Engineering bacterial symbionts to improve the biological control potential of entomopathogenic nematodes against an important agricultural pest

Inaugural dissertation of the Faculty of Science,
University of Bern

presented by

Anja Boss
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Supervisor of the doctoral thesis:

Prof. Dr. Matthias Erb, Institute of Plant Sciences, University of Bern

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Summary

The western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) is among the most damaging pests of maize plants. The larvae of this beetle feed on maize roots, and cause more than 2 billion US$ of direct economic damage per year. The western corn rootworm is currently invading Europe, including Switzerland. A promising strategy to control this pest is the use of entomopathogenic nematodes (EPNs). These tiny roundworms are used as biocontrol agents in sustainable agriculture. The nematodes harbour *Photorhabdus* bacteria in their bodies and together, they can infect and kill a variety of harmful insect pests. Yet, the western corn rootworm seems at least partly able to resist these entomopathogenic nematodes by exploiting benzoxazinoids, maize secondary metabolites. This likely limits the success of biocontrol programs. Here, I propose a strategy to improve EPNs that relies on engineering bacterial symbionts of nematodes to become resistant to the chemical defences of the target herbivore.

In chapter 1, I reported that introducing engineered bacterial symbionts into two commercially used nematode strains results in high infectivity of those newly generated nematode–symbiont pairs to kill the larvae of the WCR. WCR larvae are known to sequester benzoxazinoid secondary metabolites that are produced by maize and use them to increase their resistance to the nematodes and their symbionts. I isolated 27 *Photorhabdus* symbionts from different nematodes and increased their benzoxazinoid resistance through experimental evolution. Benzoxazinoid resistance evolved through multiple mechanisms, including a mutation in a multidrug efflux pump. I reintroduced benzoxazinoid-resistant *Photorhabdus* strains into two strains of *Heterorhabditis bacteriophora* EPNs and identified four nematode–symbiont pairs that were able to kill benzoxazinoid-sequestering WCR larvae as efficiently as the commercially available nematodes under laboratory conditions. My results suggest that modification of bacterial symbionts provides a successful and time-efficient strategy to enhance the performance of their nematode hosts.

In chapter 2, I tested four *H. bacteriophora* strains with selected and exchanged symbiotic *Photorhabdus* bacteria for their control effects on WCR larvae compared to two commercial *H. bacteriophora* nematode products (Dianem® TM, Meginem® Pro TM) and a standard soil insecticide under field conditions in Hungary. All the novel nematode-symbiont pairs similarly controlled the pest as did commercial nematode strains. This suggests that the selection

processes did not lead to any major trade off, but also that other pathogenicity factors (rather than benzoxazinoid resistance) may be more relevant for successful biocontrol under field conditions. My results suggest that modification of bacterial symbionts and targeting candidate genes to engineer better biocontrol agents provides a successful and time-efficient strategy to improve the pathogenicity of entomopathogenic nematodes against other agricultural pests.

In chapter 3, I tested if selecting *Photorhabdus* bacteria for increased tolerance to 6-methoxy-2-benzoxazolinone (MBOA) alters their tolerance to other chemicals such as other benzoxazinoids, insecticides, pesticides, secondary plant metabolites and antibiotics. My results show that MBOA-selected *Photorhabdus* strains are more tolerant to the structurally similar compounds 2-benzoxalinone (BOA) and 6-chloroacetyl-2-benzoxazolinone as well as to caffeine, nicotine and sinigrin. On the other hand, MBOA tolerant strains are more susceptible to the antibiotics ampicillin and kanamycin. The results highlight the importance of cross-resistance and collateral sensitivity assessment after directed evolution for an environmental toxin in *Photorhabdus* bacteria.

I showed that selecting a bacterial symbiont for plant toxin resistance translated into high infection potential of its nematode host against a herbivore that sequesters these toxins for self-protection. This work highlights the potential of engineering desired traits into bacterial symbionts to enhance the performance of their host and improving biocontrol against agricultural pests in the future. In addition, my work charted a map of cross-resistance and collateral-sensitivity interactions in MBOA-selected *Photorhabdus* bacteria and revealed interesting insights into resistance patterns of engineered microbes.

