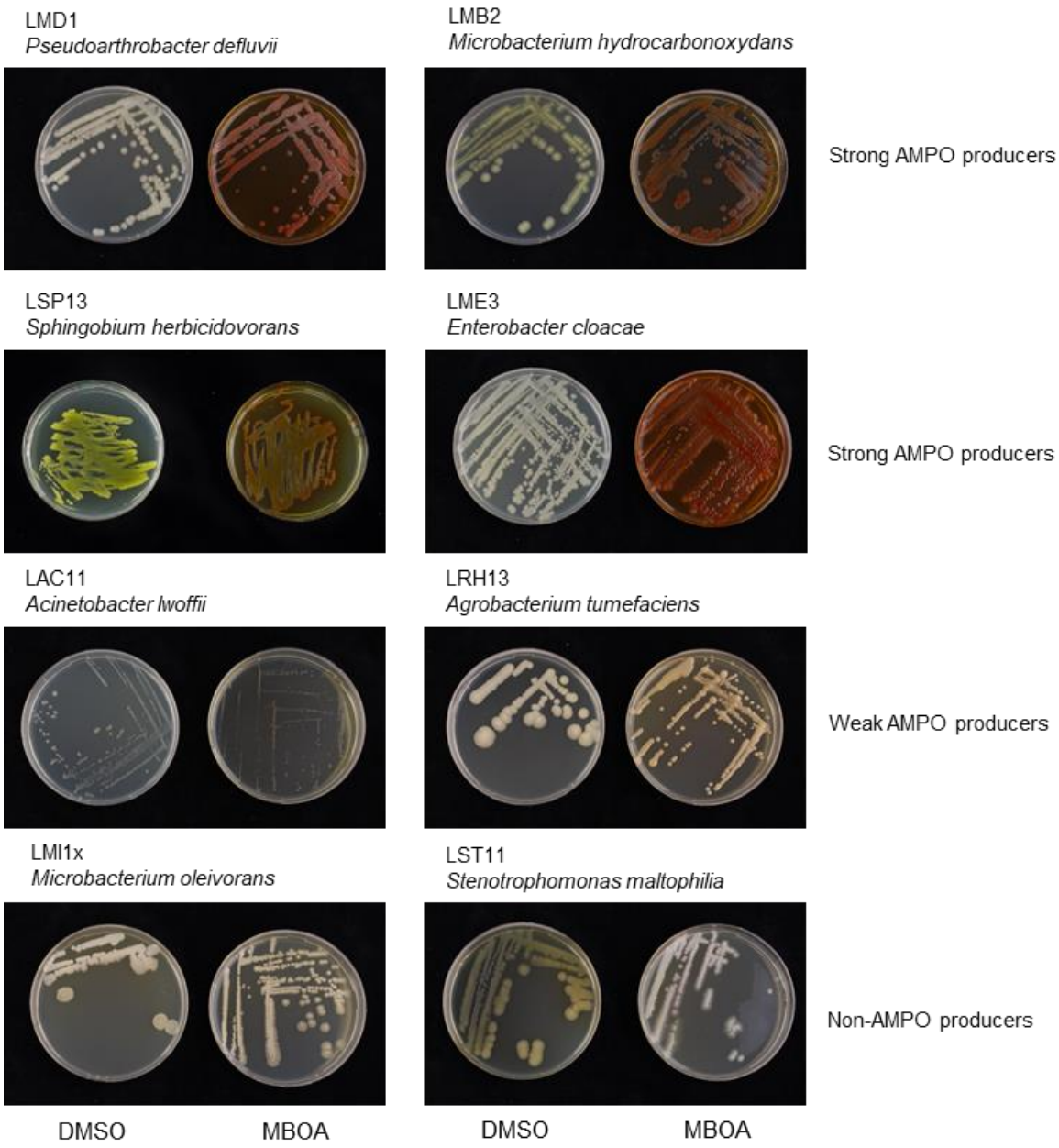


Figure S3: Rapid screening method for AMPO production. AMPO forming strains from maize root bacteria strain collection plated on medium containing DMSO (left) or MBOA and incubated for 10 days. Strong AMPO producers form a strong red colour on MBOA medium while weak AMPO producers form less. As a negative control two non-AMPO forming strains are shown.

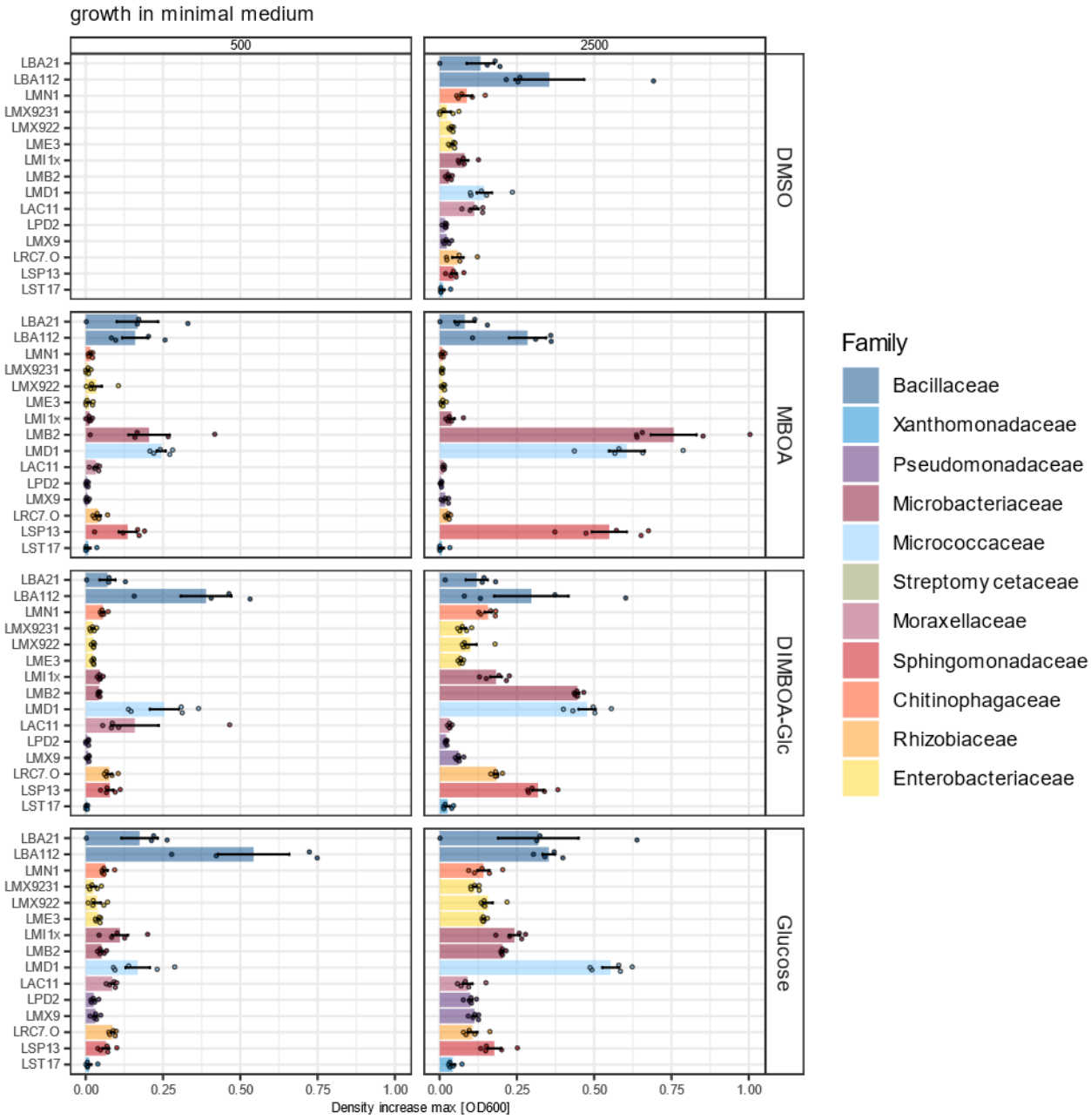


Figure S4: Bacteria use DIMBOA-Glc and MBOA as carbon source in minimal medium. Maximal density increase [OD600] of cultures grown in minimal medium supplemented with DMSO (negative control), MBOA, DIMBOA-Glc and Glucose (positive control) at 500 µM and 2500 µM for 68 hours.

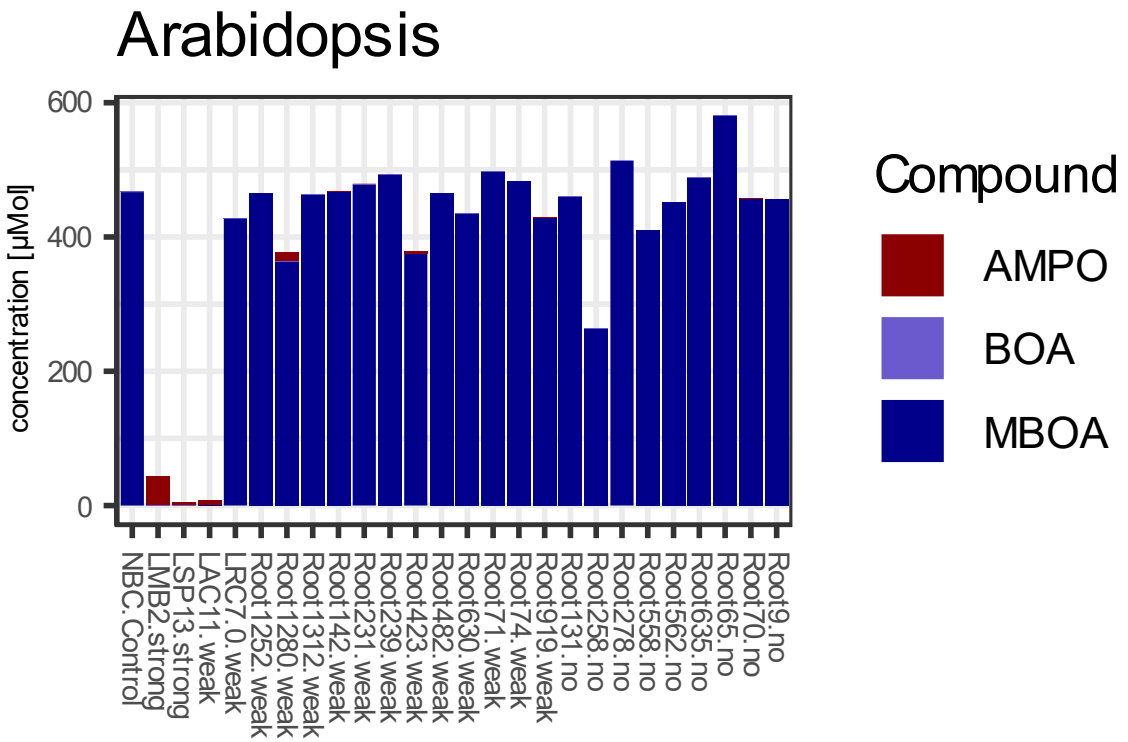


Figure S5: MBOA metabolisation by AtSphere bacteria. Metabolisation products represented in stacked bargraphs form single strains from AtSphere strain collection supplemented with MBOA. Strains which were found to show slight colour change on MBOA containing agar plates were classed as weak, while the others were referred as negative. Additionally, 4 AMPO positive maize root bacteria, two strong and two weak, were included as positive control. All measurements were made from three independently grown samples which were pooled in equal ratios prior to metabolite analysis.

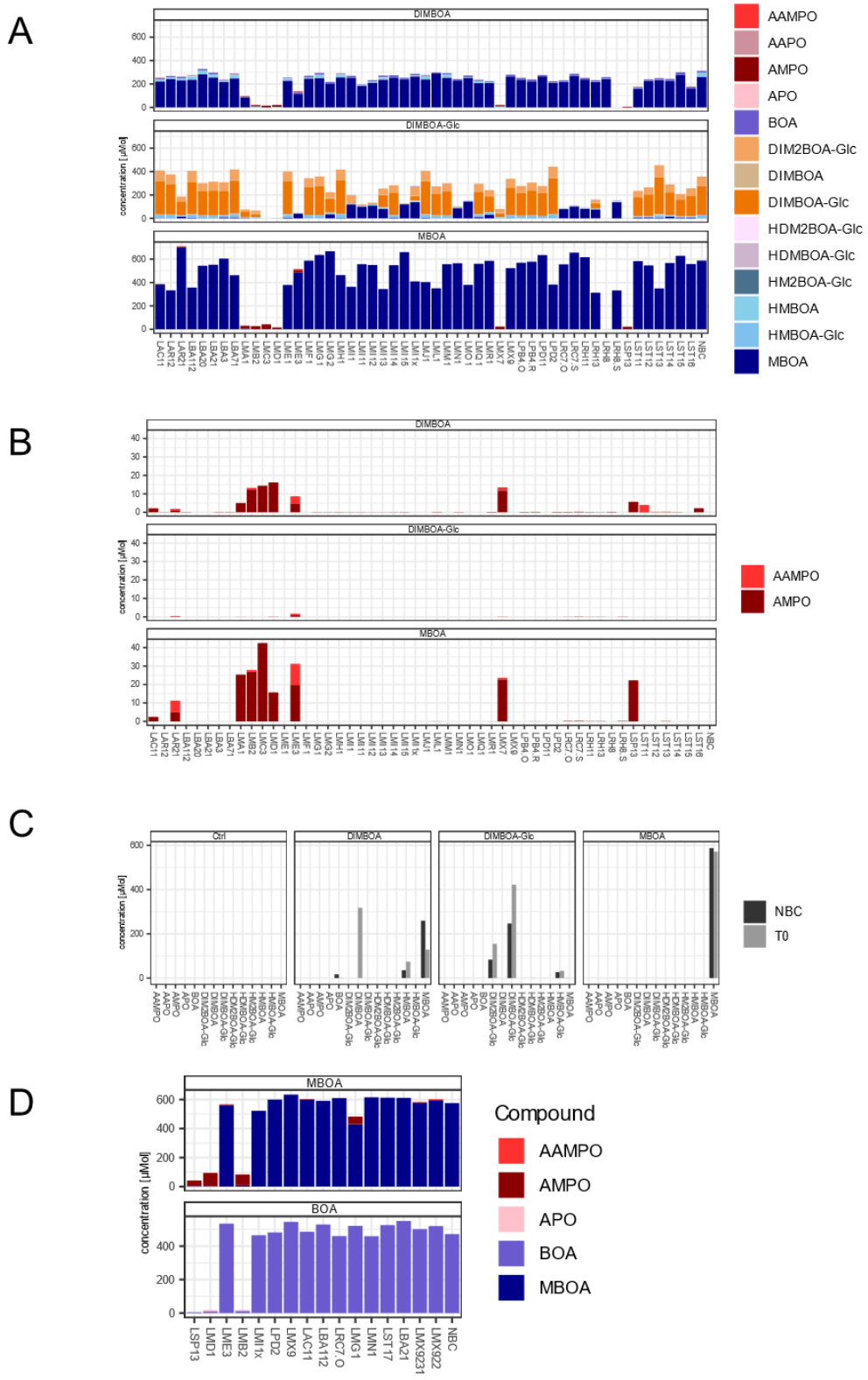


Figure S6. Complete degradation of benzoxazinoids by maize root bacteria. A) Metabolisation products represented in stacked bargraphs form single strains from MRB strain collection supplemented with DIMBOA-Glc, DIMBOA or MBOA. **B)** Stacked bargraphs displaying AMPO and AAMPO formation in the tested conditions. **C)** Concentration of DIMBOA-Glc, DIMBOA and MBOA in treatment solutions at the start of the experiment (T0). All measurements were made from three independently grown samples which were pooled in equal ratios prior to metabolite analysis.

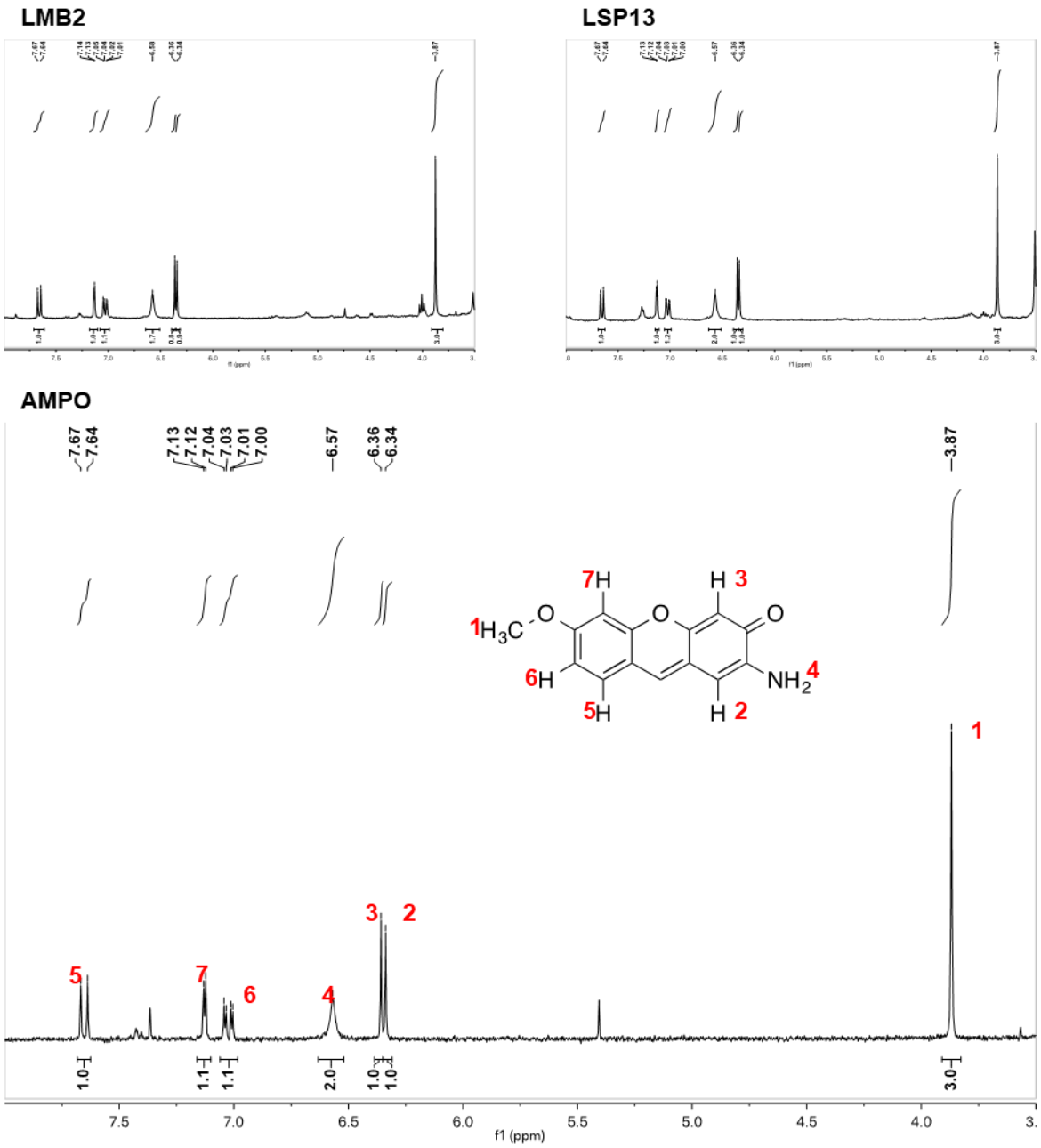


Figure S7: Confirmation of AMPO by NMR. NMR spectra of the red precipitate purified from cultures of LMB2 and LSP13 grown in MBOA for 68 hours and pure AMPO. The pattern of peaks of the red precipitate matches with pure AMPO.

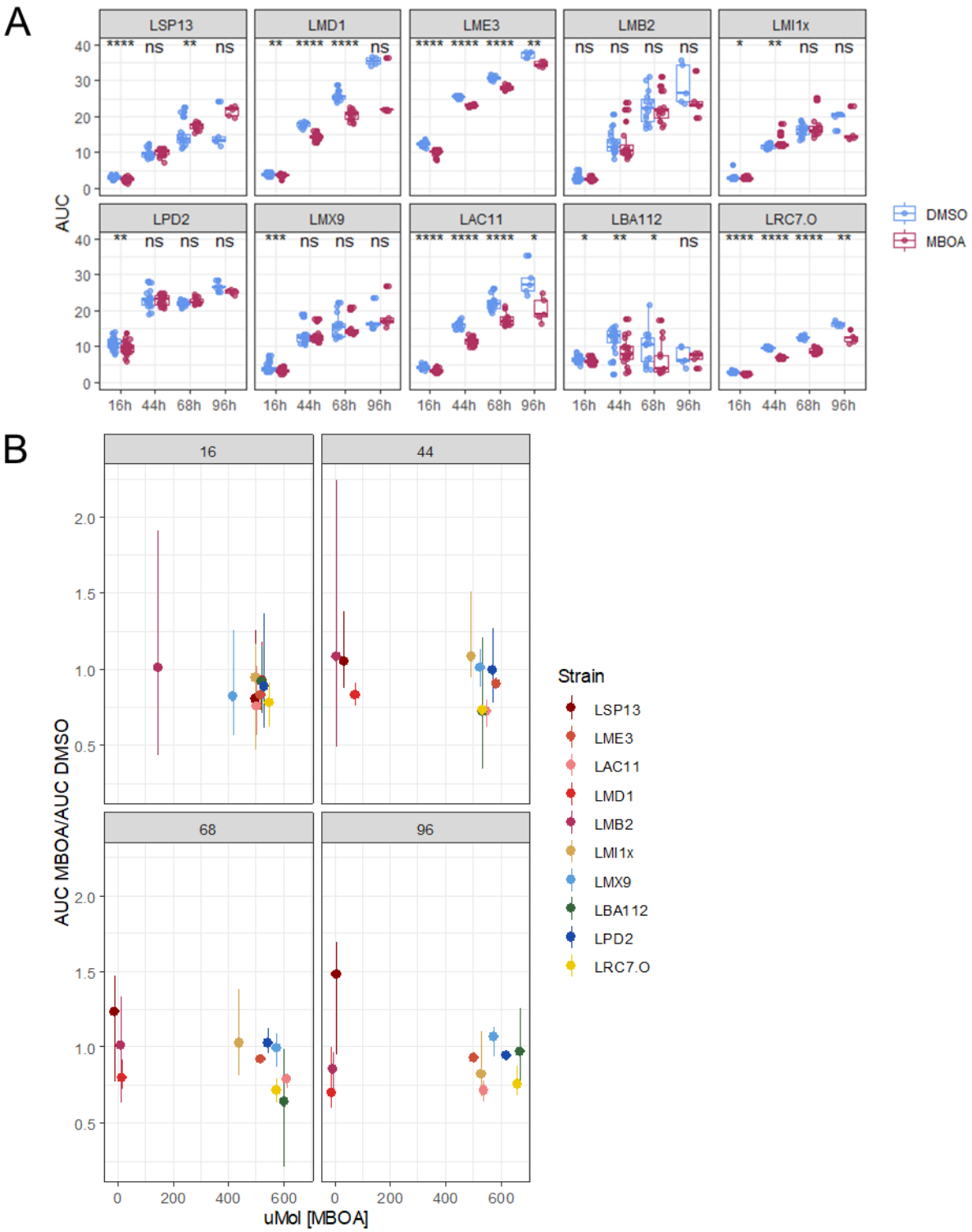


Figure S8: Bacterial growth in timeseries experiment. A) Growth of single strains over time in DMSO and MBOA. Area under the curve represents total bacterial growth at a time point and is calculated from the growth curves measured by optical density. **B)** Correlation MBOA concentration in medium with relative growth in MBOA/DMSO.