General Introduction

1. A deadly liaison: *Photorhabdus* bacteria & entomopathogenic nematodes

Photorhabdus is a genus of facultatively anaerobic, gram-negative, motile, and rod-shaped Enterobacteria belonging to the Morganellaceae family. *Photorhabdus* is the only known bioluminescent terrestrial bacterium and its name can be translated to "glowing rod" (Waterfield et al. 2009). The initial members of this genus were first identified and isolated in 1977 from the intestines of the entomopathogenic nematode *Heterorhabditis bacteriophora* (Khan and Brooks 1977, Poinar et al. 1977). The *Photorhabdus* genus underwent a significant classification in 1999, with the identification of three distinct species - *P. asymbiotica*, *P. luminescens*, and *P. temperata* - based on thorough genetic and phenotypic characterizations (Fischer-LeSaux et al. 1999). In the following two decades, several approaches were conducted to describe more species of *Photorhabdus* by using multi-locus sequence analysis (Tailliez et al. 2010, Glaeser et al. 2017). A pivotal development occurred in 2018 when Machado *et al.* utilized the substantial advancements in DNA-sequencing technologies to re-evaluate the phylogeny of the *Photorhabdus* genus. This comprehensive analysis, employing whole-genome sequencing, resulted in an expansion of the recognized species and subspecies to twenty (Machado et al. 2018). Building on this progress, subsequent studies conducted by Machado and others further solidified the taxonomy of the *Photorhabdus* genus. As of the latest information available, the *Photorhabdus* genus now consists of a total of twenty-six officially acknowledged species and subspecies (Machado et al. 2018, 2019, 2021). Since the sequencing and annotation of the type strain *P. laumondii* subsp. *laumondii* TT01 genome in 2003, *Photorhabdus* bacteria have emerged as a model to study interkingdom pathogenicity and mutualism, contributing crucial insights into the evolution and ecological roles of these bacteria in association with entomopathogenic nematodes (Duchaud et al. 2003).

Photorhabdus bacteria engage in a symbiotic association with entomopathogenic nematodes (EPNs) belonging to the genus *Heterorhabditis*. The entomopathogenic nematode *H. bacteriophora* was initially characterized in 1975, marking the discovery of a new genus, species, and family (Heterorhabditidae) within Rhabditida (Poinar 1975). The infective juvenile (IJ) stage, similar to the dauer juvenile stage of *Caenorhabditis elegans*, was identified as a carrier of a specific gram-negative bacterium (Poinar et al. 1977). This bacterium is referred

today as *Photorhabdus luminescens* subspecies *luminescens* (Fischer-LeSaux et al. 1999). Together, *Heterorhabditis* nematodes and *Photorhabdus* bacteria collectively form a highly pathogenic pair targeting soil-dwelling insects. IJs harbour *Photorhabdus* bacteria within their intestinal lumens (Ciche 2007). The non-feeding and developmentally arrested IJ is the only stage of *H. bacteriophora* found outside of insect hosts in nature and they can survive up to several weeks in the soil, seeking for suitable hosts. To locate their prey, IJs utilize sophisticated sensing mechanisms. They can sense cues such as sex pheromones or respiratory CO_2 emitted by conspecific nematodes or by insects (Dillman et al. 2012, Rivera et al. 2017). Furthermore, IJs are attracted to (E)-beta-caryophyllene, a terpene released by plant roots upon herbivore damage, indicating the potential presence of insect hosts (van Tol et al. 2001, Rasmann et al. 2005). IJs have evolved chemosensory mechanisms not only to detect insect hosts directly, but also locations where insect hosts are likely to be present. Recent research revealed that dead insects infected by EPNs emit volatiles capable of modulating the behaviour of healthy insects, attracting them to the vicinity of the cadavers and thereby increasing predation success of EPNs (Zhang et al. 2019). Upon locating an insect host, *Heterorhabditis* IJs enter through natural openings like respiratory spiracles, the mouth or anus, and releasing their bacterial symbionts into the insect's hemocoel (Kenney et al. 2019). Using green fluorescent protein- (GFP) labelled *Photorhabdus* symbionts located in the IJ intestines, it was observed that the IJs regurgitate symbiotic bacteria (Ciche and Ensign 2003). *Photorhabdus* bacteria then undergo exponential multiplication within the hemocoel, producing toxins and immunosuppressors (Bowen et al. 2000). The bacteria rapidly kill the insect, usually in less than 24 hours. The bacterium is the main cause of insect mortality since the bacterium alone has a lethal dose of less than 10 cells when injected the insect hemocoel (Milstead 1979). Notably, *P. luminescens* generates a toxin comprised of four native complexes encoded by toxin complex loci *tca*, *tcb*, *tcc*, and *tcd* (Bowen et al. 1998). Symbiotic bacteria provide a protected niche for themselves and their nematode hosts by producing insecticidal secondary metabolites, lytic enzymes and antibiotics that prevent colonialization and proliferation of saprophytic organisms such as other bacteria, protozoa or fungi (Joyce and Clarke 2003, ffrench-Constant et al. 2007). They even produce compounds that deter scavenging ants (Zhou et al. 2002). In addition, crystalline inclusion proteins that are rich in amino-acids and essential for proper *Heterorhabditis* nematodes development and reproduction are released (Richardson et al. 1988, Bintrim and Ensign 1998). Although *Photorhabdus* bacteria are the main cause for insect mortality upon EPN infection,