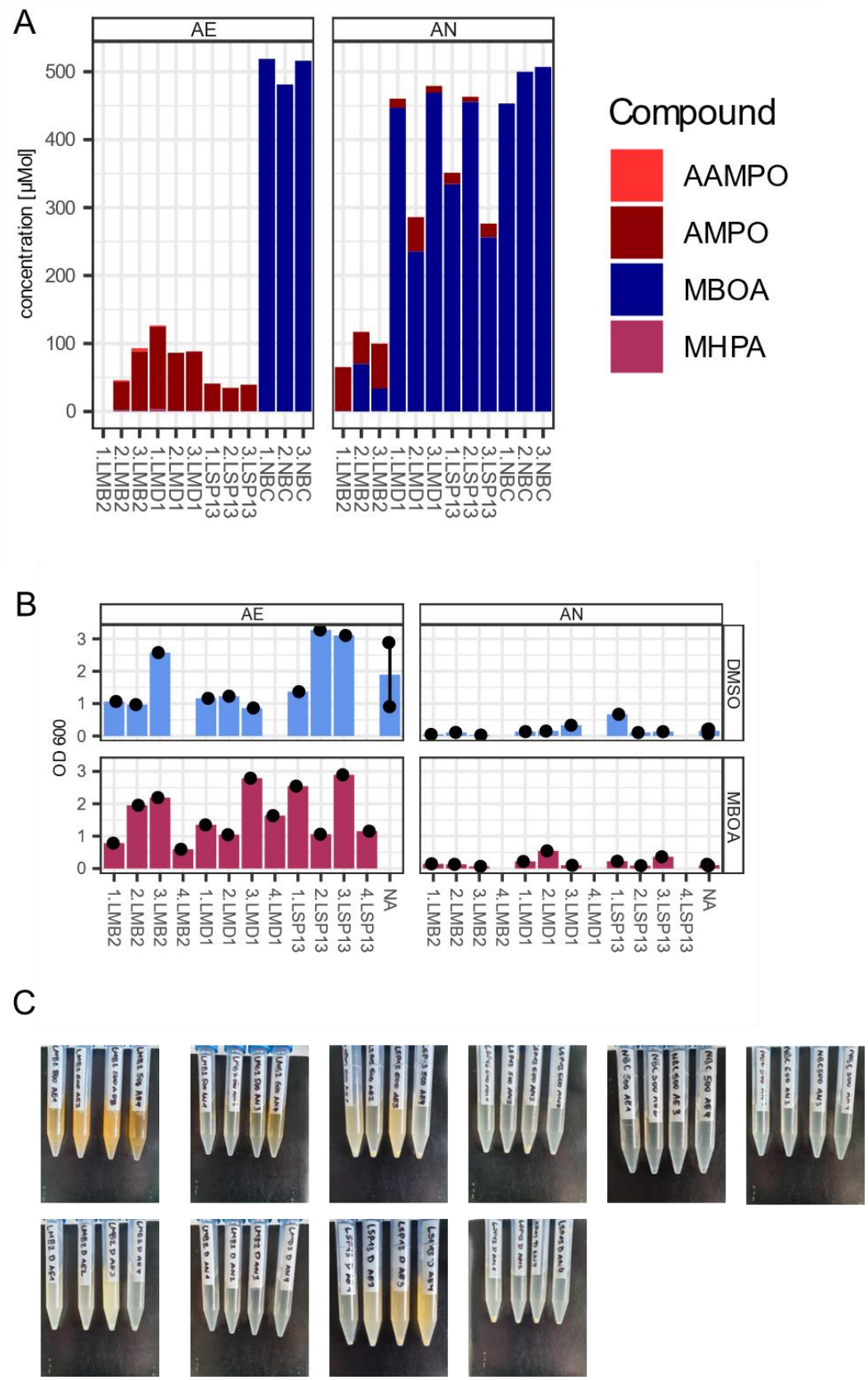


Figure S9: MBOA metabolisation by three selected strains in aerobic (AE) and anaerobic (AN) conditions. A) Metabolisation profile of strains grown in MBOA for 68 h both conditions. Replicates are shown in single bars. Concentrations shown in µMol. **B)** Bacterial growth of cultures after 68 hours (OD600) in DMSO and MBOA treatment in aerobic and anaerobic condition. **C)** Pictures of cultures at the end of the experiment.

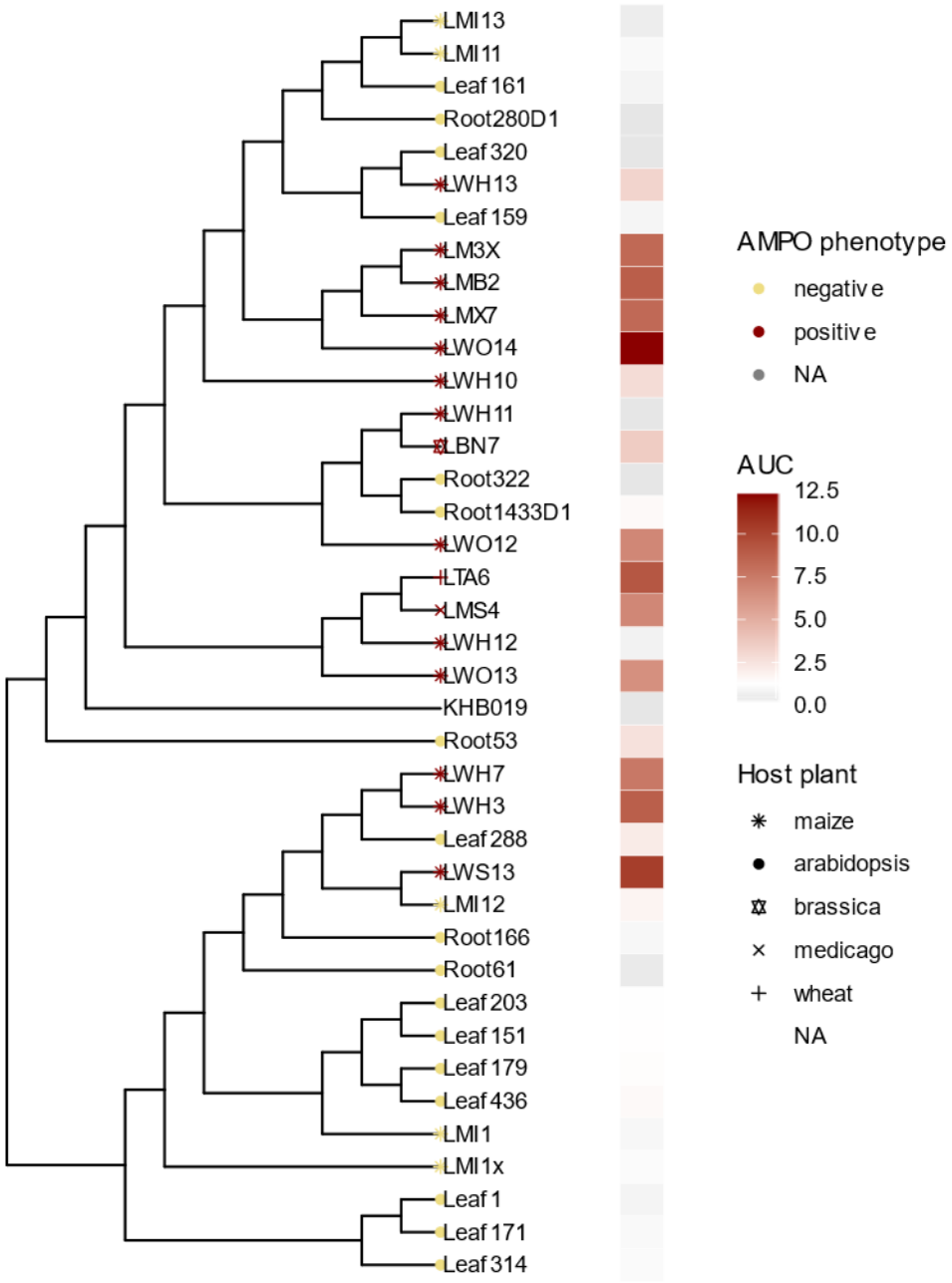


Figure S10. *Microbacteria* use MBOA as carbon source in minimal medium. Absolute growth of cultures grown in minimal medium supplemented with 500 µM MBOA for 68 hours.

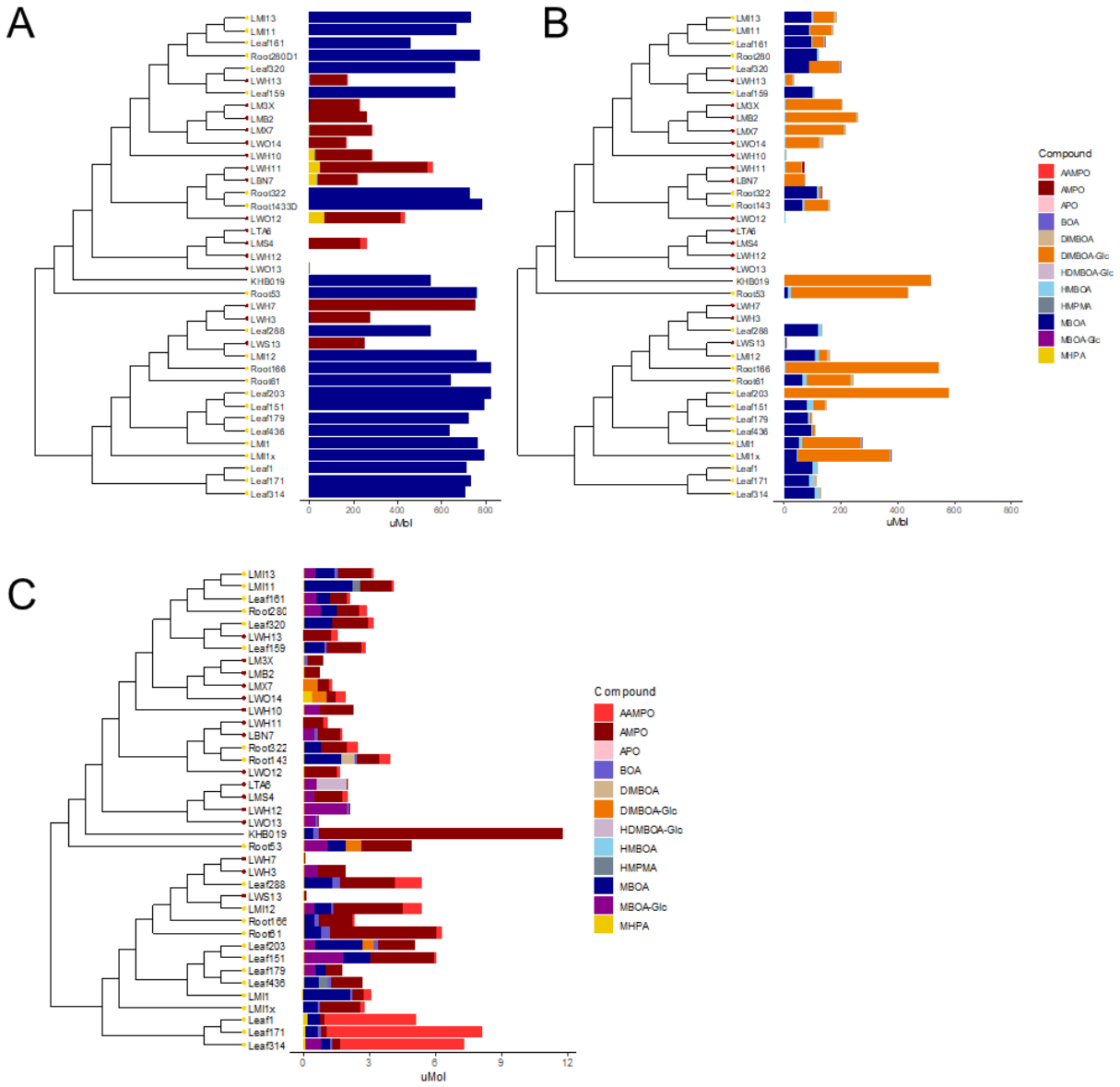


Figure S11: Benzoxazinoid metabolisation by *Microbacteria*: **A)** Metabolisation of MBOA and **B)** DIMBOA-Glc and **C)** AMPO and accumulation of degradation products. All measurements were made from three independently grown samples which were pooled in equal ratios prior to metabolite analysis.

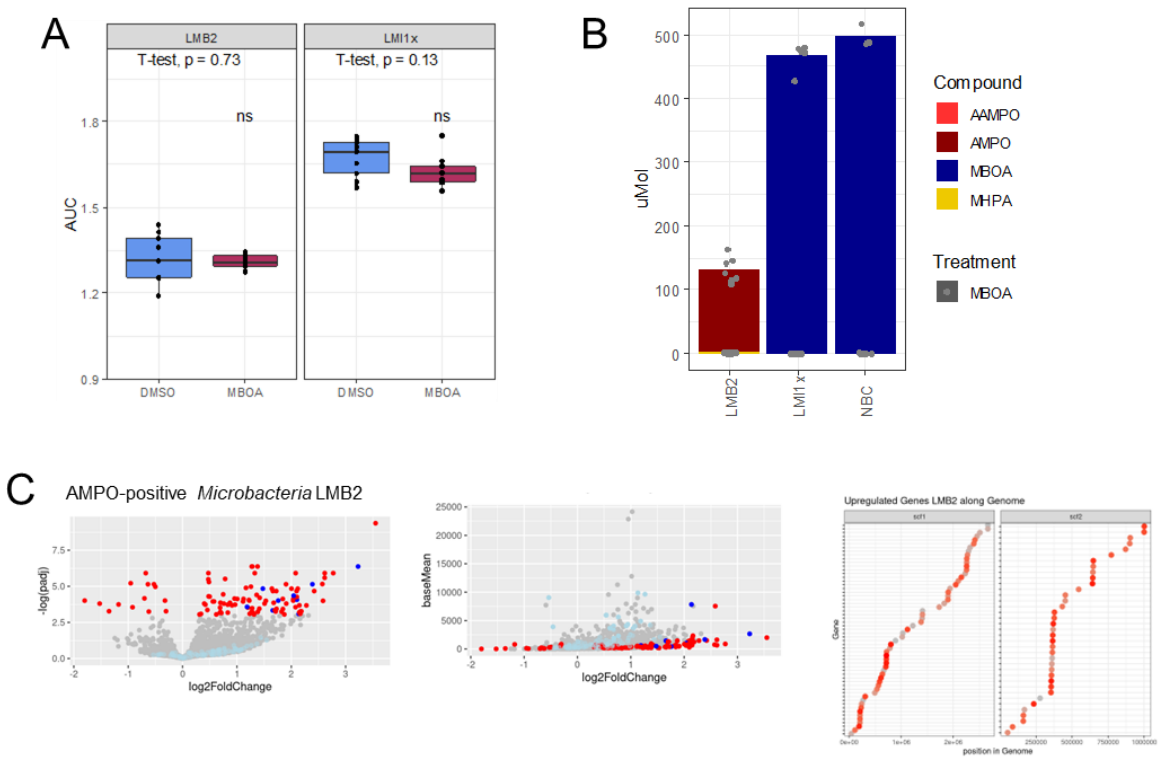


Figure S12: Transcriptomic experiment of *Microbacterium* LMB2 and LMI1x in MBOA and DMSO as control. **A)** Total growth of cultures assessed by optical density (OD600) measurements calculated to area under the curve (AUC). **B)** MBOA metabolisation profile of the two strains and the negative control without bacteria. All measurements were made from three independently grown samples which were pooled in equal ratios prior to metabolite analysis. **C)** Vulcano plot representing differentially regulated genes, a dotplot representing the expression of the differentially regulated genes and a VennDiagramm showing the overlap of genes differentially expressed in both strains. **D)** A visualization of the differentially expressed genes over the whole genome, highlighting the *bxd* gene cluster in LMB2.

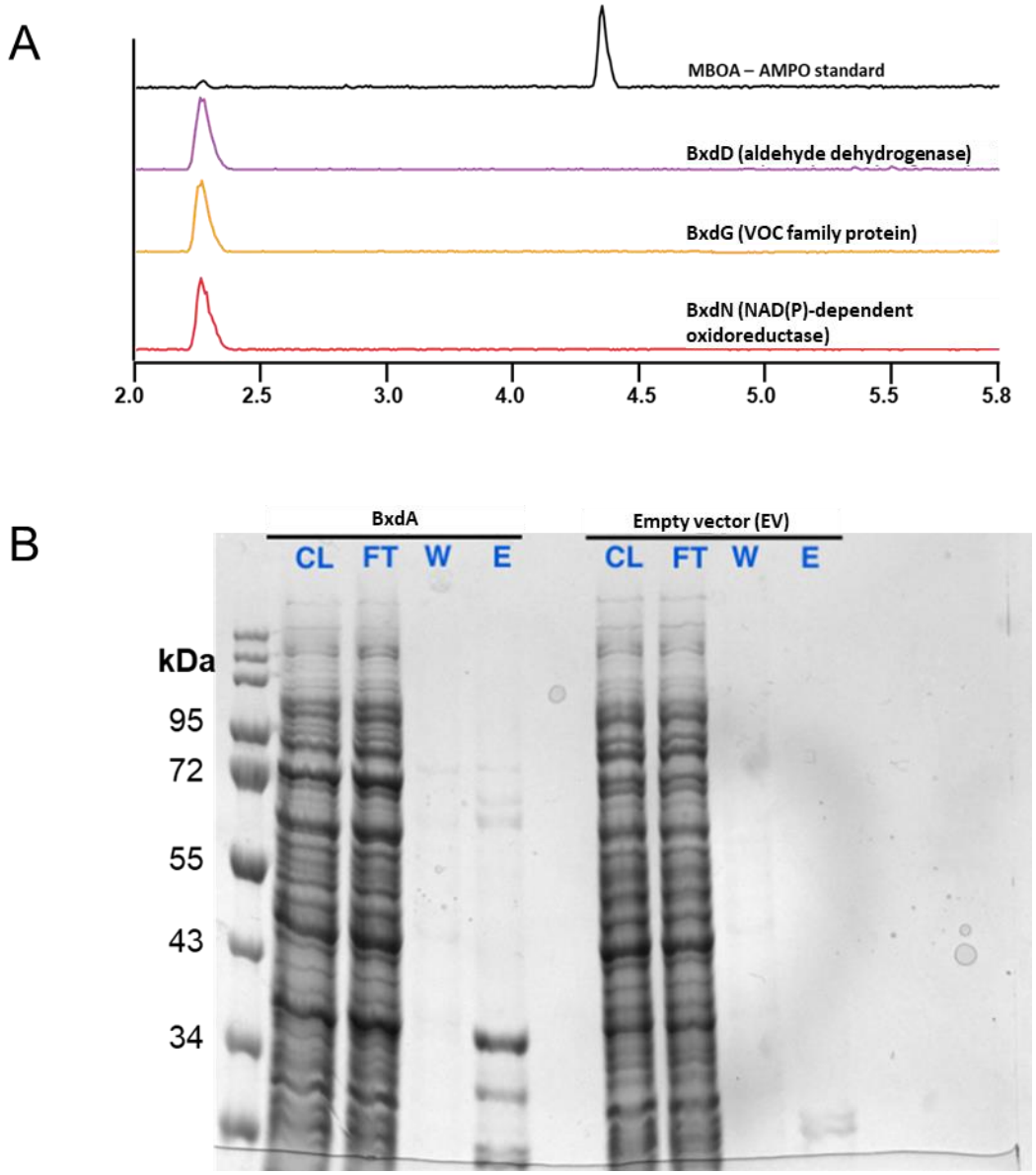


Figure S13: Heterologous expression of *bxdA*, *bxdD*, *bxdG* and *bxdN* in *E. coli* converts do not change MBOA contents in the extracts. A) High pressure liquid chromatography-mass spectrometry (HPLC-MS) full scan (positive mode) of the purified enzymes in MBOA and **B)** Protein gel of purified *bxdA* heterologously expressed protein.