Heterorhabditis IJs likely contribute to insect virulence, for example through immune suppression of the insect (Kenney et al. 2019). *Heterorhabditis* infective juveniles then recover and develop into self-fertile hermaphrodites. They lay eggs that hatch and develop through four juvenile stages (J1, J2, J3, J4) into hermaphrodites, adult females, or adult males. When egg laying ceases, worms develop inside the maternal body cavity by a process called *endotokia matricida* (Johnigk and Ehlers 1999). Intriguingly, the worms that undergo development through *endotokia matricida* predominantly exhibit the hermaphroditic IJ phenotype (Dix et al. 1992). Nematode reproduction continues for two to three generations until conditions within the insect cadaver deteriorate to such an extent (due to an increase in nematode population and the decreased availability of insect tissue and bacteria as food) that *Photorhabdus* bacteria re-associate with *Heterorhabditis* gravid females. Initially adhering to the posterior nematode intestine, a few bacterial cells progressively colonize the entire gut and the nematode progeny develop within the maternal body cavity (Waterfield et al. 2009). Following *endotokia matricida*, the newly born and developing generation of juvenile nematodes is stimulated to enter diapause and form IJs that emerge from the insect cadaver into the soil, actively seeking new prey to infect. Collectively, these characteristics render EPNs valuable biological agents in crop pest management. Various species of *Heterorhabditis* nematodes are currently commercially available for domestic or industrial applications, serving as an eco-friendly alternative to conventional pesticides (Kaya and Gaugler 1993, Koppenhöfer et al. 2020).

2. The western corn rootworm (*Diabrotica virgifera virgifera*)

The western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte is a major insect pest of field maize, *Zea mays* L. The WCR belongs to the order of Coleoptera and to the family of the Chrysomelidae. The catalogue of Wilcox lists 338 species in three groups belonging to the genus of *Diabrotica*: *virgifera*, *fucata*, and *signifera*; the first two groups include pests (Wilcox 1972). Adult WCR beetles are medium-sized, ranging from 5 to 7 mm in length, with a characteristic yellowish-green coloration and three distinctive black stripes running along their elytra. The robust mandibles are adapted for feeding on corn foliage, while the hind legs are equipped with tarsi adapted for gripping and climbing. Although the adults feed on the silk of the plant causing reduced fertilization, also resulting in yield losses, the most damaging stage of the WCR are its larvae feeding belowground on maize roots (Chiang 1973). The WCR is a univoltine species with eggs that overwinter in the soil (Krysan and Miller 1986). After maize

has germinated in the spring, the eggs soon hatch and all three larval instars feed almost exclusively on maize roots (Moeser and Hibbard 2005). Larval feeding results in the formation of characteristic feeding scars on corn roots, compromising the plant's structural integrity and making it susceptible to lodging. Severe infestations can lead to reduced plant vigour, stunted growth, and, in extreme cases, plant death (Sutter et al. 1990, Levine and Oloumi-Sadeghi 1991). Efficient WCR larval control appears difficult as the larval population hatches over a period of at least one month and feeds on maize roots for at least 2 months (Toepfer and Kuhlmann 2006). The larvae pupate in the soil and adult beetles emerge to feed on corn foliage, completing the life cycle of the WCR (Branson and Krysan 1981).