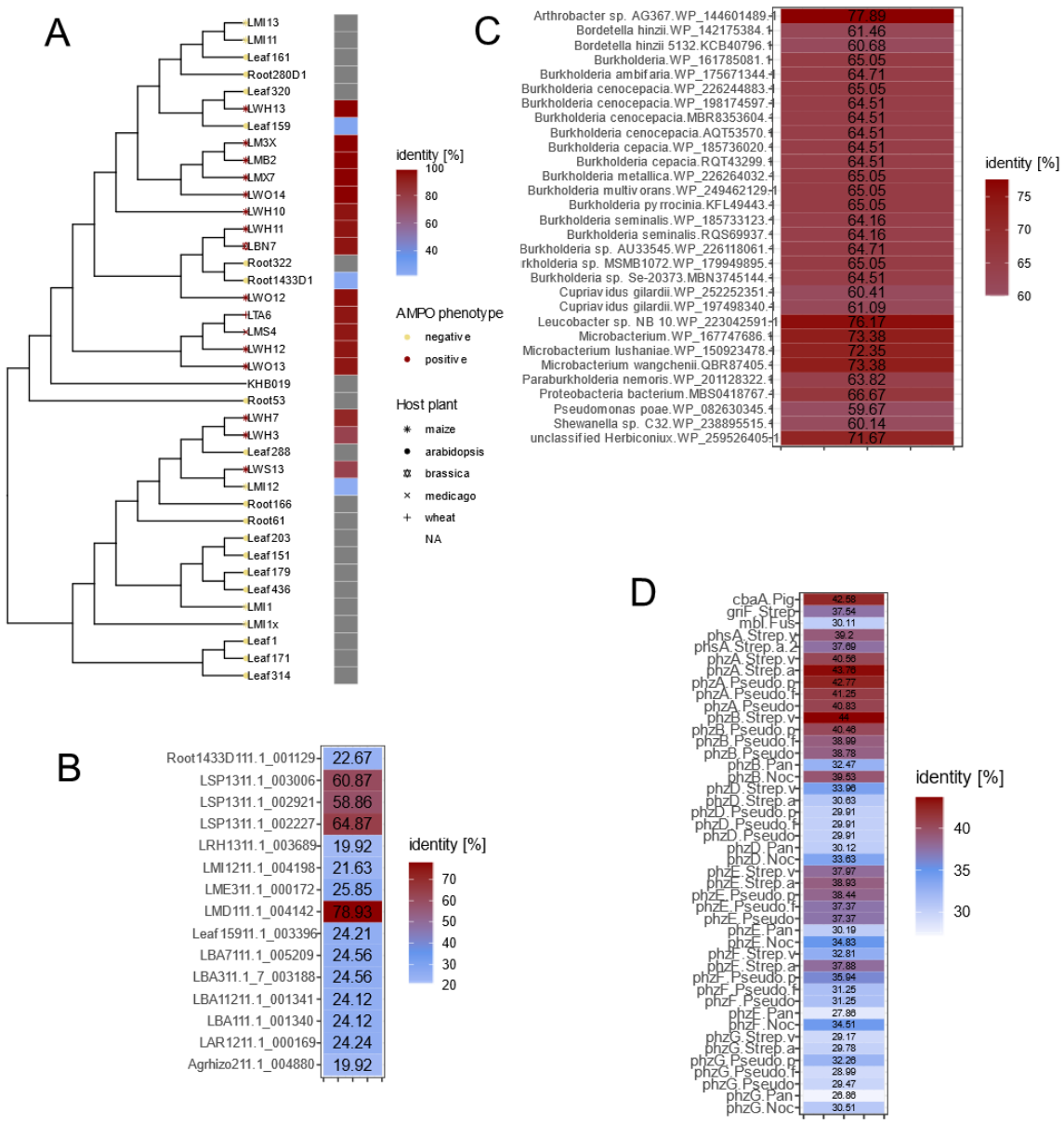


Figure S14: Lactonase similarity to other genes (Amino acid sequence). A) Similarity to Microbacteria and **B)** MRB genes identified by blast. **C)** to related genes identified by blasting the *bxdA* against the NCBI data base **D)** Similarity to sequences of phenazine synthesis genes and genes reported in BOA degradation.

Table S2: Plant growth conditions

Experiment	Samples	Soil collection	Duration	Fertilization
Plating experiment 1	Root, rhizosphere, and soil: Maize B73 WT & *bx1* Root: *Triticum aestivum (Claro), Brassica napus, Medicago sativa, Arabidopsis thaliana (Col-0)*	Changins in 2019 (Winter)	7 weeks	weeks 1 - 7
Plating experiment 2	WT & *bx1* B73 WT W22	Changins in 2019 (Summer, Valentin) & 1health soil in 2018	6 weeks	weeks 1 - 6

Greenhouse settings: 16 h light (26 °C), 8 h dark (23 °C), 50 % relative humidity, ~550 µmol m-2s-1 light

Fertilization regime:
- weeks 1 – 4: 100 ml; 0.2 % Plantactive Typ K[1], 0.0001 % Sequestrene Rapid[2]
- weeks 5 – 12: 200 ml; 0.2 % Plantactive Typ K, 0.02 % Sequestrene Rapid

[1] Hauert HBG Duenger AG, Grossaffoltern, Switzerland
[2] Maag, Westland Schweiz GmbH, Dielsdorf, Switzerland

Table S3: Stock solutions compounds for *in vitro* bacterial growth assays

Compound	Mass [g/mol]	Stock conc. [mM]	Stock [mg.ml]	solvent	Work conc. [uM]	Stock / ml [ul]
MBOA	165.1	606	100	DMSO	500	0.82
BOA	135.1	500	67.55	DMSO	500	1
DIMBOA	211.1	500	105.58	DMSO	500	1
DIMBOA-Glc	373.1	500	186.55	DMSO	500	1
AMPO	242.2	15	3.6	DMSO	30	1
DMSO	0	0	0	DMSO	2.06 ml/ml	1

Table S4: Primers used for gene cloning and colony PCR.

Name	Sequence
AMPO_K_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGGCGGAAGCCGCAAAC
AMPO_K_pF_Rv	ATGGTCTAGAAAGCTTTACTAATATGGGTTGGCAACCATTA
AMPO_0a_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGAGTGAGCGTAAAACGGAT
AMPO_0a_pF_Rv	ATGGTCTAGAAAGCTTTACTAAGTTAACAAAATCCCGGC
AMPO_C_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGGCCATAATGCGGTCCG
AMPO_C_pF_Rv	ATGGTCTAGAAAGCTTTATTAGGCCACCCAGACAGT
AMPO_D_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGGTAGAGATTATAAGAATGCC
AMPO_D_pF_Rv	ATGGTCTAGAAAGCTTTATTAAATAAGTATAGTCAGTGGATTTTC
AMPO_E_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGCCTAAGAAGAAACGCTTAC
AMPO_E_pF_Rv	ATGGTCTAGAAAGCTTTATTAGCTGCCAAAGCCTGC
AMPO_F_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGGCTGACGCTGTACG
AMPO_F_pF_Rv	ATGGTCTAGAAAGCTTTATTAGCGCTCCGGATGG
AMPO_H_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGAGCGCGTATGACCAGG
AMPO_H_pF_Rv	ATGGTCTAGAAAGCTTTATTACACTACAGGCAGAACAAAAC
AMPO_I_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGACGATGACTGAAACTCC
AMPO_I_pF_Rv	ATGGTCTAGAAAGCTTTACTACTCATCCCGTCCATAC
AMPO_K_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGGCGGAAGCCGCAAAC
AMPO_K_pF_Rv	ATGGTCTAGAAAGCTTTACTAATATGGGTTGGCAACCA
AMPO_M_pF_Fw	AAGTTCTGTTTCAGGGCCCGGTAACTACAGTAGGCTTCTTAG

AMPO_M_pF_Rv	ATGGTCTAGAAAGCTTTATCAGGACTGGCGGCG
AMPO_N_pF_Fw	AAGTTCTGTTTCAGGGCCCGATGGCCTCTATTTCGGTTATTG
AMPO_N_pF_Rv	ATGGTCTAGAAAGCTTTATTATGATCTAACTGCAAAGCC
pOPINF_Fw	TAATACGACTCACTATAGGG
pOPINF_Rv	TAGCCAGAAGTCAGATGCT

Table S5: Presence absence of genes of 10 genes upstream and 15 genes downstream (*bxd* gene cluster) of *bxdA* in other AMPO strains: *Microbacterium* LB2 as reference compared to *Pseudoarthrobacter* LMD1 and the regions corresponding to the three copies *bxdA* in *Sphingobium* LSP13

Gene	**Approach**	**LMB2**	**LMD1**	**LSP13 002921**	**LSP13 003006**	**LSP13 002227**
2-oxo acid dehydrogenase subunit E2	RNAseq_OG_kmer	1	1	0	0	0
thiamine pyrophosphate-dependent enzyme	RNAseq_OG_kmer	1	1	2	1	0
RidA family protein	RNAseq_OG	2	1	1	1	0
M24 family metallopeptidase	OG_kmer	1	1	1	1	0
NAD(P)-dependent oxidoreductase	RNAseq_OG	1	1	1	0	0
N-acyl homoserine lactonase family protein	RNAseq_kmer	1	1	1	1	1
acyl-CoA dehydrogenase family protein	RNAseq_kmer	2	1	0	0	0
aldehyde dehydrogenase family protein	RNAseq_kmer	1	0	0	0	0
VOC family protein	RNAseq	1	1	1	0	0
flavin reductase	RNAseq_kmer	1	1	0	0	0
NADPH-dependent F420 reductase	RNAseq	1	1	0	0	0
NAD-dependent succinate-semialdehyde dehydrogenase	RNAseq_kmer	1	1	0	0	0

Chapter 3

Bacterial cooperation in tolerating and metabolising plant specialized metabolites

Unpublished work

Lisa Thoenen, Marco Kreuzer, Pierre Mateo, Tobias Zuest, Micro Hecht, Gabriel Deslandes, Sophie Moreau, Mario Walthert, Rémy Bruggmann, Matthias Erb, Klaus Schlaeppi

Abstract

Root-exuded plant specialized metabolites, like the benzoxazinoids secreted by maize, shape root-associated microbial communities. While maize root bacteria vary in their tolerance to benzoxazinoids and only a subset can metabolise the antimicrobial benzoxazinoids, it is yet unknown how these traits of single strains combine in a bacterial community. In this study, we designed two synthetic communities of maize root bacteria differing in their ability to metabolise benzoxazinoids. We investigated whether bacteria would cooperate for tolerance to and metabolisation of the benzoxazinoid MBOA. MBOA shaped both communities' composition and increased the growth of MBOA-tolerant strains. As a collective trait, we found that the benzoxazinoid metabolising community showed higher MBOA tolerance. The capacity to metabolise benzoxazinoids appeared beneficial to the bacteria, not only enhancing their tolerance but also as they could use the degradation of MBOA as a sole carbon source for growth. Interestingly, the main MBOA degradation metabolite HMPAA required the cooperation of *Microbacterium* with the *Pseudomonas* strain, while this compound was not formed alone. This microbial cooperation in benzoxazinoid metabolism is a mechanism for how plant specialized metabolites affect microbial communities and possibly how bacteria have co-adapted to cope with the secondary metabolites of their host.

Introduction

Most multicellular organisms are closely associated with complex microbial communities co-adapted with their host over evolution (Baumann, 2005; Ley et al., 2008; Sprent, 2007). Each member interacts with the others in diverse natural microbial communities to efficiently use resources. Division of labour facilitates community functions to accomplish impossible tasks for monocultures (Bardone et al., 2020; Rafieenia et al., 2022). Among others, collaborative processes include the consumption of antimicrobial compounds (Lilja et al., 2016) or cooperation for complex substrate utilisation (McCarty and Ledesma-Amaro, 2019). The division of labour affects the growth of microbial populations, final product distribution, and the emergence of new phenotypes in microbial communities without any genetic modification (Diender et al., 2021). Microbial members use metabolite exchange to complement each other's biosynthetic pathways for success at the community level (Ponomarova and Patil, 2015). Specifically for pollutant degradation, soil microbial communities often share crucial functions separated among different community members (Rafieenia et al., 2022). A four-strain consortium metabolised the herbicide atrazine to cyanuric acid and utilised the compounds as a carbon source. At the same time, one member could perform the costly conversion of atrazine to cyanuric acid, which then served as a carbon source for the other community members (Billet et al., 2019). Another three-strain consortium converts the herbicide linuron more efficiently in co-culture than mono-culture since the linuron hydrolase increased in expression (Albers et al., 2018; Pileggi et al., 2020). Inspired by the numerous examples of division of labour in soil microbiome, very little is known about the role of division of labour in plant root microbial communities for root exudate metabolisation, which is often complex and antimicrobial plant metabolites.

To study the mechanisms governing the composition and functions of whole microbial communities and to investigate their interaction with the host or the environment, the synthetic microbial community (SynCom) approach is employed (Vorholt et al., 2017). A synthetic community is a microbial community designed by mixing selected strains from a strain collection isolated from a specific environment (Bai et al., 2015). It allows the detailed study of its components under controlled conditions and facilitates the establishment of causal links between microbiome composition and plant phenotypes. The significant advantage of the synthetic community approach is that organisms can be added, eliminated, or substituted at the strain level and their function can be manipulated directly (O'Banion et al., 2020). The consequences of perturbations can be monitored in the SynComs at different levels and thus enables the exploration of the role of individual organisms in the community. Although, these reduced systems do not accurately represent nature, they allow the replication of phenotypes mediated

by the microbiome (Vorholt et al., 2017). To date, there is little understanding of which host factor-mediated mechanisms control the assembly of the host-associated microbiome.

Root secreted plant secondary metabolite's structure root microbial communities. Several plant specialized metabolites have been shown to structure root microbial communities (Jacoby et al., 2020b; Lareen et al., 2016; Sasse et al., 2018). These effects were elucidated by comparing the microbial community composition on roots or rhizospheres of wild-type plants with the respective biosynthesis mutants defective in the production of specific secondary metabolites grown in natural soils or reductionist systems with synthetic microbial communities. For coumarins, which are produced by *Arabidopsis*, a SynCom was designed and used to inoculate axenically grown *Arabidopsis* wild-type plants and coumarin-deficient mutant plant. The composition of SynComs differed between the WT and the mutant, and they found coumarin tolerant strains enriched in the community (Voges et al., 2019). Benzoxazinoids (BX) structure the root microbiomes of maize in natural field soil (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019; Schütz et al., 2021). Maize root microbiome members have different levels of tolerance to benzoxazinoids which explains their correlating with their abundance on benzoxazinoid producing (BX-producing) roots (chapter 1, this thesis; Thoenen et al. unpublished). Certain bacteria can metabolise the compounds (chapter 2, this thesis; Thoenen et al. unpublished), (Schütz et al., 2019). However, in these studies only single strains were studied for their tolerance and metabolisation traits. Here we went a step ahead and tested these traits in community context.

With the aim to investigate the response of single microbiome members to plant specialized metabolites, in a recent study a collection of bacteria isolated from *Arabidopsis* roots was tested on their tolerance to the structurally similar non-methoxylated benzoxazinoids BOA and APO. These experiments revealed varying degrees among strains of tolerances to benzoxazinoids (Schandry et al., 2021). SynComs assembled from tolerant strains are overall more resilient, but the overall growth of the community was not assessed. The performance of a single isolate is not correlated with its individual performance directly but is shaped by interactions with other members of the communities and alters when benzoxazinoids are supplemented (Schandry et al., 2021). These experiments show that tolerance of strains likely has an impact on community composition in presence of benzoxazinoids, however this study was performed with an isolate collection from a non-BX producing host plant. Knowing that benzoxazinoid tolerance is strains specific, it is likely that strains isolated from BX-producing host show different responses to plant toxins. By which mechanisms plant specialized metabolites control the community composition and steer the composition and the function of host associated microbial communities is unknown.

Crops like wheat, maize and rye belonging to the *Poaceae* family produce benzoxazinoids (Niemeyer, 2009; Wouters et al., 2016). Apart from structuring maize root microbiomes, they protect the plant from insect pests, pathogens and play a role in iron uptake (Hu et al., 2018a; Niemeyer, 2009; Robert et al., 2012). Shoots and roots produce and exude them throughout plant growth (Dafoe et al., 2011; Zhou et al., 2018). Benzoxazinoids derive from indole. In maize the methoxylated benzoxazinoids dominate, in rye the non-methoxylated forms are predominant and in wheat a mixture of both is present (Belz and Hurle, 2005; Frey et al., 1997). DI(M)BOA-Glc is the main benzoxazinoid exuded by maize roots (for complete names see Table S1) (Hu et al., 2018b). Benzoxazinoids that are secreted to soil are deglycosylated by either microbial or plant derived enzymes. Spontaneously DI(M)BOA aglucons degrade to the more stable (M)BOA (Macías et al., 2004). Through microbial activity in the soil MBOA degrades to reactive aminophenols (M)AP (Kumar et al., 1993; Niemeyer, 2009; Zikmundová et al., 2002). Aminophenols are further converted on three routes to three different metabolite classes. (I) Aminophenoxazinones A(M)PO from a spontaneous reaction in presence of oxygen (Guo et al., 2022). This can be further acetylated to AA(M)PO. (II) Acetamides H(M)PAA form by an acetylation reaction from aminophenol. They may be further nitrated. (III) Malonic acids H(M)PMA form by an acylation from aminophenol (Friebe et al., 1998; Nair et al., 1990; Schulz et al., 2013; Schütz et al., 2019; Understrup et al., 2005; Zikmundová et al., 2002). (M)BOA is stable in soil for a few days while the corresponding 2-amino phenoxazine-3-ones are detectable for months (Macías et al., 2004). Even though it is evident that benzoxazinoid degradation depends on microbial activity, only a few microbes were identified which metabolise benzoxazinoids.

The soil bacterium *Acinetobacter* sp. converts BOA to APO *in vitro* (Chase et al., 1991). A more recent study identified the metal-dependent hydrolase for the degradation of the herbicide 6-Chloro-2-benzoxaziolinone (CDHB) which is a 2-benzoxazolinone (BOA) derivative to aminophenoxazinones in *Pigmentiphaga* (Dong et al., 2016). In a co-culture of the fungus *Fusarium verticillioides* with the bacterium *Bacillus mojavensis* grown on BOA, the aminophenoxazinone APO formed (Bacon et al., 2007). The acetamide HMPAA forms from BOA in presence of the soil fungus *Fusarium sambucus* (Zikmundová et al., 2002). The malonamic acid HPMA forms from BOA by a metallo-β-lactamase from the specialized maize seed endophytic fungus *Fusarium verticillioides* (Glenn et al., 2016). In *Microbacteria* isolated from the rhizosphere of BX-producing maize roots, a N-acyl homoserine lactonase converts MBOA to AMPO (chapter 2, this thesis; Thoenen et al. unpublished). Together these findings prove diverse routes for benzoxazinoid conversions by microbes. GAP: As demonstrated in with the co-culture of *Bacillus* and *Fusarium*, microbes may cooperate to degrade benzoxazinoids. Nonetheless, how

benzoxazinoids are metabolised in microbial communities and if they divide labour to metabolise benzoxazinoids remains to be investigated.

Given that benzoxazinoids shape root associated microbial communities of maize (Hu et al., 2018b), that maize root bacteria differ in their tolerance to benzoxazinoids (chapter 1, this thesis; Thoenen et al. unpublished), and that several strains can metabolise benzoxazinoids (chapter 2, this thesis; Thoenen et al. unpublished) in pure culture, we hypothesized that these traits influence benzoxazinoid tolerances and metabolisation of microbial communities. Here we aimed to test if maize root bacteria cooperate to tolerate and metabolise benzoxazinoids in small synthetic communities. We investigated I) how the abundance of single strains in SynCom compares with their benzoxazinoid tolerance as single strains, II) if microbes cooperate to metabolise benzoxazinoids and III) the effect of benzoxazinoid metabolisation (BX-metabolisation) for bacterial growth and on plant growth of host and target plants. To test this hypothesis, we designed two SynComs consisting of six core strains and one differing *Microbacterium* strain with an altered ability to metabolise MBOA. We exposed the SynComs to benzoxazinoids and measured community growth, community composition and benzoxazinoid metabolite profiles. We found the SynCom with the non-metabolising *Microbacterium* to be inhibited by MBOA and not metabolising MBOA. The SynCom with the BX-metabolising *Microbacterium* strain has a higher tolerance to MBOA and completely degraded MBOA and forms the main degradation product HMPAA. None of the single strains present in the SynCom formed HMPAA form MBOA in pure culture, but a combination of least two strains is required. By testing all possible combinations, we found that *Microbacterium* LMB2 first converted MBOA to the intermediate MAP, then the *Pseudomonas* LMX9 acetylated MAP to HMPAA. Taken together, our results revealed that maize root bacteria cooperated to tolerate and metabolise benzoxazinoids, which is beneficial for microbial growth but does not alter plant growth.

Materials and Methods

Bacterial cultures and media

Bacterial strains from the maize root bacteria strain collection (chapter 1, this thesis; Thoenen et al. unpublished) were routinely grown at 25 °C – 28 °C in tryptic soy broth (TSB, Sigma-Aldrich, St. Louis, USA) liquid, or solid medium amended with 15 g/l agar (Sigma-Aldrich, St. Louis, USA).

SynCom experiment in Erlenmeyer

To investigate how MBOA metabolising bacteria influence community dynamics, we performed an *in vitro* SynCom experiment in liquid cultures and supplemented MBOA. Cultures were composed of six core strains (*Stenotrophomonas* LST17, *Bacillus* LBA21, *Pseudomonas* LMX9, *Streptomyces* LMG1, *Chitinophaga* LMN1, *Enterobacter* LMX9231) and either an AMPO producing *Microbacterium* (LMB2) or another closely related strain which does not form AMPO (LMI1x). All the strains were previously isolated from maize roots (chapter 2, this thesis; Thoenen et al. unpublished). The six core strains chosen for the experiment represent the greatest diversity of maize root bacteria strains not degrading MBOA. They map to taxonomic units in the maize microbiome from the field (Hu et al. 2018, Cadot et al. 2020) and thus it is possible to differentiate them with 16s sequencing. Prior to the setup of the experiment, pure strains were pre-grown in 50% TSB overnight at 28 °C with 180 rpm shaking. Optical density of the single cultures was adjusted to OD 0.01 they were mixed. The cultures were washed twice with buffer ($MgCl_2$ 10 mM) by centrifugation (5 min at 3600 rpm) and resuspended in 35 ml final volume to SCS (LMI1x) and SCR (LMB2).