Because of the geographical distribution of most other diabroticites and the close association of the WCR with maize, the species is commonly considered as originating from Mexico, or possibly Guatemala, where its original native host was probably *Tripsacum*, a close wild relative of maize (Smith 1966, Branson and Krysan 1981, Gray et al. 2009). It is hypothesized that the WCR then fed on early domesticated maize, and incidentally followed the dissemination of the plant into southwestern North America and the Great Plains, so that the history of the WCR tracks the history of maize into those regions (Branson and Krysan 1981, Gray et al. 2009). Since its first introduction into Serbia in 1992, it has spread across the European continent, resulting in well-established populations in approximately nineteen countries (Kiss et al. 2005, Gray et al. 2009, Meinke et al. 2009, EC 2012) (http://extension.entm.purdue.edu/wcr/). It is expected that this invasive pest species will expand further in the EU, but also globally (Aragón et al. 2010, Ciosi et al. 2011, Aragón and Lobo 2012, Kriticos et al. 2012). The WCR is one of the world's most devastating maize pests which causes more than $2 billion of direct economic damage every year in the United States (Wechsler and Smith 2018). Maize yield losses in Europe were estimated to account for 472 million euros, if no control measures were implemented (Wesseler and Fall 2010).

The main pest managements against the WCR include (1) cultural control of the larvae through crop rotation, (2) synthetic insecticides such as granular or fluid soil insecticides as well as seed coatings against larvae, or foliar sprays against adults, (3) transgenic maize that express insecticidal proteins in the roots, or (4) entomopathogenic nematodes targeting the larvae of the pest (Levine and Oloumi-Sadeghi 1991, Van Rozen and Ester 2010). One successful management strategy against the WCR is rotation of maize with non-host crops as the species overwinters in the egg stage usually on the fields where maize has been grown (Branson and

Ortman 1970). This option is however not the most economically viable and is not suitable for large monoculture farms. The WCR also adapted to crop rotation by changing its egg laying behaviour, especially in the USA. Increased oviposition was observed in soybean, pumpkin, sunflowers, sugar beet and other non-host plants and jeopardize the effect of crop rotation (Levine et al. 2002, Mabry et al. 2004, Moeser and Vidal 2004, Foltin and Robier 2014). Such a crop rotation-resistant WCR variant has not evolved in the EU yet. Further, the application of synthetic insecticides into the furrow during sowing has been documented to diminish larval-induced damage to roots and mitigate the risk of plant lodging, including the insecticides tefluthrin, chlorpyrifos ethyl, clothianidin, and λ-cyhalothrin (Sutter et al. 1990, Blandino et al. 2017). However, soil as well as foliar insecticides occasionally fail in sufficiently reducing WCR populations. The challenge persists, with insecticide resistance emerging as a contributing factor to the inconsistent performance of pesticides in the control of adult WCR. In the case of adult control, variations in pesticide efficacy are frequently linked to insecticide resistance: WCR adults have exhibited resistance to certain chlorinated hydrocarbons since the 1960s, as well as to methyl-parathion and carbaryl since the mid-1990s (Ball and Weekman 1962, Ciosi et al. 2009, Meinke et al. 2009). Furthermore, the mobility of WCR beetles poses a considerable challenge, allowing them to migrate from untreated fields into already treated ones (Levine and Oloumi-Sadeghi 1991, Gray et al. 1992). While the resistance of larvae to pesticides remains less explored, there is evidence suggesting that adult resistance to pesticides is passed on to the next generation, diminishing their susceptibility to specific pesticides (Wright et al. 2000). This phenomenon has been demonstrated in larvae originating from North American adults that were resistant to methyl-parathion, terbufos, chlorpyrifos, carbofuran, and tefluthrin, as well as bifenthrin (Wright et al. 2000, Pereira et al. 2015). Furthermore, several studies showed that the residuals of the systemic neonicotinoids used for seed coating are found in water, soil, sowing dust, pollen and nectar and can have negative effects on non-target pollinating insects. These findings push the research to the development of more sustainable management practices (Blacquière et al. 2012, van der Sluijs et al. 2013, Wood and Goulson 2017). Genetically modified (GM) maize transformation events expressing insecticidal *Bacillus thuringiensis* (*Bt*) toxins against corn rootworms offer additional means of control against the WCR. The mode of action of *Bt*-toxins is to bind selectively to specific receptors on the epithelial surface of the midgut of larvae of susceptible insect species, leading to death through pore formation, cell burst and subsequently septicemia (Sanahuja et al. 2011, Vachon et al. 2012,

Bravo et al. 2013). Several *Diabrotica*-active *Bt*-maize events are currently grown commercially in Argentina, Brazil, Canada and the USA. The cultivation of *Diabrotica*-active *Bt*-maize can reduce the use of insecticides that are more harmful to the environment, given that less or no treatments with soil or foliar broad-spectrum insecticides are needed. But there is a concern that the widespread, repeated, and exclusive deployment of the same *Bt*-maize transformation event will result in the rapid evolution of resistance in the WCR (Siegfried et al. 1998).

In summary, the development of resistance against insecticides and transgenic events, human safety issues and environmental concerns push the research to develop new management practices (Levine and Oloumi-Sadeghi 1991). Improved biological control solutions are thus urgently needed to control the WCR in an environmental friendly manner (Kuhlmann and van der Burgt 1998, Babendreier et al. 2006). The WCR with its intricate life cycle and adaptability, presents a persistent challenge to global corn production. A holistic understanding of its taxonomy, morphology, life cycle and ecological significance is crucial for developing sustainable management strategies for Europe that balance economic considerations with environmental impact. A three-year field study conducted in the focal points of the invasion (Hungary, Croatia and Yugoslavia) revealed that any of the life stages of the WCR were attacked by indigenous natural enemies in the European maize agro-ecosystem (Toepfer and Kuhlmann 2004). Based on these findings, two options are available for biological control of the WCR in Europe. The first option is a classical biological control approach, where specific natural enemies from the area of origin of the WCR are selected (Kuhlmann et al. 2005). A possible candidate would be the tachinid *Celatoria compressa* with a restricted host range to diabroticite beetles (Kuhlmann et al. 2005). The second option (to be used either alone or in combination with the first option) is an inundative biological control approach using commercially available natural enemies, such as entomopathogenic nematodes (Kuhlmann and van der Burgt 1998, Ehlers 2003b).

3. EPN and symbiotic bacteria as biological control agents against *D. v. virgifera* – and the problem of sequestration by the western corn rootworm

For a pest like the WCR, that is most damaging at the larval stage, soil-dwelling entomopathogenic nematodes are considered as strong candidates for use in a biological control strategy (Toepfer et al. 2010). EPNs have a relatively wide host range including larvae of the WCR and they actively search for a host (Jackson 1996; Toepfer et al. 2005; Grewal,

Ehlers, and Shapiro-Ilan 2005). EPNs can be mass produced, both *in vivo* and *in vitro*, they have potential for application in integrated pest management programs and are safe to humans and the environment (Ehlers 2003a). In Europe, *Heterorhabditis bacteriophora* Poinar (Nematoda: Rhabditida) is one of the most common biological control agents against WCR larvae (Toepfer et al. 2005). The EPN strain attacks all three larval stages as well as to some extent the pupal stage of WCR (Jackson 1996, Kurtz et al. 2009). In a study by Toepfer *et al.* on virulence of different EPN species, *H. bacteriophora* that harbours *Photorhabdus* symbionts, was the most promising candidate out of 8 tested EPN species to control WCR larvae (Toepfer et al. 2005).