The treatments solutions were prepared by adding stock solutions of MBOA dissolved in DMSO (10 mg/ml, Sigma) or DMSO (Sigma-Aldrich, St. Louis, USA) at the same volume to TSB to reach concentrations 500 µM (MBOA low) or 2500 µM (MBOA high) and DMSO. One milliliter of the premixed SynComs was inoculated to 29 ml of treatment solutions in 50 ml Erlenmeyer flasks. As a negative control the same volume of $MgCl_2$ 10 mM buffer was added. The experiments were performed with 5 replicates per treatment and SynCom combination. Assembled cultures were grown at 28°C with 180 rpm shaking for 68 hours. At the start of the experiment, 1 ml aliquots of undiluted SynComs were stored for amplicon sequencing. Pure treatment solutions were mixed in a in 3:7 ratio with MeOH 100% + 0.7% formic acid for metabolite analysis. All samples were stored -80°C until further analysis. Sterility of the treatment media was confirmed by plating on TSB plates. After 68 hours of growth, cultures were visually examined, OD_{600} was measured, and cultures were aliquoted in samples for further downstream analysis. One milliliter of the cultures was aliquoted and flash frozen in liquid nitrogen for amplicon sequencing and samples for

metabolite analysis were taken as described above. Viable cell counts (CFU) were assessed by plating serial dilutions on TSB agar plates and counted after 24 hours of incubation.

High-throughput SynCom experiments

For the timeseries, the pairs, the dropouts, and the growth of SynComs and single strains in different benzoxazinoids, the bacteria were grown in a high-throughput system. Prior to the setup of these assays, we prepared liquid pre-cultures in a 96-well format from fresh plates. Pre-cultures from plates were prepared with freshly picked isolates and inoculated with an inoculation needle (Greiner bio-one, Kremsmünster, Austria) to 1 ml of 50% liquid TSB in 2 ml 96-well deep-well plates (Semadeni, Ostermundigen, Switzerland), covered with a Breathe-Easy membrane (Diversified Biotech, Dedham, USA) and grown until stationary phase for 4 days at 28°C and 180 rpm.

Before the setup of the assays, the SynComs were assembled by mixing equal volumes of the precultures. Complete SynComs S and R included all the 6 core strains and the respective *Microbacterium* strain. For the pairs, *Microbacterium* LMB2 was mixed with one of the core strains. The dropout communities were mixed by including all core strains except one. To set up the assays we inoculated bacterial cultures into the medium with benzoxazinoid compounds. For the stock solutions, we dissolved pure DIMBOA-Glc, MBOA, BOA (Sigma-Aldrich, St. Louis, USA) in DMSO. Pure DIMBOA-Glc and AMPO were isolated from natural source in our laboratory. Stock concentrations differed depending on their solubility. For the treatment solutions, we added stock solutions to 50% liquid TSB. The concentration for all compounds is 500 µM and the DMSO concentration is constant. We filled the treatment solutions to 200 µl 96-well microtiter plates (Corning, Corning, USA) using an 8-channel pipette or a liquid handling system (Mettler Toledo, Liquidator 96™, Columbus, USA). Shortly before the start of the assay, we inoculated 4 µl of the pre-cultures into the plates. We piled the plates with a lid and inserted them into a stacker (BioStack 4, Agilent Technologies, Santa Clara, United States). The plate reader (Synergy H1, Agilent Technologies, Santa Clara, United States) connected to the stacker measures optical density (absorbance at 600 nm) every 100 min over 68 hours. Before each measurement, the reader shook the plates for 120 s. To check for contamination, we included "no bacteria controls" on each plate. Additionally, we included a plate containing only medium. To measure MBOA metabolisation over time, we removed plates from the stack after 16 h, 24 h, 44 h, 68 h and 96 h. For the other reactions we harvested the cultures after 68 h.

DNA extraction, library prep and sequencing

The DNA was extracted from two technical replicates per Erlenmeyer sample. For DNA extraction, 1 ml of the culture was pelleted at 13'000 rpm, then the first buffer was directly added

to the pellet. Then the extraction was performed following the manufacturer's instructions. The DNA concentration was quantified with the AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit (Biotium, Fremont, USA). DNA was diluted to 0.2 ng/µl for subsequent amplification. A two-step PCR was performed, where the first PCR the DNA is amplified and in the second PCR the PCR products are tagged with custom barcodes. The bacterial PCR reaction mix was composed of 5 µl DNA template, 0.4 µl of 10 µM 515-F (GTGYCAGCMGCCGCGGTAA, (Ul-Hasan et al., 2019) and 806*-R (GGACTACNVGGGTWTCTAA) primers each, 8 µl 5Prime HotMasterMix (Quantabio, Beverly, USA), 2 µl 3% BSA and 4.6 µl autoclaved MilliQ water to a final reaction volume of 20 µl. The cycling profile was 94°C for 3 minutes, 25 cycles of 94°C for 45 seconds, 50°C for 60 seconds, 72°C for 90 seconds, and 65 °C for ten minutes. All PCR reactions were purified with SPRIselect beads (Beckman Coulter Life Sciences, Indianapolis, USA) following manufactures instructions. The reactions from the first PCR were amplified with unique barcoded primer pairs for each sample. The barcoded PCR were purified SPRIselect bead purified, DNA was quantified with the Qubit™ dsDNA BR kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and the samples were pooled equimolar using a Myra Liquid Handler (Bio Molecular Systems, Upper Coomera, Australia). The pooled library (BE11) was bead purified and sequenced by MiSeq v2 500 cycle nano sequencing kit at the Next Generation Sequencing Platform (University of Bern) using the 2x350 bp pair-end sequencing protocol (Illumina Inc., San Diego, USA).

Metabolite extraction from bacterial cultures

At the end of the experiment, we examined colour changes in the cultures by eye. To fix bacterial cultures, we added 150 µl bacterial cultures to 350 µl of the extraction buffer (100% methanol + 0.14% formic acid) in non-sterile round bottom 96-well plates (Thermo Fisher Scientific, Waltham, USA). We stored the fixed samples with a final concentration of 70% methanol and 0.1% formic acid at -80 °C. To reduce the number of samples, we pooled three replicates of the same culture. We diluted the pooled sample by mixing 50 µl to 700 µl MeOH 70% + 0.1% FA. We filtered the cultures through regenerated cellulose membrane filters (CHROMAFIL RC, 0,2 µm, Macherey-Nagel, Düren, Germany) by centrifugation (6200 rpm for 2 min) to remove bacterial debris. To avoid any residual particles, we centrifuged the cultures at 13'000 rpm for 10 min at 4 °C. We aliquoted the supernatants in glass vials (VWR, Dietikon, Switzerland) and stored the samples for a few days at 20°C until analysis.

Profiling benzoxazinoid degradation products in bacterial cultures

Using an Acquity I-Class UHPLC system (Waters) coupled to a Xevo G2-XS QTOF mass spectrometer (Waters) equipped with a LockSpray dual electrospray ion source (Waters) we quantified benzoxazinoids in samples of filtered bacterial cultures. Gradient elution was

performed on an Acquity BEH C18 column (2.1 x 100 mm i.d., 1.7 mm particle size (Waters Corporation, Milford, USA) at 98–50% A over 6 min, 50-100% B over 2 min, holding at 100% B for 2 min, re-equilibrating at 98% A for 2 min, where A = water + 0.1% formic acid and B = acetonitrile + 0.1% formic acid. The flow rate was 0.4 ml/min. The temperature of the column was maintained at 40°C, and the injection volume was 1 µL. The QTOF MS was operated in sensitivity mode with a positive polarity. The data were acquired over an m/z range of 50–1200 with scans of 0.1 s at collision energy of 6 V (low energy) and a collision energy ramp from 10 to 30 V (high energy). The capillary and cone voltages were set to 2 kV and 20 V, respectively. The source temperature was maintained at 140°C, the desolvation temperature was 400°C at 1000 L/hr and cone gas flows was 100 L/hr. Accurate mass measurements (<2 ppm) were obtained by infusing a solution of leucin encephalin at 200 ng/mL at a flow rate of 10 µL/min through the Lockspray probe (Waters). For each expected benzoxazinoid compound, four standards with concentrations of 10 ng/ml, 50 ng/ml, 200 ng/ml and 400 ng/ml were run together with the samples (DIMBOA-Glc, DIMBOA, HMBOA, MBOA-Glc, MBOA, BOA, AMPO, APO, AAMPO, AAPO, HMPMA) or 40 ng/ml, 200 ng/ml, 1 µg/ml and 10 µg/ml for MHPA and MAPH)

Root assay

To assess the effect of SynComs on the plant growth, we performed an axenic *Arabidopsis* root growth assay. *Arabidopsis* (Col-0) were surface sterilized with 70% EtOH + 0.1% Triton-X100 for 1 min, then with 5% bleach + 0.1% Triton-X100 for 12 min and rinsed three times with autoclaved MilliQ. For sowing, the sterilized seeds were immersed in 0.2% sterile agar and with a 200 µl pipette the 100 seeds were distributed per 50% MS + sucrose plates (Duchefa Biochemie, Haarlem, Netherlands). Plates were wrapped with parafilm and kept for 4 days @ 4°C for seed stratification. Then the plates were transferred to the sterile climate room (16 h light and 18/22 °C). After 8 days, the germinated seedlings were transferred to 50% MS agar plates inoculated with SynComs.

The bacterial strains for the SynCom were grown as pure culture overnight in 30 ml of 0.5 TSB medium to a 50 ml Erlenmeyer at at 28°C with 180 rpm shaking. Then bacteria were harvested by centrifugation of 25 ml culture at 3'600 rpm for 10 min. Pellets were resuspended in 10 ml MgCl2 (10 mM), the centrifugation and the washing were repeated twice. Then the cultures were diluted to OD600 = 0.02 per strain. For SynComs equal volumes per strain were pooled in 3 ml final volume in 10 ml MgCl2 (10 mM). 100 µl of the SynComs (ca. 10^8 CFU/ml) were spread on ½ MS agar plates. Per plate, 5 pregerminated seedlings were transferred and placed 2 cm from the top. Then the plates were wrapped with parafilm and placed in the sterile climate room for 12 days. Upon harvest, root length and root elongation were measured manually with a ruler. For biomass measurements, roots and shoots were separated and the five plants

grown on the same plate were pooled in a pre-weighed microcentrifuge tube. Fresh weight was immediately assessed, then the biomass was dried for 48 hours at 60 °C before measuring dry biomass. In further analysis, additional parameters were calculated: root_growth_rate = (root_length - root_initial) / root_initial, shoot watercontent = shoot fresh weight – shoot dry weight, root watercontent = root fresh weight – root dry weight, root to shoot ratio = Root_FW/Shoot_FW.

Plant assays

Soil and plant material: The field soil used for the experiments was collected by Selma Cadot in Changins (Nyon, Switzerland). The soil was sieved and mixed with sand (20%) and sterilized by X-Ray (Synergy Health AG, Däniken, Switzerland). Maize seeds (B73 WT and *bx1*) were sterilized by washing with sterile water for 5 min, 70% ethanol for 2 min followed by a bleach solution containing 29 ml sterile water, 15 ml bleach (Migros) and 1 ml Tween (Sigma-Aldrich, St. Louis, USA) for 20 min. At the end, they were washed five times for 15 min with sterile water. All washing steps were performed in the dark. To pregerminate, the seeds were placed on wet filter paper and kept in the dark for 48 h.

Bacterial cultures: SynComs were assembled as described above for the Erlenmeyer experiment. SynComs were adjusted to an OD = 0.14. The pregerminated seeds were inoculated with 40 ml of bacterial culture in a 50 ml autoclaved pipette box (Sarstedt). They were slowly shaken for 3h. To quantify the CFU, cultures were serially diluted, plated on TSB agar plates and counted after a few days.

Maize growth: For each treatment and genotype (WT and *bx1*) twelve replicates were grown. The autoclaved 180 ml pots (Semadeni, Ostermundigen, Switzerland) were equally filled with sterilized soil. All pots were pre-watered with 13 ml sterile distilled water using a 5 ml pipette. A hole was made in every pot using a 5 ml pipette tip (to 1.5 ml scale). The pre-germinated bacteria-inoculated seeds were carefully placed in the hole with the root facing downwards. On top of the planted seed 7 ml of bacterial solution or $MgCl_2$ (NBC) were added. All the pots were randomized and then placed in the Percival growth chamber with the conditions 16 h light at 26 °C, 8 h dark at 22 °C, 60% relative humidity, irradiance of 400 µmol m−2s−1 (described by Planchamp et al.). Plants were watered with sterilized tap water by weight every two to three days. Chlorophyll content and the height of the plants were measured every seven days. The chlorophyll was measured at three different places on the youngest fully grown leaf with a SPADE-502 chlorophyll meter (Konica Minolta, Japan) and the mean value was recorded. After a growth period of 26 days the plants were harvested. The shoot was cut off and its fresh weight was determined. The roots were harvested and washed in sterile water twice by vigorously

shaking 30 s, dried, weighed, and dried. Both shoots and roots were dried for 48 h at 80 °C, subsequently dry weight was assessed.

Data analysis community profiling

The dada2 pipeline was applied to get high-quality 16S sequences from raw reads (Callahan et al., 2016). First the raw reads were filtered and trimmed with filterAndTrim. The 19 bp long primer sequences were removed (trimLeft and trimRight = 19) and low-quality reads (truncQ=2), reads matching PhiX and sequences containing N were discarded. From the quality filtered reads errors were learned using learnErrors. Using derepFastq filtered sequences were dereplicated. Using dada2 algorithm, the reads were denoised. Read pairs were merged using mergePairs with a mininmal overlap of 20 bp. Chimeric sequences were removed using removeBimeraDenovo. Next, the taxonomic assignment was done in two searches against the SILVA database until the genus level (Callahan, 2017). A phyloseq object was exported for downstream analysis. The 16s sequences of the isolates included in the SynComs were mapped to the amplicon sequences using usearch (Edgar, 2010) with an identity of 0.97. Reads were rarefied using phyloseq (McMurdie and Holmes, 2013). Prior to the analysis the abundance of the strains was normalized with the estimated copy number of 16s genes in the strain. These normalized abundances were for downstream analysis. Effects on community composition were tested by permutational analysis of variance (PERMANOVA, 999 594 permutations) on Bray-Curtis distances in the R package vegan (Oksanen et al., 2019). We tested for differences between genotypes (model: beta diversity ~ treatment * SynCom). We plotted the the Canonical Analysis of Principal coordinates (CAP) of the betadiverstiy using the R package phyloseq (McMurdie and Holmes, 2013).

Statistical analysis

Data analysis was performed in R version 4.0 (R core Team, 2016). Data management and visualization was performed using the tidyverse package collection (Wickham et al., 2019). We calculated the area under the bacterial growth curve using the function auc from the MESS package (Ekstrøm, 2016) and normalized with the growth of in control treatment (AUC norm). Raw bacterial growth data (AUC) were analysed by comparing the growth of the strain in the control treatment and the respective concentration of a compound using analysis of variance (one-sample t-tests). Further packages used for the data analysis are the following: Broom (Robinson, 2014), DECIPHER (Wright, 2016), DESeq2 (Love et al., 2014), emmeans (Lenth et al., 2019), ggthemes (Arnold, 2019), multcomp (Hothorn et al., 2008), phytools (Revell, 2012) in combination with some custom functions.

Results

MBOA structures SynComs

In previous research we identified maize root bacteria capable of metabolising benzoxazinoids. In this study, we specifically investigated how the capacity of benzoxazinoid metabolising strains affects the dynamics of a bacterial community. To this end, we designed two SynComs consisting of 7 strains. These SynComs contained a common set of six bacteria, which were selected because they were taxonomically diverse, distinguishable by 16s rRNA amplicon sequencing and they could not metabolise MBOA. As a seventh strain, *Microbacterium* strains differing in their ability to metabolise MBOA were included. In SynCom S (SCS) the *Microbacterium* LMI1x is not capable of metabolising MBOA and in SynCom R (SCR) the *Microbacterium* LMB2 can metabolise MBOA (Fig. 1a). We grew the SynComs in liquid cultures exposing them to low (500 µM) or high levels of MBOA (2500 µM) or a control treatment (DMSO) for 68 hours. Upon harvest we measured total community growth, community composition and benzoxazinoid metabolite profiles.

To investigate the effect of MBOA metabolisation on total community growth and to test if the two SynComs differ in their MBOA tolerance, we assessed total community growth by optical density and plating of colony forming units (CFU). The optical density of SynComs was reduced in presence of MBOA, but SCS suffered more than SCR in both treatments (Fig. 1b). CFU plating confirmed that SynCom with the capacity of metabolising MBOA had a larger community size in presence of the compounds (Fig. S1). Thus, SCS is more susceptible to MBOA while SCR can tolerate it better. These differences in community size demonstrated that the MBOA metabolising *Microbacterium* LMB2 was key to confer MBOA tolerance to the SCR.

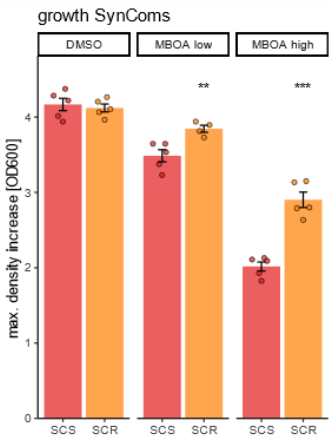
To test whether the capacity of MBOA metabolisation would not only alters the size of the community but also its composition, we profiled the cultures with 16S rRNA gene amplicon sequencing. The treatment had a significant effect ($p = 0.001$) on community composition but not the SynCom type (Fig. 1c). Community composition did not differ between SynComs and was unaffected in absence of MBOA (control) and at low levels of MBOA. At high levels of MBOA however, both communities changed relative to control and the two communities differed significantly between each other ($p = 0.001$). These differences in composition are caused by the increase of tolerant and depletion of susceptible SynCom members in presence of MBOA depending on the treatment and the SynCom type (Fig. 1d, Fig. S2a). In the SCS, the *Stenotrophomonas* LST17 was strongly reduced in the MBOA high treatment, while *Pseudomonas* LMX9 and *Chitinophaga* LMN1 increased in abundance. In the case of *Stenotrophomonas* LST17, the reduced growth is explained by weak growth of LST17 in high MBOA concentrations (Fig. 4b, chapter 1). In line with this, *Pseudomonas* LMX9 increasing in abundance was MBOA tolerant. The

composition of SCR exposed to high MBOA, is more comparable the SCR grown in the control treatment (Fig. 1c). Similar as in SCS, the abundance of *Stenotrophomonas* LST17 is reduced while *Pseudomonas* LMX9 and *Chitinophaga* LMN1 are more abundant, but the differences were weaker than in SCS. Contrary to SCS, *Bacillus* LBA21 was completely absent in SCR in high MBOA. These differences in community composition among the SynComs, indicated that the *Microbacteria*'s presence in these communities and their capacity to degrade MBOA altered community structure in presence of high levels of MBOA. Differences in MBOA tolerance of the single strains (Fig. 4b, chapter 1) did not completely explain altered abundances in the communities, pointing to more complex mechanisms responsible for these dynamics. It is well possible that the MBOA metabolising *Microbacterium* LMB2 in SCR degrades the toxic MBOA and thus MBOA susceptible strains like LST17 grow better. Further interactions among microbes like the formation of antibiotics, differences in growth rates or metabolic cross-feeding may influence community compositions of SynComs in MBOA. In conclusion, MBOA controlled community size and shaped community composition of the two SynComs tested.

A

SCS	SCR	Strain	Genus	MBOA metabolization	MBOA tolerance
X	X	LMX9231	*Enterobacter*	weak	Intermediate
X	X	LMN1	*Chitinophaga*	No	Susceptible
X	X	LST17	*Stenotrophomonas*	No	Susceptible
X	X	LMX9	*Pseudomonas*	No	Intermediate
X	X	LMG1	*Streptomyces*	No	Susceptible
X	X	LBA21	*Bacillus*	No	Intermediate
X		LMI1x	*Microbacterium*	No	Tolerant
	X	LMB2	*Microbacterium*	Strong	Tolerant

B



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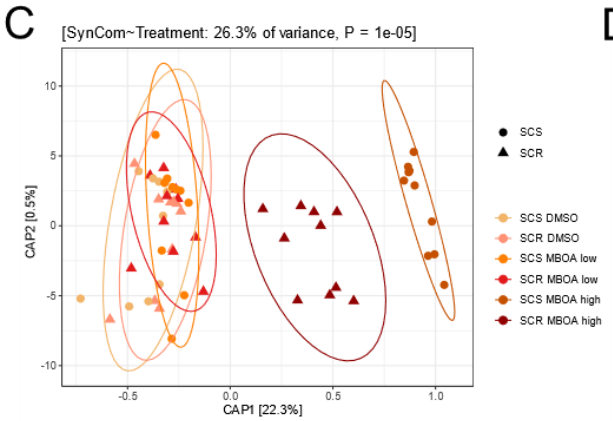

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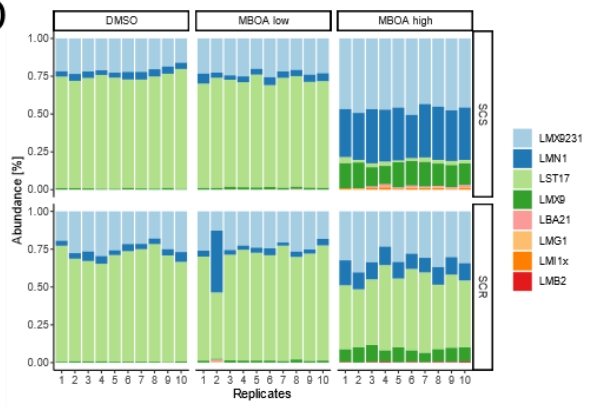

	All MBOA	MBOA high	MBOA low
Treatment	0.001 ***	0.001 ***	0.661
SynCom	0.278	0.107	0.743
Treatment:SynCom	0.085	0.057	0.810

Figure 1. MBOA shapes composition of SynComs *in vitro*. A) SynComs consist of 6 core strains and one *Microbacterium* differing in MBOA metabolisation, in *Microbacterium* LMI1x in SCS does not metabolise MBOA while *Microbacterium* LMB2 in SCR metabolises MBOA. **B)** Growth of SynComs exposed to the three DMSO, MBOA low (500 µM) and MBOA high (2500 µM) treatment, optical density measured at 600 nm. means ± SE, bargraphs and individual datapoints are shown and t-test are included (n = 5). **C)** Relative abundance of ASVs corresponding to the strains in the two SynComs grown in the three treatments. Each bar represents one sequenced sample, two technical replicates per Erlenmeyer sample (n = 10) **D)** Constrained Analysis of Principal Coordinates (CAP) confirming the treatment effects found in the PERMANOVA, axis labels indicate percentage of explained variance (n = 10).

SynComs differ in their ability to metabolise MBOA

To investigate how the capacity of metabolising MBOA affected the degradation of benzoxazinoids by the SynComs, we measured their complete benzoxazinoid metabolite profiles at the end of the growth. SCS converted a small fraction of MBOA to a little bit of AMPO (Fig. S3). In SCR MBOA was absent, indicating that the community completely metabolised this compound. Small amounts of AMPO and AAMPO were detected but they explained a small fraction of the original levels of MBOA. We checked the complete metabolite profiles using untargeted metabolomics and detected an abundant compound forming in SCR and absent in SCS appearing at retention time 2.88 min and mass of 181.19 g/mol. We compared this compound with available literature and by running the samples along the standard, we identified this compound as HMPAA. Its quantification revealed that it explained ca. half (250 µM) of the initial amounts of MBOA (500 µM; Fig. 2a). Thus, HMPAA is the dominant MBOA degradation product of the SCR.

To understand the dynamics of MBOA metabolism in the two SynComs, we performed a time series experiment. Opposite to the first experiment, this experiment was conducted in a high-throughput system and the cultures were grown in 200 µl microplates. As observed before, SCS formed little AMPO from MBOA (Fig. 2a). In SCR, little HMPAA was formed already after 16 h and accumulated steadily until the end of the experiment after 96 h. At this timepoint, no MBOA was present any more in the culture, confirming the previous observations from the experiment in the Erlenmeyer cultures. In parallel, we also measured the capabilities of MBOA metabolism of the individual SynCom strains (Fig. 2b). All strains, except *Microbacterium* LMB2 were unable to metabolise MBOA after 68 hours. Only the *Microbacterium* LMB2 degraded MBOA completely (500 µM) and formed approximately 100 µM AMPO. In pure cultures of LMB2, MBOA is first degraded to the intermediate MAP and then dimerized to form AMPO (Fig. 2c). In the time series experiment, we detected a compound accumulating in SCR with a mass of 139.06 g/mol, which corresponds to the mass of the intermediate MAP (Fig. S3b). The formation of HMPAA requires an acetylation of MAP which may be catalysed by an enzyme present in one of the six other SynCom strains. These experiments show that in SCR MBOA metabolisation yields the HMPAA as a major degradation product. Given that only one of the SCR strains metabolised MBOA, none of those strains formed HMPAA in pure culture and that HMPAA accumulated only in the SCR community, indicated that the formation of HMPAA required a combination of strains with

Microbacterium LMB2 and at least one other SynCom strain. Together these experiments demonstrate that the SynCom strains cooperate in BX-metabolisation to form the degradation product HMPAA, which single strains would not form.

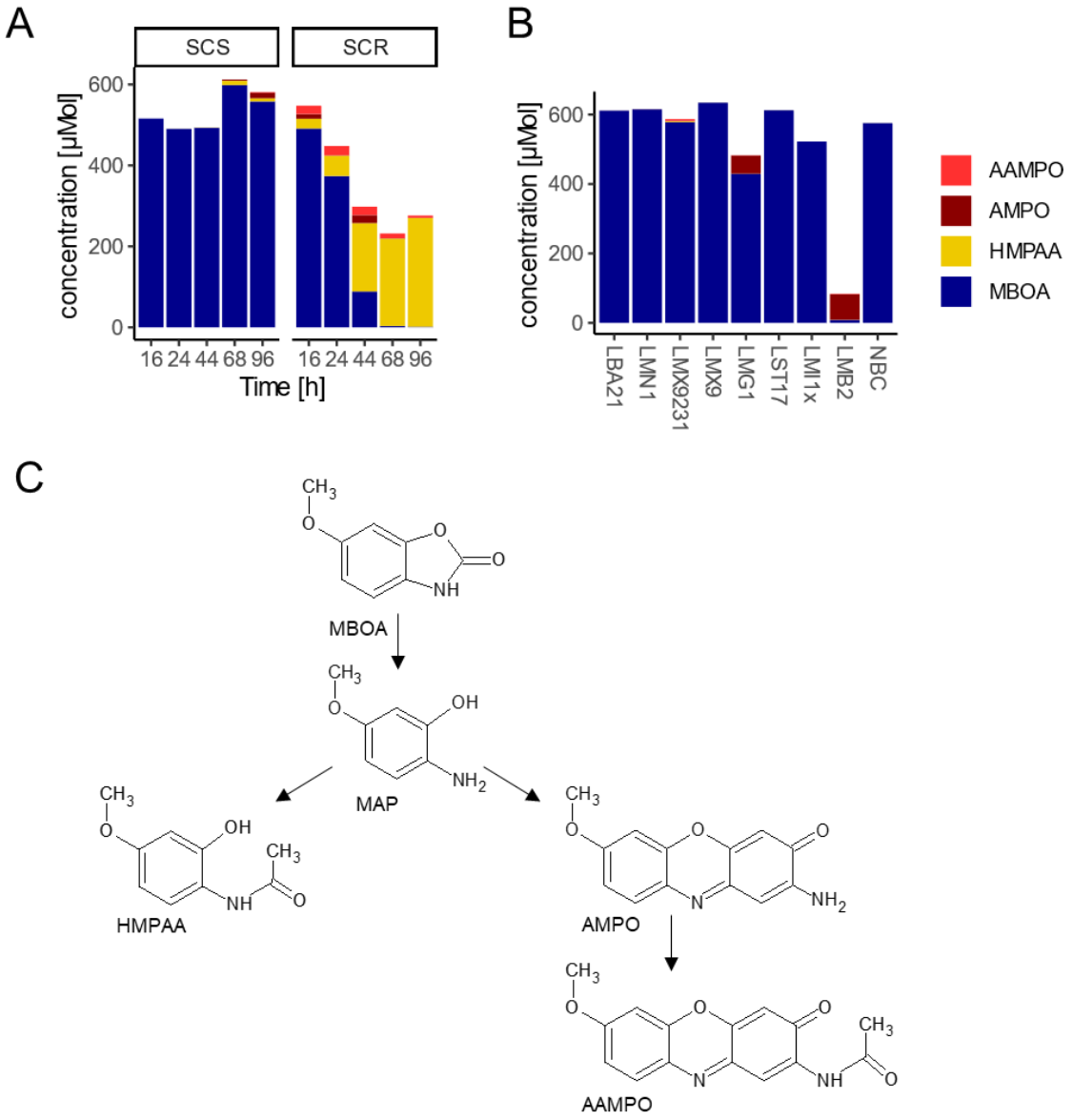

Figure 2. SynComs differ in their ability to metabolise MBOA. A) Benzoxazinoid metabolites measured over five time points in the two SynComs grown in MBOA. **B)** Benzoxazinoid metabolites of single strains grown for 68 hours in MBOA. Each bar represents a pooled sample consisting of three independent samples. **C)** Schematic representation of MBOA metabolisation routes in SCR.

A combination of MBOA-metabolising *Microbacterium* LMB2 with another strain is required for HMPAA formation

Next, we tested combinations of core SCR strains together whether they can form HMPAA. The combinations were tested as pairs and dropout communities. For the pairs, we combined *Microbacterium* LMB2 with one other SynCom strain in equal ratios and grew LMB2 and SCR

along as control. In combination with the *Bacillus* LBA21, *Chitinophaga* LMN1 and *Streptomyces* LMG1, while high amounts of AMPO were formed, almost no HMPAA was detected (Fig. 3a). The latter observation indicated that these strains were not the suspected partners of *Microbacterium* LMB2 to form HMPAA. However, when LMB2 was grown in co-culture with *Enterobacter* LMX9231, *Pseudomonas* LMX9 and *Stenotrophomonas* LST17, we detected high proportions of HMPAA. To confirm the requirement of one or several of these strains for HMPAA formation in SCR, we grew dropout communities where in each community one strain was excluded (Fig. 3b). Only communities missing *Enterobacter* LMX9231 or *Stenotrophomonas* LST17 – these were the dominant species in the SynComs (Fig. 1d) – metabolite profiles are comparable to the complete SCR. This suggested that the trait to acetylate MAP was among the remaining four strains, which were all low abundant strains in a full SCR. Indeed, removing either *Bacillus* LBA21, *Chitinophaga* LMN1, *Streptomyces* LMG1 and *Pseudomonas* LMX9 from the communities, not all MBOA was degraded and importantly, less HMPAA was formed compared to the complete SCR. Together with the strain pair data, this suggested LMX9 as the key partner of *Microbacterium* LMB2 to form HMPAA. The underlying rational of HMPAA formation in combination of LMB2 and LMX9 is that in dropout communities missing LMX9, the least amounts of HMPAA were formed.

Chemically, the formation of HMPAA requires an acetylation of the intermediate MAP (Fig. 2c). In *Pseudomonas chlororaphis* an arylamine N-acetyltransferase (NAT) was reported to convert AP to AAP (Guo et al., 2020). Since AP and AAP are structural analogous of MAP and HMPAA only differing in one methoxy group, we hypothesized that a similar acetyltransferase that catalyses the acetylation of MAP to HMPAA might be present in *Pseudomonas* LMX9. Thus, we searched the amino acid sequence of the NAT of *P. chlororaphis* in the genomes of our strains including LMX9. In *Pseudomonas* LMX9, we found a corresponding enzyme with 65% similarity in amino acid sequence. In contrast the most similar enzymes in the other and non-HMPAA producers *Bacillus* LBA21, *Streptomyces* LMG1 and *Stenotrophomonas* LST17 only low sequence similarities were identity (23.58%, 24.29% and 41.61%, respectively. Future experiments are needed to proof whether the N-acetyltransferase in *Pseudomonas* LMX9 is catalysing to acetylation of MAP to HMPAA.

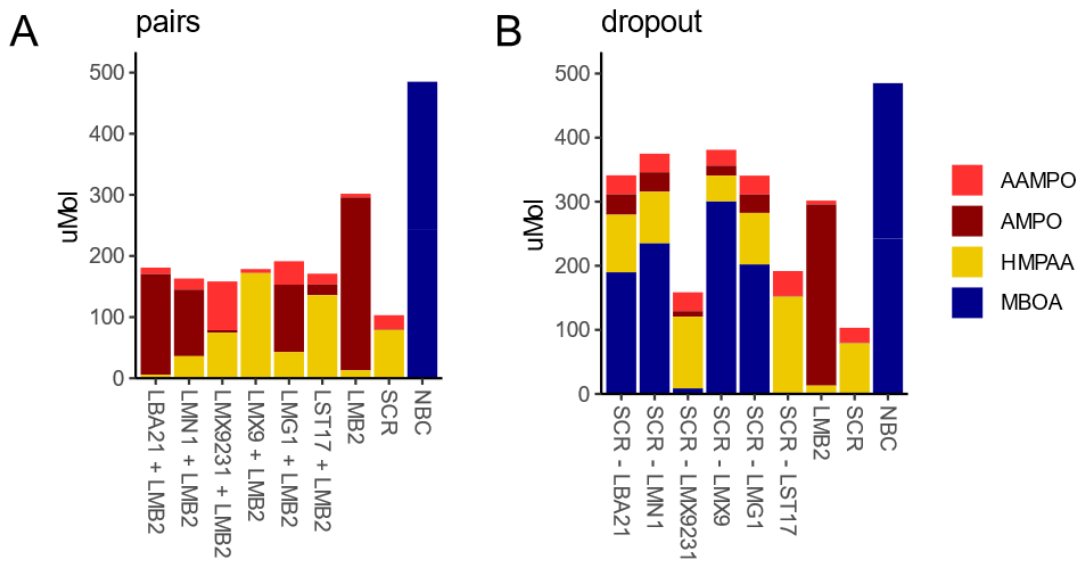

Figure 3: A combination of strains is required for the formation of HMPAA. **A)** Benzoxazinoid metabolites of pairs combinations of *Microbacterium* LMB2 with core SynCom strains **B)** dropout communities missing one strain. Metabolite profiles of LMB2 alone, complete SCR and the no bacteria control are shown in both graphs. Metabolite profiles were measured form cultures grown for 68 hours in MBOA. Each bar represents a pooled sample consisting of three independent samples.

Differences in tolerance of SynComs is the same for other benzoxazinoids tested

The two SynComs differ in their ability to metabolise MBOA and their level of tolerance to MBOA. To further understand whether the ability to metabolise MBOA was causal for their better tolerance to benzoxazinoids (SCR), we tested if the SynComs differed in their tolerance to other benzoxazinoids, too. To this end, we grew the two SynComs and the pure strains in the full growth medium TSB supplemented with BOA, DIMBOA-Glc (each at 500 µM and 2500 µM) and AMPO (at 30 µM and 180 µM) and again with MBOA for reference. As observed previously for MBOA, the community size of the SCR was less inhibited in BOA (Fig. 4a). While community size of both SynComs was unaffected by the MBOA degradation product AMPO, interestingly, they grew better with increasing levels of DIMBOA-Glc. In general, the growth responses of SCS resembled the growth response of the fast-growing strains *Enterobacter* LMX9231, *Pseudomonas* LMX9 and *Chitinophaga* LMN1 (Fig. 4b). For SCR however, the growth pattern of SCR resembled mostly the growth of the pure strain *Microbacterium* LMB2. These findings with other benzoxazinoids indicated that probably not the ability to metabolise MBOA per se but rather a general tolerance to these compounds increased their tolerance to MBOA and other benzoxazinoids. Specifically in SCR when MBOA is metabolised and thus the MBOA sensitive strains *Stenotrophomonas* LST17 could increase in abundance (Fig. 1d). These growth assays demonstrate that BX-metabolisation increases the tolerance of a SynCom in various benzoxazinoids.

To test if the metabolisation of MBOA metabolisation enables the community to access benzoxazinoids as carbon source for bacterial growth, we performed the same experiments with the two SynComs and the pure strains in minimal media supplemented with different benzoxazinoids. As positive control, we supplemented the cultures with glucose concentrations equal to the tested benzoxazinoids (500 µM, 2500 µM) and saturating to provoke maximal growth (30 000 µM). Compared to the control, the SCR grew significantly better in high levels of MBOA and DIMBOA-Glc (Fig. 4c). On contrary, the SCS only grew better at high levels of DIMBOA-Glc, albeit weaker than the SCR. This indicated to us that the SCR, by metabolising MBOA, could benefit from its use as a carbon source for growth. The better growth of both SynComs in DIMBOA-Glc is probably due to one or more of the six shared members that have the ability to cleave the glucose moiety. The improved growth of the SCR is probably explained by full metabolisation of DIMBOA-Glc including a further degradation of MBOA. The reduced growth of SCS on DIMBOA-Glc compared to SCR, might be explained they these bacteria could just use the carbon from the glucose moiety for growth. This interpretation is supported by the results of similar growth of SCS in 2500 µM of DIMBOA-Glc and 2500 µM of glucose alone. Among the single SynCom strains, *Enterobacter* LMX9231, *Pseudomonas* LMX9, *Chitinophaga* LMN1 and *Microbacterium* LMI1x grew to similar levels both in DIMBOA-Glc as well as in glucose (Fig. 4d). This suggested that these strains can de-glycosylate the benzoxazinoids. Interestingly, *Microbacterium* LMB2 was the only strain that grows much better in presence of DIMBOA-Glc 2500 µM and MBOA 2500 µM, which is consistent with its ability to degrade MBOA, but also indicating that it is capable to de-glycosylate and therefore, can benefit from the complete molecule as a carbon source. Taken together, these experiments demonstrated that SynComs profit from metabolising the benzoxazinoids by using them as nutritional compounds for their own growth.

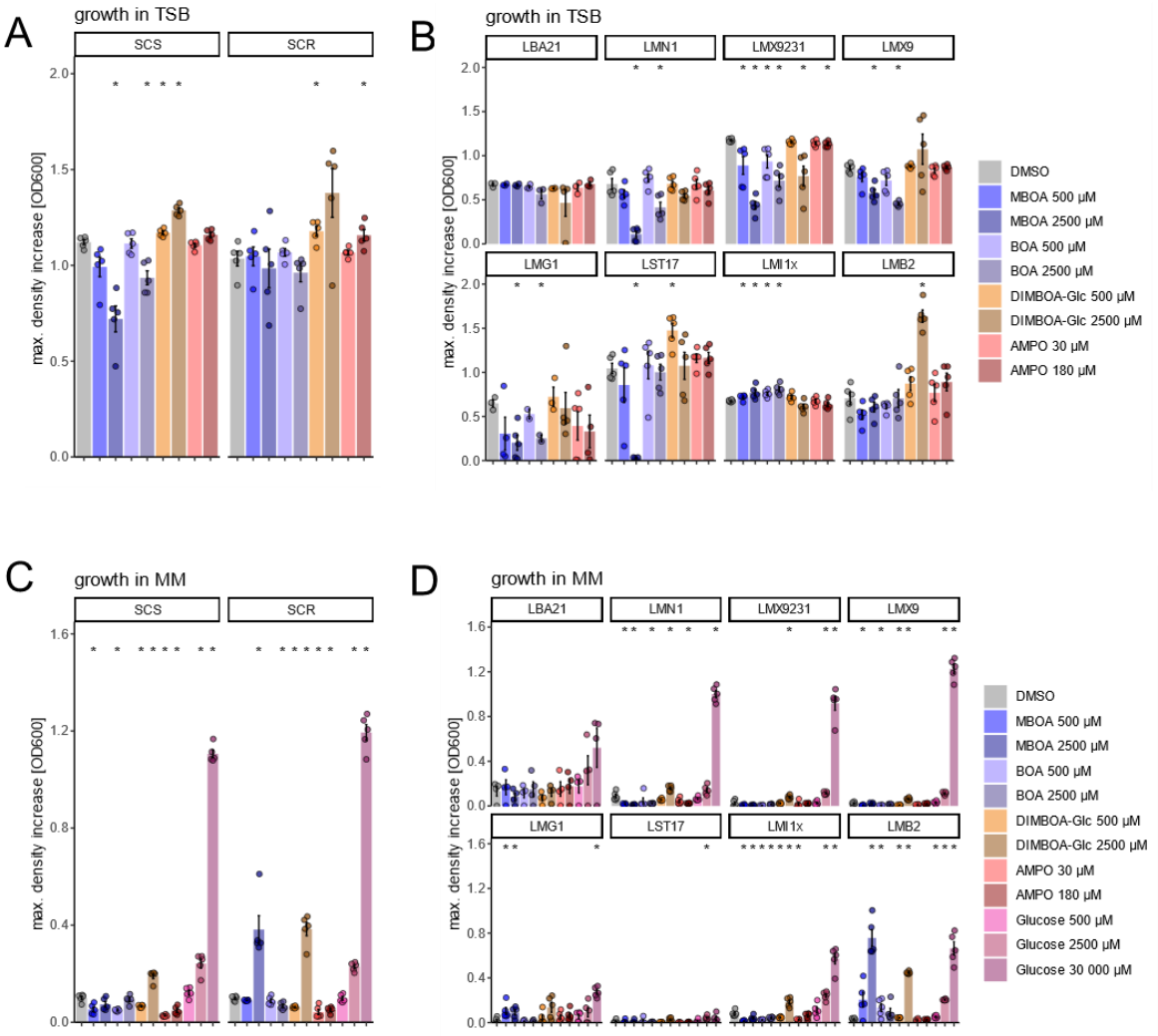

Figure 4. SynComs differ in their ability to tolerate and different benzoxazinoid compounds. A) Growth of SynComs **B)** and single strains in full medium (TSB) supplemented with MBOA, BOA, DIMBOA-Glc and AMPO. means ± SE, bargraphs and individual datapoints are shown and t-test are included (n = 5) **C)** Growth of SynComs **D)** and single strains in minimal medium (MM) supplemented with MBOA, BOA, DIMBOA-Glc and AMPO and glucose showing their ability to use the compounds as carbon source for microbial growth.