Unfortunately, the WCR is to some extent resistant to EPNs due to its capacity to exploit the defensive mechanisms of maize for its own benefit (Robert et al. 2017). Accumulation of benzoxazinoids (BX) occurs predominantly in the haemolymph of the insect and renders WCR larvae highly resistant to EPNs and their entomopathogenic symbiotic bacteria. Metabolite analysis revealed highest concentrations of BXs for the glucosides 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one *O*-glucoside (HDMBOA-Glc, >100 mg/g FM) and 6-methoxy-2-benzoxazolinone *N*-glucoside (MBOA-Glc, >25 mg/g FM). The resulting increased resistance of the WCR against EPNs was associated with these two chemicals and their two different modes of action (Robert et al. 2017). First, WCR larvae release the relatively stable MBOA-Glc via the frass and exudates, which acts as a repellent for host-searching infective juvenile nematodes. Second, WCR larvae accumulate HDMBOA-Glc and release a breakdown product, 6-methoxy-2-benzoxazolinone (MBOA), upon nematode infection, which acts toxic on the nematodes as well as on the released symbiotic bacteria (Figure 1). WCR larvae that accumulate MBOA and HDMBOA-Glc reduce therefore the survival and the infectivity efficiency of the nematode and its *Photorhabdus* bacterial symbionts by more than 50% compared to benzoxazinoid-deficient WCR larvae (Robert et al. 2017). These experiments showed that BX-dependent resistance of the WCR against EPNs is associated with the sequestration of HDMBOA-Glc and MBOA that are highly toxic to both the nematode and its endobiotic bacterium. Sequestered and reactivated BXs protect WCR against predation by the third and the fourth trophic levels (Figure 1).

Natural enemies of herbivores are expected to adapt to the defence strategies of their preys or hosts. It is very likely that herbivore natural enemies attacking sequestering hosts have evolved mechanisms to resist or tolerate plant defence metabolites that they frequently encounter in their environment in parallel with their hosts (Ode 2006). It has recently been postulated that, although EPNs are often generalists, they are restricted in their mobility,

resulting in populations confined to small geographical regions (Hazir et al. 2003). This limitation is believed to exert significant pressure on each population, prompting them to adapt to local plant hosts and the associated secondary metabolites (Zhang et al. 2019).