Differences in metabolism of SynComs is the same for other benzoxazinoids tested

To further investigate if the metabolisation capacities of the SynComs were restricted to MBOA or may be expanded to other benzoxazinoids, we analysed their metabolisation profiles when exposed to different benzoxazinoids in TSB medium. None of the SynComs metabolised BOA (Fig. 5a). As for MBOA, *Microbacterium* LMB2 was the only single strain metabolising BOA (Fig. 5b). DIMBOA-Glc, the precursor of MBOA, was metabolised by both SynComs. SCS partially degraded DIMBOA-Glc and formed low levels of MBOA (ca. 100 µM). In contrast the SCR, DIMBOA-Glc was completely degraded and converted to MBOA, AMPO and HMPAA. The molar sum of these degradation products did not equal the initial amounts of DIMBOA-Glc in the cultures, suggesting that either degradation to other undetected metabolisation products or full degradation, e.g. to simple carbon units for primary metabolism. Among the single strains, *Enterobacter* LMX9231

was the only strain that completely metabolised DIMBOA-Glc, thereby identifying the key bacterium capable to initiate the breakdown of DIMBOA-Glc in the context of the SynComs. Exposing the SynComs to AMPO, yielded similar amounts of the further metabolisation product AAMPO by both SynComs. The analysis of single strains revealed that the SynCom members, *Enterobacter* LMX9231, *Pseudomonas* LMX9, *Streptomyces* LMG1 and *Stenotrophomonas* LST17 were capable to acetylate AMPO to AAMPO in pure culture. Interestingly, the other strains *Bacillus* LBA21, *Chitinophaga* LMN1, *Microbacterium* LMI1x and LMB2, of which the former three are unable to degrade MBOA (Fig. 5b), they were capable to degrade AMPO, a degradation product of MBOA. The conversion of AMPO to AAMPO requires an acetylation of the NH_2 group, which is the same position that is acetylated for HMPAA formation. The same acetylation reaction might be responsible for the conversion of MAP to HMPPA, supported by the observation that the same three strains *Enterobacter* LMX9231, *Pseudomonas* LMX9 and *Stenotrophomonas* LST17 in combination with *Microbacterium* LMB2 form HMPAA. Interpreting these individual findings in a community context of SCR suggests that *Enterobacter* LMX9231 initiates breakdown of DIMBOA-Glc to MBOA, *Microbacterium* LMB2 will further metabolise it to AMPO that then is acetylated by *Pseudomonas* LMX9, *Enterobacter* LMX9231, *Stenotrophomonas* LST17 and *Streptomyces* LMG1 to form AAMPO. In summary, the two SynComs differ in tolerance to various benzoxazinoid, their ability to use different benzoxazinoids as carbon source and to metabolise different benzoxazinoids.

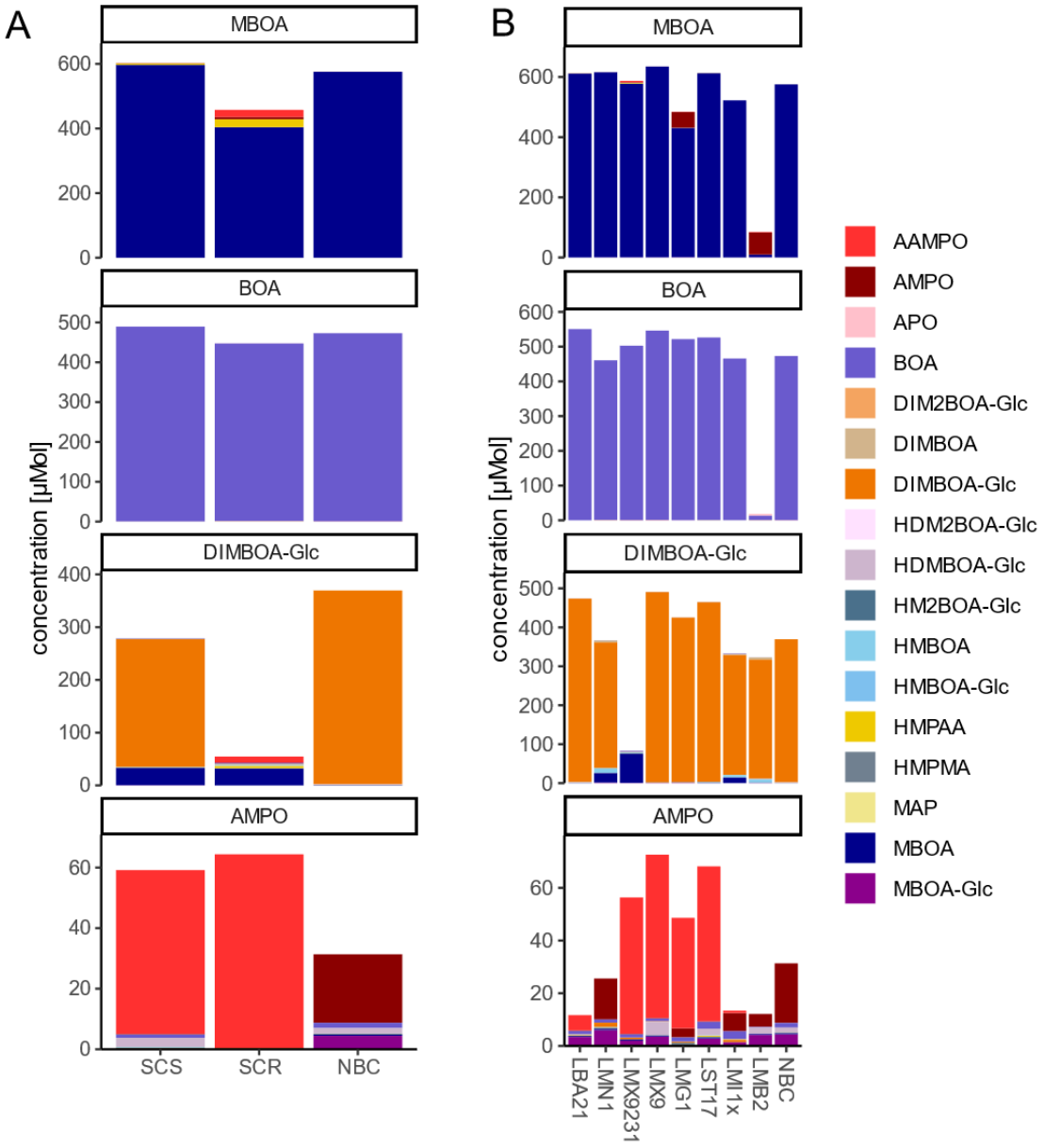

Figure 5. Metabolite profiles of SynComs and single strains exposed to different benzoxazinoids. A) SynComs and **B)** single strains grown for 68 hours in MBOA, BOA, DIMBOA-Glc and AMPO. Each bar represents a pooled sample consisting of three independent samples.

SynComs do affect plant growth but independent of benzoxazinoid metabolisation

With the knowledge of the tolerance and metabolisation phenotypes of the SynComs in presence of different benzoxazinoids, we sought to test the effects of the SynComs on plant growth in presence of benzoxazinoids. *Arabidopsis* seedlings were placed on control or MBOA containing agar and inoculated either with the SynCom S, the SCR and a no-bacterial control treatment and we scored plant growth parameters after 12 days. The two SynComs differed in their effects on shoot dry weight in MBOA plates, while this was not seen in absence of the compound (sig. interaction effect of treatment:SynCom, p = 3.80E-02; Fig 6a). Although both

SynComs rescued the inhibitory effect of MBOA on shoot dry weight, the growth promotion by the non-metabolising SCS was stronger compared to the MBOA-metabolising SCR. Like the shoot, also root dry weight was differentially affected by the two SynComs an only in presence of MBOA. In general, the bacteria promoted root growth by SCS and less by the SCR when MBOA was present (Fig. S5d). These observations can be explained by accumulation of the phytotoxic AMPO from the MBOA metabolising SynCom R. The other root phenology measures (growth rate, elongation, or length) were affected by the presence of the SynComs but unlike the biomass measures, they were independent of the SynCom's differential ability to metabolise MBOA (Fig. 6b, S5ef). Taken together, we find that bacterial SynComs rescued the MBOA-inhibited growth of *Arabidopsis*, but significantly less when they metabolise MBOA what results in accumulation of degradation products (HMPAA and AAMPO).

Complementary to the direct *in vitro* exposure of *Arabidopsis* to the chemicals we tested for the effects of differential BX-metabolising SynComs with plant-exuded benzoxazinoids in soil. We performed an experiment where we grew BX-producing WT and the BX-deficient *bx1* maize lines in sterilized field soil and inoculated them with the SynComs or with non-bacterial control treatment. Examining the effects on maize growth we found the SCS - but not the BX-metabolising SCR forming the metabolites HMPAA and AAMPO *in vitro* - specifically increased the shoot dry weight of WT maize while this was not the case in absence of benzoxazinoids in *bx1* (Fig. 6c). SynCom inoculated did not alter root biomass nor chlorophyll content (Fig. S6ab). Taken together, we find that SynComs improved the growth of *Arabidopsis* and maize in presence of benzoxazinoids, however, this growth promotion was reduced in a SynCom that was able to metabolise MBOA to HMPAA and AAMPO.

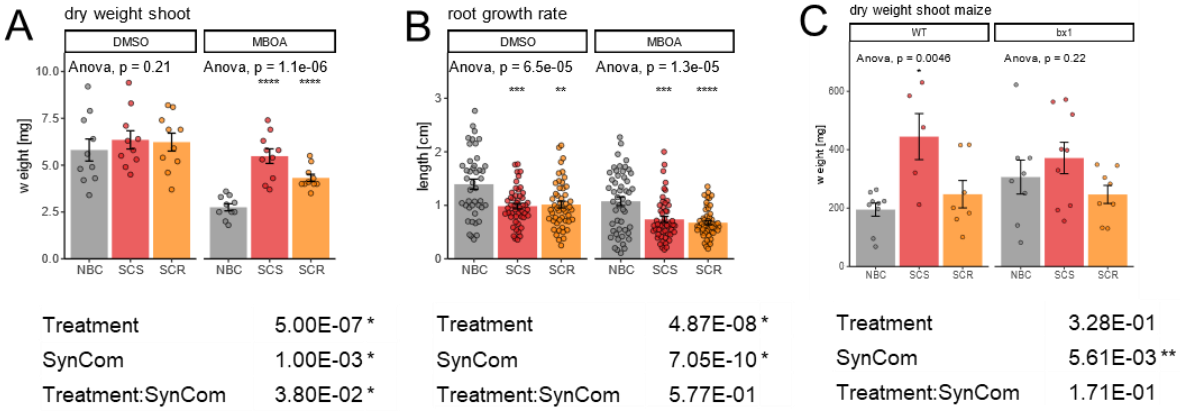

Figure 6. Effect of SynComs on plant growth. **A)** Shoot dry weight (n = 10) and **B)** root growth rate (n = 50) of *Arabidopsis* grown on axenic agar. **C)** shoot dry weight of maize grown in sterilized soil inoculated with SynComs (n = 8-10). Means ± SE bargraphs and individual datapoints are shown. Results of ANOVA and pairwise t-test between NBC and treatment is shown inside the panels, p-value < 0.05 = *.

Discussion

Root exuded plant specialized metabolites structure microbial communities (Jacoby et al., 2020b). Yet, the underlying mechanisms of how bacterial members of the communities cope with plant specialized metabolites are largely unknown. Several isolates from the maize root bacteria strain collection can metabolise benzoxazinoids (chapter 2, this thesis). To understand the role of benzoxazinoid metabolising bacteria in a community, we designed two 7-member synthetic communities. They contain six core strains plus either a benzoxazinoid metabolising or a non-metabolising *Microbacterium* strain. Here we show that bacteria associated with maize roots cooperate to metabolise benzoxazinoids. Microbial cooperation alters benzoxazinoid metabolite profiles in communities. This increases community benzoxazinoid tolerance and alters community composition. It appears that metabolising benzoxazinoids is beneficial to bacteria to use them as the sole carbon source for growth. But differences in benzoxazinoid metabolism do not alter effects of SynComs on plant growth. Microbial cooperation in benzoxazinoid degradation is a mechanism for how plant specialized metabolites affect microbial communities.

Benzoxazinoids control community size and shape communities *in vitro*

To investigate how benzoxazinoids shape microbiomes and if responses of single microbes to benzoxazinoids explain their abundance in the community, we measured community size and community composition at the end of the experiment. We find the SynCom including the BX-metabolising strain, is more tolerant to MBOA. In the two SynComs, MBOA exposure led to different community composition. In SCS, MBOA reduces *Stenotrophomonas* LST17, while *Pseudomonas* LMX9 and *Chitinophaga* LMN1 increase in abundance. For *Stenotrophomonas* LST17, the inhibition is explained by its low MBOA tolerance. Our experiments are performed in a reduced system with SynComs of a limited number of microbes. They represent a small fraction of the microbial diversity in the community. Further all experiments were performed in sterile growth systems under optimal growth conditions, ignoring environmental complexity in the soil. However, SynComs are the tool to understand mechanisms governing the assembly of complex microbial communities (Vorholt et al., 2017). The fact that benzoxazinoids shape SynComs in vitro, underline the importance of benzoxazinoids shaping root associated microbial communities on BX-producing maize roots which was demonstrated by recent studies (Cadot et al., 2021b; Cotton et al., 2019; Hu et al., 2018b; Kudjordjie et al., 2019). A similar experiment revealed that SynComs assembled from bacteria isolated from *Arabidopsis* roots grown in vitro and exposed to benzoxazinoids BOA and APO alter their community composition (Schandry et al., 2021). This effect depends on the tolerance of the single strains in the pure compounds. SynComs assembled from tolerant strain show overall a higher resilience, i. e. less variance explained by treatment. Even though our communities only differ in one strain with altered ability to

metabolise benzoxazinoids, these observations go in line since in our case the non-metabolising SCS shows a stronger difference in community composition when exposed to MBOA than the BX-metabolising SCR. This is probably explained by MBOA detoxification in SCR, leading to a reduced toxicity. This is supported by the fact that in SCR the MBOA susceptible LST17 has a higher abundance than in SCS. Contrary to the study by Schandry et al. using *Arabidopsis* root bacteria, in our experiment, we employed root bacteria isolated from BX-producing maize roots (chapter 1, this thesis; Thoenen et al. unpublished). The advantage of our approach is that these bacteria are adapted to the host secondary metabolites and evolved mechanisms to cope with the benzoxazinoids. One mechanism of dealing with benzoxazinoids is tolerance, another is the use as carbon source and the third is metabolisation of the compounds (chapter 2, this thesis; Thoenen et al. unpublished). In the *Arabidopsis* collection we did not find any isolates metabolising MBOA while it is an abundant trait in the maize root bacteria collection. This highlights the importance of adaption to plant specialized metabolites both to steer community composition and for own benefits of the microbes. Our findings provide e a new mechanism governing the structuring of microbial communities by benzoxazinoids extending on benzoxazinoid tolerance by single strains. Benzoxazinoid metabolism by a community is a mechanism in a community to tolerate benzoxazinoids and structures communities. As several plant specialized metabolites shape community composition of root associated microbiomes, it is likely that metabolisation of plant specialized metabolites steers community composition. The root microbiome of Arabidopsis is shaped by triterpenes and single microbiome isolates can metabolise triterpenes (Huang et al. 2018). There it is well possible that the ability of single strains to metabolise triterpenes may affect community composition.

Microbial cooperation in benzoxazinoid metabolisation

Given that single SynCom members can metabolise benzoxazinoids and that the composition of the two SynComs differ, we hypothesized that SynCom strain cooperate to metabolise benzoxazinoids. The metabolite analysis of the two SynComs grown in MBOA, revealed that the BX-metabolising SynCom degrades MBOA to form HMPAA as the main metabolite. Yet, no SynComs strain formed this metabolite in pure culture when exposed to MBOA. Among those strains, *Microbacterium* LMB2 is the only strain metabolising MBOA to form AMPO. Thus, we hypothesized that HMPAA formation requires at least two strains. Since we did not detect MBOA degradation in the other SynCom, it was clear that *Microbacterium* LMB2 is involved in the formation of HMPAA. Testing many strain combinations as pairs and dropout communities, revealed that HMPAA forms in combination of *Microbacterium* LMB2 with *Enterobacter* LMX9231, *Stenotrophomonas* LST17 and *Pseudomonas* LMX9. Testing dropout communities revealed that the absence of LMX9 prevents HMPAA formation. Together these

results prove that HMPAA formation requires *Microbacterium* LMB2 and *Pseudomonas* LMX9. We tested the strain combinations required for HMPAA formation by growing combination of strains as pairs or growing dropout communities missing one strain. Microbial dynamics in these communities may vary to the full SynComs, since strains have different growth rates, produce different antibiotics, and have different levels of MBOA tolerance. Further niche differentiation by different substrate utilization and metabolic cross-feeding of essential metabolites and vitamins defines the coexistence of strains in the community (Krumbach et al., 2021). Specifically in a stable seven-member community assembled from maize root bacteria, an *Enterobacter* dominates, mainly because it has a fast growth rate, has the broadest substrate utilization profile and is auxotrophic for vitamin B. The removal of this *Enterobacter* decreases the use of carbohydrates by the community and thus decreases community stability pointing to its role as keystone species. Similarly, in our SynComs *Enterobacter* LMX9231 dominates, which may be related to similar mechanisms. This SynCom was also grown in maize extracts and specifically *Enterobacter*, *Curtobacterium* and the complete SynCom depleted a group of compounds which includes sugar conjugates of flaovnoids and benzoxazolones. Thus, the dominance of *Enterobacter* may ensure community stability and be a characteristic for maize derived SynComs.

Given that several genera can metabolise benzoxazinoids in pure culture (chapter 2, this thesis) and that combination of different strains can yield HMPAA, it is likely that microbial cooperation to degrade benzoxazinoids is widespread. A similar example of microbial cooperation in benzoxazinoid metabolism is reported for a co-culture of the fungus *Fusarium verticillioides* with the bacterium *Bacillus mojavensis* (Bacon et al., 2007). In pure culture *Fusarium* metabolises BOA to HMPA, but in co-culture with *Bacillus* the metabolisation is redirected to form APO. Since the *Fusarium* is susceptible to APO, this microbial cooperation is a way how the *Bacillus* controls the pathogenic fungus. Yet, the underlying genetic mechanisms were not investigated. Thus, microbial cooperation may change the metabolite dynamics in rhizosphere altering biological functions. Similar mechanisms may be responsible for the metabolisation of other plant specialized metabolites by root associated microbial communities. Single microbiome isolates can metabolise triterpenes (Huang et al. 2018) and it is well possible that the strains may cooperate to metabolise triterpenes leading to altered rhizosphere metabolite profiles.

Genetic mechanisms responsible for microbial cooperation in benzoxazinoid metabolisation

Since the formation of HMPAA requires *Microbacterium* LMB2 and *Pseudomonas* LMX9, it is likely that *Microbacterium* converts MBOA to the intermediate MAP which is then acetylated by *Pseudomonas* to HMPAA. In *Microbacterium* LMB2 a N-acyl homoserine lactonase handled the

conversion of MBOA to AMPO (chapter 2, this thesis; Thoenen et al. unpublished). Here we found a homologous gene of the arylamine N-acetyltransferase identified in *Pseudomonas chlororaphis* catalysing the formation of AP to AAP (Guo et al., 2020). Thus, it is likely that in *Pseudomonas* LMX9 this enzyme handles the conversion of MAP to HMPAA. Yet, this observation is only based on blasting a candidate sequence. To understand the chemical reactions, it should be tested if LMX9 metabolises MAP to HMPAA. To disentangle the genetic basis of microbial cooperation in BX-metabolisation in SynComs, an insightful experiment is to combine *Microbacterium* LMB2 with a knock-out in the lactonase gene and *Pseudomonas* LMX9 with a knock-out in the acyltransferase. A transcriptome analysis of the communities or pairs growing in MBOA would shed light in the genetic response of benzoxazinoid degradation in the SynComs. Our SynComs provide a tool to investigate the genetic basis of microbial cooperation for benzoxazinoid metabolism.

Effect of benzoxazinoid metabolisation for bacterial growth and on plant growth of host and target plants

It is an open question why microbes metabolise benzoxazinoids, is it to detoxify them, to profit from them as carbon source, may they use degradation products as defense against other microbiota members or may it be beneficial for the plant growth? Having established the phenotypes of the two SynComs, we investigated the role of BX-metabolisation of the SynComs for bacterial growth and effect on plant growth. The benzoxazinoid metabolising SynComs is more tolerant to BOA and grows on BOA, MBOA and DIMBOA-Glc as sole carbon source. The non-BX metabolising SynCom is susceptible to BOA and can only use the glucose moiety of the DIMBOA-Glc molecule as carbon source. Thus, BX-metabolisation is beneficial for bacterial growth. This exemplifies the specific adaption of host microbiomes to tolerate and metabolise host secondary metabolites. In line microbial cooperation is also a way for rhizosphere bacteria to access an extra carbon source to sustain growth which is advantageous to thrive in the rhizosphere (Huang et al., 2019; Krumbach et al., 2021).

In the axenic *Arabidopsis* growth system, on MBOA containing plates both SynComs, independent of their ability to metabolise benzoxazinoids increased plant biomass but reduced root length, indicating an effect on root branching. The limitation of this experiment is the use of the axenic growth system which does not reflect the complexity of a natural system. Yet, it provides a first insight in the effects of the SynComs on plant growth in presence of benzoxazinoids and allows more experiments to disentangle the molecular mechanisms. Thus, BX- metabolisation in these SynComs does not affect their plant growth promotion abilities. Yet, this is probably due to the characteristic metabolite profile dominated by HMPAA. Other benzoxazinoid degradation metabolites like the aminophenoxazinone AMPO and APO were

shown to have strong phytotoxic effects (Venturelli et al., 2015). Thus, it may be beneficial for the plants to steer the composition of the microbial composition to redirect the BX-metabolisation to one or the other way. The effects of the microbes on root architecture may be triggered by bacterial auxin production. This has been described for Similar root phenotypes were found to be triggered by auxin formed by an actinobacterium *Micrococcus luteus* (García-Cárdenas et al., 2021). Thus, it is possible that SynComs strains form auxin (IAA), for example *Pseudomonas* LMX9 since other *Pseudomonas* species produce auxins and change root architecture (Ortiz-Castro et al., 2020). To test the involvement of auxin signalling for the root phenotypes caused by the SynComs, root assays with *Arabidopsis* mutants defective in auxin signalling could be tested. To further investigate the implications of BX-metabolisation for the growth of SynComs in benzoxazinoids, SynComs with *Microbacterium* and *Pseudomonas* mutants may be tested as described above. To disentangle the mechanisms of plant growth promotion of SynComs in presence of benzoxazinoids, using the same system single strains or combinations, different benzoxazinoid metabolites or Arabidopsis mutant lines may be tested. One option may be to test the role of auxin in plant growth promotion on MBOA plates. Further they may be a crosstalk between benzoxazinoids in the substrate with auxin formation in the bacteria.

Conclusion

Natural microbial communities are diverse and single members interact to fulfil a common task or to use resources efficiently (Rafieenia et al., 2022). Here we report that maize root bacteria cooperate to tolerate and metabolise benzoxazinoids. The cooperation of maize root bacteria to tolerate and metabolise benzoxazinoids increases their performance as a community. Microbial cooperation in benzoxazinoid degradation is a mechanism of how plant specialized metabolites affect microbial communities and highlights the importance of the division of labour in microbial communities. Our findings highlight the importance of studying plant specialized metabolites mediating chemical communication in plant-microbiome interactions in more complex synthetic or natural microbial communities.

Author statement

Experiments were planned and conducted by L.T. Co-authors contributed to the library preparation (Fig. 1cd), the analysis of the amplicon sequencing data (Fig. 1cd), the running of the metabolite samples on the HPLC and the Arabidopsis root assay (Fig. 6ab) and the maize growth assay (Fig. 6c).

Acknowledgements

We thank Sarah Dolder for plant maintenance. Further we thank Thomas Roder for the development and support with the open genome browser. We thank Dr. Pamela Nicholson from the Next generation sequencing platform at the University of Bern. This work was supported by the Interfaculty Research Collaboration “One Health” of the University of Bern.

Supplementary figures

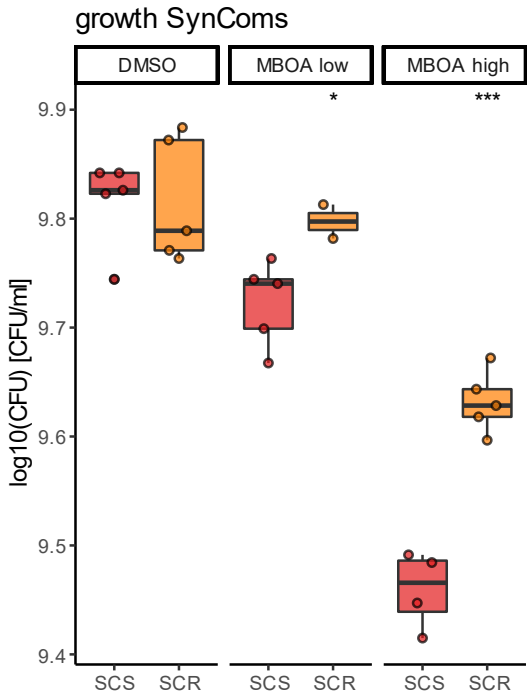

Figure S1: Growth of SynComs in three treatments. DMSO, MBOA low (500 uM) and MBOA high (2500 uM), log transformed CFU counts are shown. means ± SE, boxplots and individual datapoints are shown and t-test are included (n = 5).

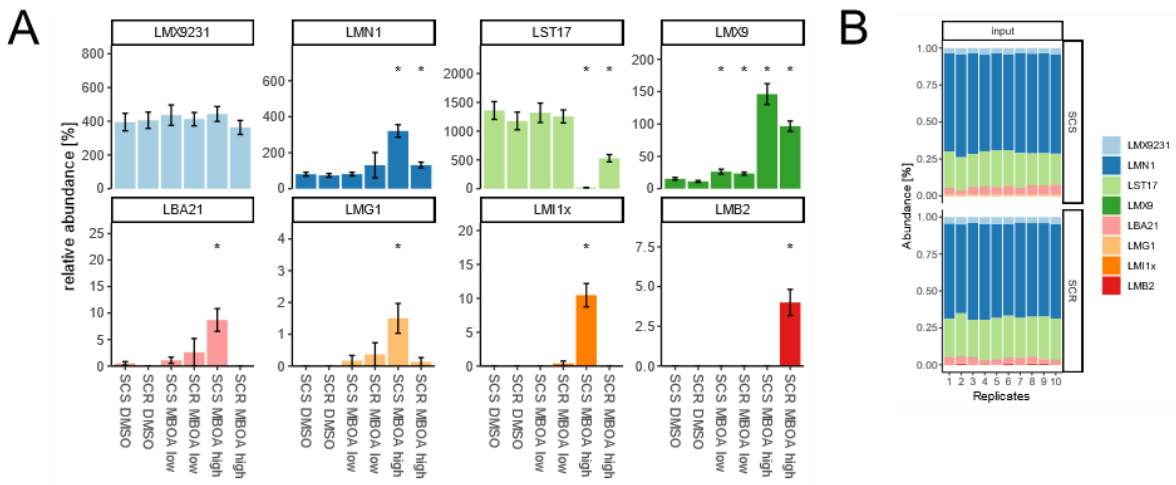

Figure S2: Abundance of ASVs in SynComs. **A)** Single ASVs abundance in the two SynComs exposed to the three treatments. means ± SE, bargraphs and individual datapoints are shown and t-test are included (n = 10). **B)** Relative abundance of ASVs corresponding to the SynComs strains in the two SynComs grown in the input community. Each bar represents one sequenced sample which are two technical replicates per sample.

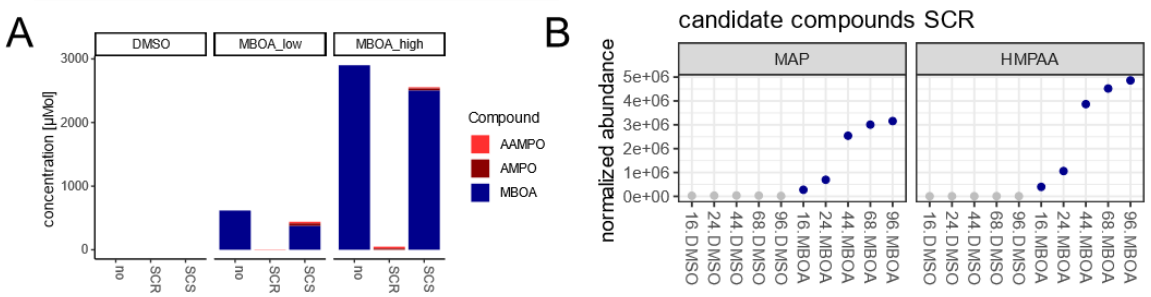

Figure S3: Metabolite profiles of cultures grown in Erlenmeyer. A) Benzoxazinoid metabolite profiles of SynComs grown for 68 hours in MBOA. Each bar represents the mean of five individually grown samples. **B)** normalized abundance of a compound with a retention time of 2.92 min and a mass of 139.0640 g/mol potentially corresponding to MAP and a compound with a retention time of 2.96 and a mass of 181.0740 g/mol confirmed to correspond to HMPAA.

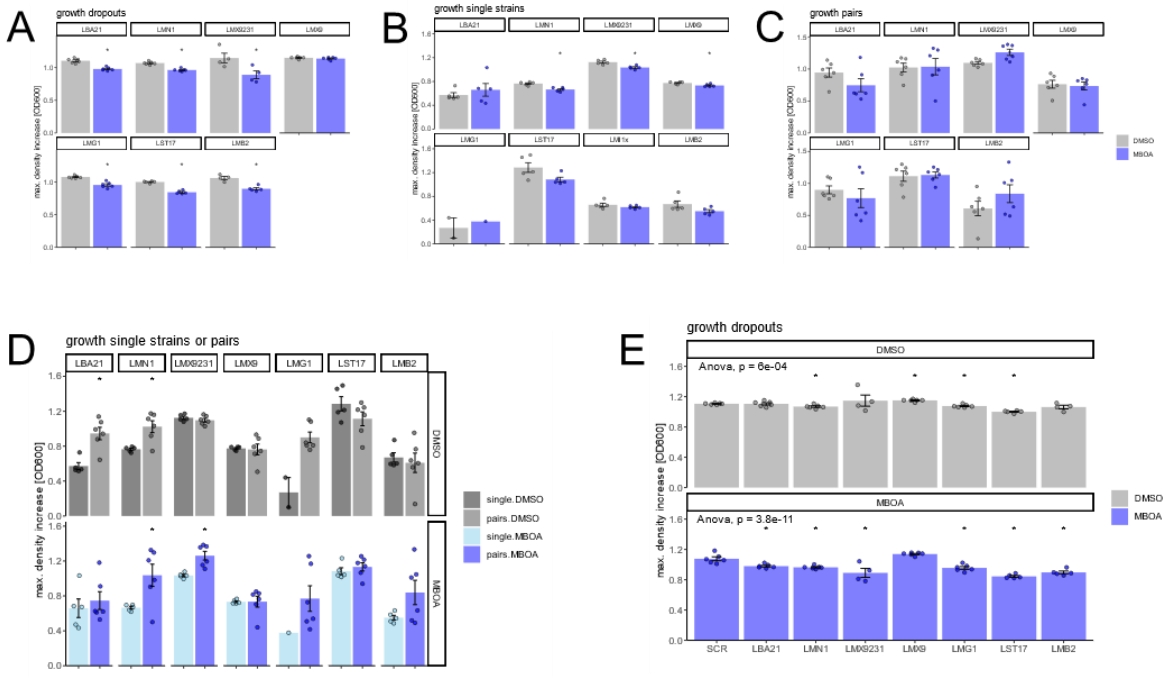

Figure S4: Bacterial growth of dropout communities, single strains, and pairs in MBOA. **A)** Dropout communities **B)** single strains, **C)** pairs grown in DMSO and MBOA low. max. optical density (OD600) is shown, means ± SE, bargraphs and individual datapoints are shown and t-test are included (n = 5). **D)** same data of growth as single strains or pairs and. **E)** same data of growth as dropout communities are shown as a direct comparison.

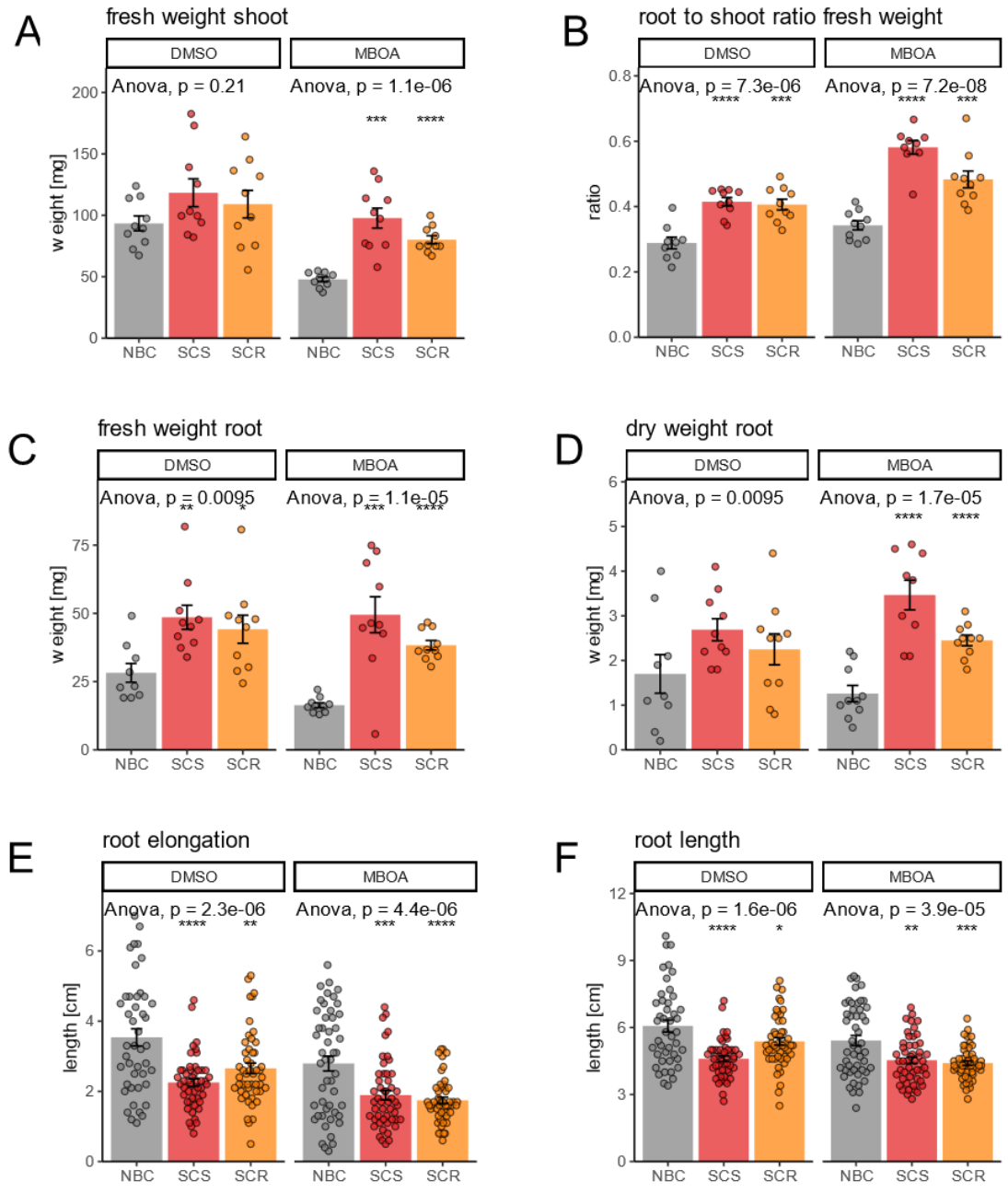

Figure S4: Plant growth parameters of Arabidopsis grown in plate assays. A) Shoot fresh weight (n = 10) and **B)** root-to-shoot ratio of fresh weight (n = 10), **C)** root fresh weight (n = 10), **D)** root dry weight (n = 10), **E)** root elongation (n = 50) and root length (n = 50) of *Arabidopsis* grown on axenic agar. Means ± SE bargraphs and individual datapoints are shown. Results of ANOVA and pairwise t-test between NBC and treatment are shown inside the panels, p-value < 0.05 = *.

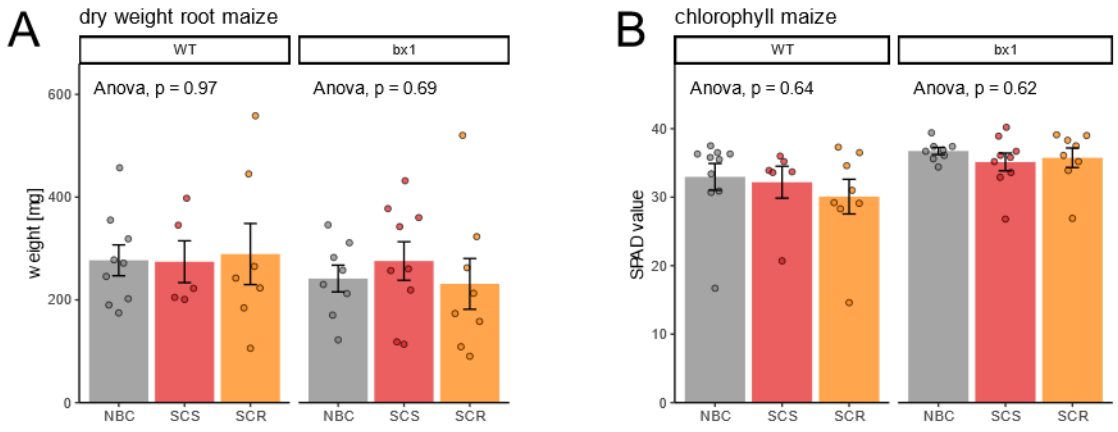

Figure S6: Plant growth parameters of maize. A) root dry weight and **B)** chlorophyll content of maize grown in sterilized soil inoculated with SynComs (n = 8-10). Means ± SE bargraphs and individual datapoints are shown. Results of ANOVA and pairwise t-test between NBC and treatment are shown inside the panels, p-value < 0.05 = *.

General Discussion

With this thesis, we investigated the mechanisms of how plant specialized metabolites like the benzoxazinoids of maize structure the root-associated microbial communities. To study the direct effects of benzoxazinoids on maize root microbiome members, we established a strain collection of maize root bacteria. We tested their ability to tolerate and metabolise benzoxazinoids as single strains and as synthetic communities. Using a high-throughput *in vitro* growth system and metabolomics, we found that the maize root bacteria differentially tolerated benzoxazinoids and that several strains could metabolise benzoxazinoids. Both bacterial traits were enriched in communities colonizing roots of BX-producing plants. Mechanistically, we reveal that bacterial tolerance to benzoxazinoids was associated with cell wall architecture. For metabolisation, we identified a lactonase enzyme specifically present in maize root-associated bacteria that converts the main benzoxazinoid MBOA to AMPO. In addition, we have detected that bacteria cooperate to tolerate and metabolise benzoxazinoids in synthetic communities. Together we demonstrate that the ability of maize root bacteria to cope with benzoxazinoids, especially to tolerate and to metabolise the compounds, explains the structuring of the maize root microbiome by benzoxazinoids. Here, we discuss the methodological approach, the mechanisms, and the biological context of these findings. Finally, we discuss the implications of the findings for future research and their implications for using plant-microbe interactions to improve plant fitness.

Benzoxazinoids mediate plant-microbiome interactions

This part of the discussion focuses on the idea that the mechanisms of how benzoxazinoids structure the microbiome evolved with a mutual interest of the microbes to survive in the rhizosphere in the presence of the compounds. In other words, the exuded plant specialized metabolites would favour, enrich or promote beneficial microbes. At the same time, the growth of pathogenic strains would be restricted, collectively resulting in a healthy microbiome with a neutral or beneficial effect on the plant (Koprivova and Kopriva, 2022).

Adaption of microbiome members to benzoxazinoids shapes the maize root microbiome

Supporting the above outlined idea, some beneficial microbes are known to be attracted to plant roots by the carbon-rich environment created by root exudates. Still, at the same time, microbial growth is inhibited by antimicrobial compounds present in root exudates. In line, tolerance to plant specialized metabolites enables them to grow better in the rhizosphere. This concept has been proven for certain specialized plant metabolites with a limited number of root microbiome isolates (Harbort et al., 2020; Stringlis et al., 2018; Voges et al., 2019). For example, bacterial tolerance to coumarins *in vitro* explained their increased abundance on wild-type roots,

indicating that the antimicrobial activity of coumarins shapes root microbiomes. Similarly, triterpenes selectively inhibit the growth of some root microbiome isolates. Bacteria with high tolerance to triterpenes were also more abundant on *Arabidopsis* roots (Huang et al., 2019). Here we show that benzoxazinoid tolerance of single strains explained benzoxazinoid-dependent colonisation of maize roots by the corresponding taxonomic groups. In summary, we propose that tolerance to host-secreted antimicrobial compounds is an important mechanism determining root microbiome composition.

Another mechanism for bacteria to cope with antimicrobial compounds is metabolisation (Blair et al., 2015). To further increase bacterial fitness, bacteria can completely catabolize antimicrobial compounds and use them as carbon sources (Cycoń et al., 2019). *Sphingobium*, is enriched on tomato roots in the presence of tomatine and can use the compounds as carbon source (Nakayasu et al., 2021). Certain strains from *Arabidopsis* roots catabolize triterpenes to use them as carbon source, enabling them to be abundant microbiome members (Huang et al., 2019). We found that bacteria which can metabolise benzoxazinoids are enriched on benzoxazinoid-producing maize roots. Further, these maize root bacteria can use benzoxazinoids as the sole carbon source for growth. Together this indicates that the metabolisation of plant specialized metabolites does not only serve its detoxification but also serves these microbes as additional carbon source for microbial growth together enabling better root colonization. Thus, the capacity to metabolise secondary metabolites directly structures the root microbiomes. Given the high degree of microbiome specificity and the widespread nature of plant specialized metabolites, we propose that this mechanism may structure root microbiomes across the plant kingdom.

In microbial communities members need to interact with the others to fulfil a common task or to use resources efficiently (Rafieenia et al., 2022). It is well known that microbes cooperate to degrade inhibitory compounds or decompose complex substrates, which would otherwise affect the growth of microbial populations. For instance, a 7-member community of maize root bacteria cooperate to metabolise primary metabolites in root exudates. Further, the growth of certain bacteria is sustained by cross-feeding on metabolites produced by others (Krumbach et al., 2021). A co-culture of the fungus *Fusarium verticillioides* with the bacterium *Bacillus mojavensis* converts BOA to APO. Conversely in mono-culture Fusarium metabolises BOA to another product, HMPA (Bacon et al., 2007). While *Fusarium* forfeited from this cooperation, as it is susceptible to APO, the cooperation benefitted the *Bacillus* by controlling the growth of the pathogenic fungus. Here we investigated bacterial benzoxazinoid tolerance and metabolisation in a community context. The single-strain analysis revealed that the traits for both tolerance and metabolisation were distributed among the different tested strains, suggesting that in a

community context, they would need to cooperate for full performance of both functions. Indeed, we found that the bacterial strains cooperated to tolerate and metabolise the benzoxazinoids in our SynCom experiments. Thus, in a community bacterial cooperation among BX-metabolising members is important to improve the ability of the community to tolerate the compounds. These findings highlight the importance of studying plant specialized metabolites mediating chemical communication in plant-microbiome interactions in more complex synthetic or natural microbial communities.

Mechanisms of microbial adaptions to benzoxazinoids in the maize root microbiome

Microbes need to overcome the toxicity of these antimicrobial compounds to colonise the plant roots. Possible mechanisms include tolerance, which may be mediated by preventing access to the target, changes in antibiotic targets, modification of targets or direct modification of the antibiotic compounds (Blair et al., 2015). Several benzoxazinoids compounds were reported to have antimicrobial activity due to their ability to intercalate to DNA (Hashimoto and Koichi, 1996). The mechanisms of MBOA tolerance were investigated using experimental evolution in the genus *Photorhabdus*, the endosymbiotic bacterium of entomopathogenic nematodes. Mutations in MBOA-tolerant strains fell into the categories of DNA transcription, membrane architecture and membrane channels (Machado et al., 2020). Mutations in genes regulating these processes are often related to antibiotic resistance in bacteria (Delcour, 2009; Fernández and Hancock, 2012). MBOA tolerance is achieved by inactivating an aquaporin-like channel, *AqpZ*. This restricts the diffusion of the toxin into the cell and thus prevents access to the target organism. In chapter 1, we found that cell wall architecture, affects benzoxazinoid tolerance in maize root bacteria. Gram-positive bacteria were generally more tolerant to the benzoxazinoids MBOA and BOA, while they were more susceptible to aminophenoxazinones than gram-negative bacteria (chapter 1, Figure 4f-j). These findings partially align with the literature where gram-negative bacteria are often more tolerant to antibiotics since the outer membrane restricts prevents the entrance of antibiotics to the cell. Through mutations in porins they can create new resistances (Breijyeh et al., 2020). Taken together, these results show that tolerance to benzoxazinoids can be mediated by cell wall structure, but the mode of action of benzoxazinoids on bacterial growth remains to be investigated.

Finding benzoxazinoid metabolising bacteria enriched on maize roots, indicates that bacteria evolved specific mechanisms to metabolise benzoxazinoids. Mechanistically, genes encoding the enzymes which metabolise plant specialized metabolites in bacteria are often located in a degradation operon. Examples are the alcohol dehydrogenase *hcdE* in the hydroxycoumarin degradation gene cluster in *Pseudomonas mandelii*, the monooxygenase *fdeE* in the flavonoid degradation operon in the rhizobacterium *Herbaspirillum seropeidaceae* or the

metal-dependent hydrolase *cbaA* in the 2-amino-5-chlorophenol gene cluster in *Pigmentiphaga* (Dong et al., 2016; Krikštaponis et al., 2021; Marin et al., 2013). In the genus of *Microbacteria,* we identified a benzoxazinoid degradation gene cluster with the first enzyme being an N-acyl homoserine lactonase termed as BxdA. BxdA metabolises MBOA to AMPO. Interestingly we found this lactonase only in bacteria associated with maize roots, while this gene was absent in genomes of *Arabidopsis* root bacteria. This finding suggests that the maize root bacteria are specifically adapted by the evolution of a benzoxazinoid degradation gene cluster to metabolise the secondary metabolites of their host. Similar adaptions to plant specialized metabolites may also be present in other plant microbiomes which were shown to be structured by other root exudates (Jacoby et al., 2020a).

Biological relevance of microbial adaptions to benzoxazinoids

The exudation of specialized plant metabolites is an important tool for plants to steer the assembly of a healthy microbiome (Hong et al., 2021). Many plant specialized metabolites are known to inhibit the growth of pathogens in the rhizosphere (Pascale et al., 2020). Coumarins released by *Arabidopsis* roots in iron-limiting conditions impeded the growth of fungal pathogens such as *Fusarium oxysporum* (Stringlis et al., 2018) or of the bacterial pathogen *Ralstonia solacnacearum* (Yang et al., 2016). Benzoxazinoids inhibit the virulence of pathogenic bacterium *Agrobacterium tumefaciens* (Maresh et al., 2006). At the same time plant specialized metabolites can promote the growth of beneficial bacteria (Hong et al., 2021; Nakayasu et al., 2021; Neal et al., 2012; Stringlis et al., 2018; Yu et al., 2021). For example the flavonones exuded by maize roots enrich for the growth promoting *Oxalobacteraceae* (Yu et al., 2021). Benzoxazinoids were shown to attract beneficial microbes to maize roots (Neal et al., 2012) and selectively act to structure the maize root microbiome (Hu et al., 2018b). We found that benzoxazinoids inhibited the growth of taxa belonging to well-known plant pathogens (*Xanthomonas* and *Agrobacerium*) (Maresh et al., 2006). Benzoxazinoid tolerant isolates belong to families with well-known plant beneficial strains (*Pseudomonas* and *Bacillus*) (Neal et al., 2012; Neal and Ton, 2013; Santos et al., 2020). Hence the metabolic heterogeneity of root exudates may provide a basis for communication and recognition of selecting beneficial microbial communities to improve plant health.

The association of plants with benzoxazinoid metabolising bacteria alters the profile of benzoxazinoid and related compounds in the rhizosphere (Schütz et al., 2019). When bacteria metabolise benzoxazinoids they form the phytotoxic aminophenoxazinones (chapter 2, this thesis). They likely do not change the growth of the host plant, but they inhibit the growth of neighbouring plants (Venturelli et al., 2015). Thus, it is beneficial for maize to select for benzoxazinoid metabolising root bacteria producing a natural herbicide in the rhizosphere soil, which is under competition by adjacent plants.

Together, our findings provide evidence that benzoxazinoids directly impact bacterial members of the root microbiome. Bacteria have various ways to cope with benzoxazinoids: either tolerate them, and/or they metabolise them to other, eventually less toxic, compounds or they completely catabolize them to use as a carbon source. Recent research showed, that bacteria also alter motility, biofilm formation (Guo et al., 2016) and chemotaxis (Neal et al., 2012) in response to benzoxazinoids. These mechanisms alter the composition of the maize root microbiome either directly or indirectly through altered plant-microbe interactions. Using benzoxazinoids, plants probably to select a healthy microbiome favouring beneficials and inhibiting pathogenic members (chapter 1). Further microbial activity alters the soil's chemical profiles, affecting microbial growth, thus microbe-microbe interactions, and impacting community composition (chapter 2). Both altered chemical profiles and altered structure of microbial communities will also affect their functions. Functionally this will lead to the altered phenotype of the microbial community, for example, changing its ability to grow in various substrates because the functional metabolic capacity of the community had changed. Further, the tolerance to toxins, metabolite profiles and nutrient acquisition potential is altered. These functional changes in the microbial community phenotypes will, together with altered community composition where the abundance of beneficial and pathogenic bacteria may have changed, lead to a different function of the microbiome for plant fitness. Specifically, plant growth may be altered because of the abundance of beneficial bacteria or pathogens, the presence of plant growth-inhibiting compounds or limited nutrient availability. Further, plant tolerance to abiotic and biotic stressors may be impacted through these mechanisms.

Next steps to understand the mechanisms of benzoxazinoids in microbiomes

In this thesis, we investigated benzoxazinoids' direct effect on the maize microbiome's bacterial members *in vitro*. Benzoxazinoid tolerance and metabolism are important traits for bacteria to be abundant members in the maize root microbiome. To extend our knowledge on these mechanisms, future research should aim to uncover underlying genetic mechanisms and ecological functions of these mechanisms.

Investigation of molecular mechanisms of benzoxazinoid bacteria interactions

To date the mode of action of benzoxazinoids on bacterial growth is not fully uncovered and only a few tolerance genes are known (de Bruijn et al., 2018; Machado et al., 2020). Possibilities to identify candidate genes of benzoxazinoid tolerance include comparative genomics for instance using a collection of closely related bacteria that have a heterogeneous distribution of phenotypes. An option is to use the kmer approach, which we used to unravel the genetic basis of benzoxazinoid metabolism in bacteria (chapter 2). Candidate genes then need to be confirmed either using knock-out mutants in the natural host strain. For certain wild strains

belonging to well-studied genera like *Pseudomonas*, established mutagenesis and transformation protocols exist (Huang et al., 2017). However, for others like *Microbacteria*, suitable protocols still need to be developed first which may be challenging since they belong to the *Actinobacteriota*. One option would adopt protocols established in *Pseudomonadota* to replace a gene with a kanamycin resistance cassette (Ledermann et al., 2016) or by removing the gene by homologous recombination (Santos et al., 2022). Another option is to use an orthologous approach and test the functions of candidate genes in knock-out mutants. Since *E. coli* is a well-studied model organism *E. coli*, mutant libraries single-gene deletions of all nonessential genes exist (Baba et al., 2006). Furthermore a possibility is to work with *Sphingobium* strains, which have highly similar lactonase genes, could be genetically modified by homologous recombination (Kaczmarczyk et al., 2012). The tolerance phenotype of bacterial mutants can be tested using our high-throughput bacterial growth system. Efforts to create knock out genes involved in benzoxazinoid tolerance will give an insight to the mode of action of benzoxazinoids in bacteria.

Metabolisation of plant specialized metabolites enables bacteria to detoxify the compounds, use them as carbon source or form metabolites to supress of other microbes (Blair et al., 2015; Cycoń et al., 2019). Given that AMPO has a selective antimicrobial effect (chapter 1) and that maize root bacteria which metabolise MBOA to AMPO can use MBOA as sole carbon source while still producing AMPO, raises the question whether MBOA to AMPO metabolisation is a detoxification process or if AMPO is just produced as a side product of the reaction but bacteria just want to access the carbon source. Similar gene clusters are known to catalyse the conversion and subsequent complete catabolization of coumarins, flavonoids and herbicides related to benzoxazinoids in soil bacteria. For these gene clusters the biochemical steps of the metabolisation of the initial compound to several intermediates and subsequent use in the tricarboxylic acid cycle are described (Dong et al., 2016; Krikštaponis et al., 2021; Marin et al., 2016). Benzoxazinoid degradation in *Microbacteria* is mediated by the *bxdA* gene located in the benzoxazinoid degradation gene cluster. This includes in total 15 genes, of which the function in benzoxazinoid degradation remains to be investigated. For the genes in the *bxd* gene clusters genes similar predicted functions are predicted as in the gene cluster for coumarin degradation, such as the dehydrogenases *bxdC*, *bxdD* and *bxdI*. Additionally, the *bxdF* 2-oxo acid dehydrogenase subunit E2 was reported to have a role in the citrate cycle (Knapp et al., 1998). One follow-up question is whether among these genes is one that codes for an enzyme responsible to (i) metabolise the intermediate MAP or AMPO further or (ii) that fully degrade to intermediates which will be further catabolized to use in the tricarboxylic acid cycle. Since certain genes of the *bxd* gene cluster also are present in the other gene clusters described for complete degradation of plant specialized metabolites, it is well possible that the other genes in the gene cluster catalyse the complete metabolisation of benzoxazinoids to use in the tricarboxylic acid cycle. The

functions of these genes can be tested using heterologous expression in *E. coli.* These experiments would inform about further functions of the *bxd* gene cluster for benzoxazinoid metabolisation and use as carbon source. However, to test the function of benzoxazinoid metabolisation for tolerance in *Microbacteria* would require the establishment of a protocol for genetic manipulation of *Microbacteria*.

The ecological function of bacteria benzoxazinoid interactions

To test the ecological role of benzoxazinoid tolerance an informative assay would be to test benzoxazinoid tolerance mutants have a reduced ability to colonize maize roots. Along with that, it would be interesting to test if benzoxazinoid tolerance impacts the abundance of such mutant bacteria in communities like SynComs. Combined with such experiments, these would allow to demonstrate the fitness benefit of benzoxazinoid tolerance for root bacteria thriving on maize roots.

For benzoxazinoid metabolisation, the first thing, which must be tested, is weather benzoxazinoid metabolising root bacteria do not only metabolise benzoxazinoids *in vitro* but if they also alter benzoxazinoid profiles in soil. To further investigate the functional importance of benzoxazinoid metabolising maize root bacteria, there are two promising strategies to follow. To further investigate ecological relevance of AMPO-forming bacteria, their distribution, and the specific recruitment to BX-producing plants a screen for AMPO-forming bacteria, either in strain collections or in root extracts from other fields with differing physio-chemical properties could give insights. On one hand having identified a gene, e.g. the lactonase *bxdA* responsible for AMPO formation, makes it possible to screen for the distribution of this gene in natural soil microbiomes by assays combining plating of root microbiota extracts combined with qPCR or directly by cultivation independent methods like qPCR and/or sequencing. Such experiments would allow to reveal how widespread and abundant such a trait is on roots of different plant species and in different field soils, thereby providing information whether the trait is specifically associated with plant species or specific environmental conditions.

On the other hand, it's necessary to study the ecological importance of this trait that bacteria metabolise the benzoxazinoid MBOA to AMPO. AMPO inhibits the growth of neighbouring plants (Venturelli et al., 2015). Further it acts antimicrobial and selectively inhibits the growth root microbiome members (Schandry et al., 2021) as well as plant pathogens (Maskey et al., 2003; Niemeyer, 2009). AMPO functions as a natural herbicide so for instance, AMPO forming bacteria are interesting candidates to apply for sustainable weed and pathogen control. One possibility is to inoculate AMPO-forming bacteria to maize plants co-cultivated with a weed plant such as *Arabidopsis* and investigate if these bacteria inhibit the growth of the target plant (Qasem, 2017). Since no mutants in wild-type AMPO-forming bacteria exist to date, these

experiments could be performed using closely related *Microbacteria* strains. Thus, understanding the ecology of AMPO formation may open new perspectives for biotechnological applications.

Implications for plant-microbe interactions to improve plant fitness

In recent years, it has been recognised that the plant microbiome has great potential to improve agricultural yields (Batista and Singh, 2022). Plants have evolved with their microbiome to form a functional unit, the plant holobiont (Vandenkoornhuyse et al., 2015). Plants use specific metabolites to recruit a species-specific microbiome. Thereby bacteria co-adapted with the plant to cope with specialised metabolites and they form a specific pair. In addition to being a key to structure the root microbiome, the genetics of the biosynthesis of plant specialized metabolites is well studied. This makes plant specialized metabolites are a promising plant trait to engineer to steer microbiota structure to harness beneficial functions (Batista and Singh, 2022; Hong et al., 2021). Benzoxazinoids which control the microbiome through antimicrobial activity and mediating microbial conversions are interesting candidates. The compounds may be directly applied, or plants may be bread for increased benzoxazinoid exudation (Hong et al., 2021). Benzoxazinoid conditioned microbiomes have been shown to improve plant defence (Hu et al., 2018b). Here we reveal mechanisms how benzoxazinoids shape the maize root microbiome and propose benzoxazinoids as candidate metabolites to steer the maize root microbiome.

Another option to make use of microorganisms in agriculture, is to apply them directly to the plants. Traditionally single microbial strains have been inoculated to plants and often it is observed that the inoculants fail to establish on plant roots, possibly due to environmental factors or microbe interactions (de Oliveira et al., 2017; Li et al., 2022). Another factor however, might be that they do not tolerate the host-secreted secondary metabolites (Batista and Singh, 2022). The inoculants may have evolved on another host than the now inoculated crop. Following up that hypothesis that inoculants must be adapted to secondary metabolites of their target plant, it would be necessary to test experimentally whether benzoxazinoid tolerant bacteria are more successful in colonizing benzoxazinoid producing roots and ultimately if they outperform un-adapted strains with their effects on the plant growth. Here we identified that benzoxazinoid tolerance as an important mechanism determining root colonisation of maize. Thus, for the application of microbial inoculants on maize plants, it may be worth selecting inoculants based on their adaptation to benzoxazinoids and testing their ability to tolerate and/or metabolise them or even exploiting them chemotactically. Another option to select for microbes adapted to host secondary metabolites is to directly isolate them from their host plant and the suitable environmental conditions. Further on existing biocontrol's strains could be genetically engineered to enhance their tolerance to benzoxazinoids. Apart from the application of single strains, synthetic communities consisting of several microbes with complementary functions are

promising since they combine several microbial traits. This provides a broader range of responses to different conditions (Yin et al., 2022). SynComs may be combined with the application of critical metabolites, for example, benzoxazinoids (Batista and Singh, 2022). Implementing the knowledge on mechanisms how bacteria cope with plant specialized metabolites may thus improve the success of microbial inoculants in the field.

In this study, we identified a few interesting microbes as candidates for the application in the field. Namely, *Pseudomonas* are abundant in the maize root microbiome and are tolerant to benzoxazinoids and aminophenoxazinones and this makes it likely that they establish in the rhizosphere (chapter 1). Furthermore, *Pseudomonas* are prime candidates as inoculants since they have rapid growth rates and many isolates are known to be plant-growth-promoting agents as well as some strains that can induce systemic resistance (Ahmad et al., 2019; Haney et al., 2018; Li et al., 2022; Neal et al., 2012; Neal and Ton, 2013). *Enterobacter* is also abundant in the microbiome, MBOA and AMPO tolerant and we found them dominating our 7-member SynComs independently of benzoxazinoid metabolisation. They were further were reported to be important in a SynCom colonizing maize roots to inhibit the fungal pathogen *Fusarium* through antagonistic activity (Niu et al., 2017). In future research should investigate the ability of these strains to promote plant growth or to protect the plant against fungal pathogens on maize.

Like benzoxazinoid tolerance, also benzoxazinoid metabolism is an interesting trait for application as inoculants. *Sphingobium* is abundant in the rhizosphere of field-grown maize (Cadot et al., 2021b) and can readily metabolise benzoxazinoids to AMPO (chapter 2). Interestingly, *Sphingobium* is specifically associated with maize root microbiomes (Xiong et al., 2020). Members of the *Sphingomonadacee* are known to promote plant growth by producing phytohormones such as auxin, and gibberellin and alleviating drought stress (Luo et al., 2019; Santiago et al., 2017). AMPO is known to inhibit the growth of fungal pathogens (Bacon et al., 2007; Maskey et al., 2003) and the reduce the growth of neighbouring plants (Venturelli et al., 2015). Thus, applying *Sphingobium* to the soil or increasing its abundance by increased benzoxazinoid contents may improve pathogen and weed control in proximity of benzoxazinoid producing maize plants.

Conclusion

Plant-microbe interactions play an inevitable role for plant health. In recent years it has been recognised that the plant microbiome has a great potential to improve agricultural yields. Thus, it is important to understand the mechanisms how the plant steers microbiome composition. A key factor are plant specialized metabolites, such as benzoxazinoids which shape the maize root microbiome (Hu et al., 2018b). Our work shows that maize benzoxazinoids structure the maize root microbiome by favouring bacteria that can tolerate and metabolise benzoxazinoids. The antimicrobial activity of benzoxazinoids may be a tool for the plant to assemble a healthy microbiome by inhibiting the pathogenic bacteria and facilitate the establishment of the beneficial bacteria which are mostly benzoxazinoid tolerant. Bacteria carrying the *BxdA* metabolisation gene shape the plant's chemical environmental footprint. In a microbial community bacteria cooperate to tolerate and metabolise benzoxazinoids. mechanism for how plant specialized metabolites affect microbial communities and possibly how bacteria have co-adapted to cope with the secondary metabolites of their host. Together these findings demonstrate the importance of direct effects of benzoxazinoids in structuring maize root microbiomes and shaping the microbial and chemical footprint of the plant. In a next step, responsible genes for benzoxazinoid tolerance and microbial cooperation need to be identified. By creating knock-out mutants of genes encoding for tolerance, metabolisation and microbial cooperation allows testing the functional consequences of these bacterial traits for plant growth. Ultimately benzoxazinoids can be used to create stable and healthy microbiomes which promote agricultural productivity.

A few important new questions emerge from this work for future research:

Can the mechanisms observed *in vitro* be observed in natural systems as well?

We identified bacterial isolates capable of metabolising benzoxazinoids *in vitro*, but it needs to be tested if the same metabolisation takes place in the natural soil environment.

What is the ecological function of benzoxazinoid tolerance and/or metabolisation by maize root bacteria?

It remains to be experimentally demonstrated if benzoxazinoid tolerance improves root colonization of maize root bacteria on benzoxazinoid producing maize roots. Further the ecological consequences of benzoxazinoid metabolisation in the maize root microbiome also need to be tested. Lastly, microbial cooperation in benzoxazinoid tolerance and metabolisation was observed in a reduced community grown in an *in vitro* system. It remains to be tested how microbes cooperate to tolerate and metabolise benzoxazinoids also in natural soil microbiomes.

Are these mechanisms widely distributed in maize fields?

Our study focused on maize root bacteria isolated from one Swiss maize field. To test for the importance of the mechanisms identified, benzoxazinoid tolerance and metabolism need to be assessed in maize root microbiomes form other fields. For AMPO-formation this could be done by simple plating assays and counting AMPO-forming colonies coupled to qPCR analysis to amplify the lactonase *bxdA.*

Are similar mechanisms responsible for plant secondary metabolite mediated structuring of root microbiomes?

Apart from benzoxazinoids, several other plant specialized metabolites like coumarins and flavonoids have been shown to structure root microbial communities and likely it is known that certain bacteria can tolerate and metabolise these compounds. Therefore, it would be interesting to test if these mechanisms also explain the plant secondary metabolite dependent structuring root microbiomes in other plant species.

Answers to these questions will provide further insights in the mechanisms how plant specialized metabolites structure plant microbiomes and which functional consequences these interactions have for plant growth. This will also give explanations why these complex interactions between hosts and microbes evolved. Further understanding of the mechanisms steering the composition and the functions of plant microbiomes will make it possible to harness the functions for improved crop production in future agriculture.

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Declaration of consent

on the basis of Article 18 of the PromR Phil.-nat. 19

Name/First Name:	**Lisa Thönen**
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Title of the thesis:	**Maize root bacteria cooperate to tolerate and metabolise host-secreted plant specialized metabolites**
Supervisor:	**Prof. Dr. Matthias Erb**
	Prof. Dr. Klaus Schläppi

I declare herewith that this thesis is my own work and that I have not used any sources other than those stated. I have indicated the adoption of quotations as well as thoughts taken from other authors as such in the thesis. I am aware that the Senate pursuant to Article 36 paragraph 1 litera r of the University Act of September 5th, 1996 and Article 69 of the University Statute of June 7th, 2011 is authorized to revoke the doctoral degree awarded on the basis of this thesis. For the purposes of evaluation and verification of compliance with the declaration of originality and the regulations governing plagiarism, I hereby grant the University of Bern the right to process my personal data and to perform the acts of use this requires, in particular, to reproduce the written thesis and to store it permanently in a database, and to use said database, or to make said database available, to enable comparison with theses submitted by others.

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