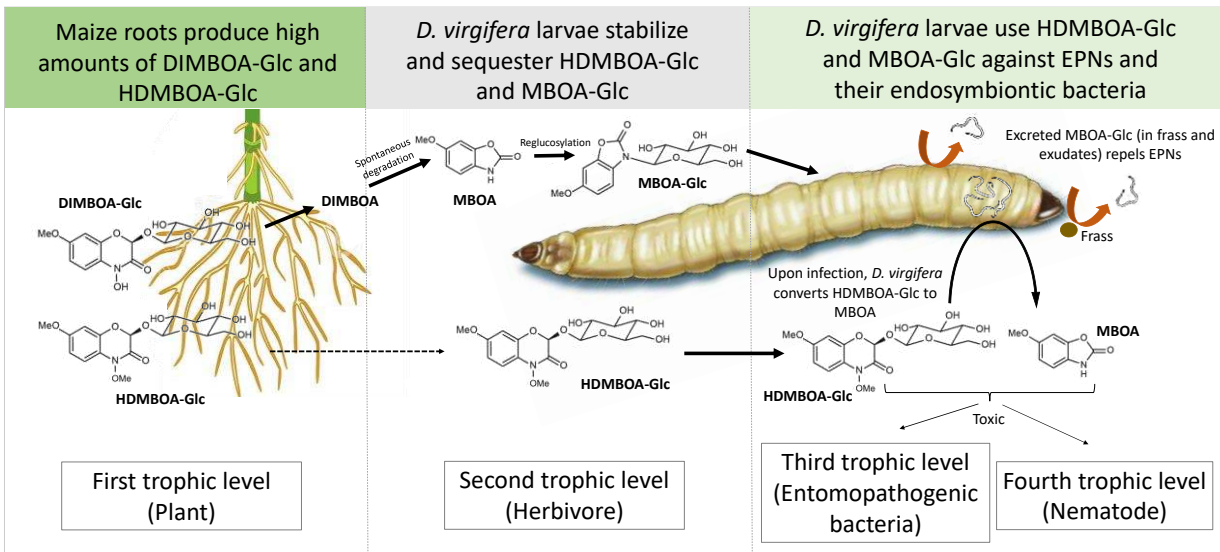

Figure 1: A model illustrating how BX sequestration and activation of plant toxins protects *Diabrotica v. virgifera* larvae from their enemies at multiple trophic levels. MBOA-Glc, released in the frass and on the exoskeleton, repels infective juvenile entomopathogenic nematodes. Upon infection by nematodes and their symbiotic entomopathogenic bacteria, HDMBOA-Glc is activated to produce MBOA. Both HDMBOA-Glc and the activated MBOA reduce the growth of the symbiotic bacteria and kill EPNs. Figure was adapted from Robert et al. 2017.

It is hypothesized that EPN populations that coexisted with benzoxazinoids for a long time are expected to evolve some tolerance or resistance to these compounds. When exposed to WCR fed on benzoxazinoid-containing maize seedlings, almost all *H. bacteriophora* isolates from Mexico were equally capable to kill sequestering larvae. This suggests that prolonged coexistence in Central America between two species may result in enhanced adaptive capabilities of EPNs to their local hosts and the associated plant secondary metabolites (Bruno et al. 2020). Tested in the laboratory, the exposure of a benzoxazinoid-susceptible nematode strain to WCR over 5 generations results in higher behavioural and metabolic resistance and benzoxazinoid-dependent infectivity toward WCR larvae (Zhang et al. 2019). Thus, natural enemies that are exposed to a plant defence sequestering herbivore can evolve both behavioural and metabolic resistance to plant defence metabolites, and these traits are associated with higher infectivity toward a defence sequestering herbivore. In addition, a previous study has shown that it is also possible to improve the efficacy of EPNs toward the WCR by engineering benzoxazinoid-resistant bacterial symbionts (Machado et al. 2020). Five *Photorhabdus* symbionts from different nematodes were isolated and their MBOA resistance

was increased by 40 up to 183% through experimental evolution. By sequencing the whole genome of these evolved symbionts, it was proved that benzoxazinoid resistance evolved through multiple mechanisms, including a mutation in the aquaporin-like channel gene *aqpZ*. The benzoxazinoid-resistant *Photorhabdus* strains were then reintroduced into their original EPN hosts and one nematode carrying MBOA-selected symbionts killed more benzoxazinoid-containing WCR larvae than nematodes carrying their ancestral symbionts under lab conditions (Machado et al. 2020). This work highlights the potential of engineering desired traits into bacterial symbionts to enhance the performance of their host.

4. Improving bacterial symbionts by targeted engineering

Bacterial symbionts provide many important services to their hosts related to development, nutrient uptake, reproduction and speciation, defence against natural enemies, immunity and stress tolerance (Piel 2002, Braendle et al. 2003, Oliver et al. 2003, Koropatnick et al. 2004, Baumann 2005, Bäckhed et al. 2005, MacDonald and Monteleone 2005, Scarborough et al. 2005). Given this wide spectrum of associations and functions, symbiotic microorganisms constitute promising and mostly untapped sources for potential applications in medicine, bioremediation, industrial processes and agriculture. But in most of the cases so far, bacterial symbionts have only been applied directly to biomedical and agricultural problems by targeted inoculation or transplantation. For example, inoculation of soybean fields with nitrogen-fixing rhizobia is used to increase soybean yields (Yang et al. 2018). Furthermore, several *Photorhabdus* species, symbiotic partners of entomopathogenic nematodes, produce a toxin with high oral toxicity to *Manduca sexta* and therefore represents a potential alternative to the insecticidal *Bt*-toxin for transgenic deployment (Bowen et al. 1998). In contrast, the direct engineering of bacterial symbionts remains largely untapped and only a few direct examples are found in the current literature. For example, engineered rhizobia bacteria expressing an algal *arsM* gene can methylate and volatilize arsenic, providing a proof of concept for potential future use of legume-rhizobia symbionts for arsenic bioremediation (Zhang et al. 2017). By genetically engineering *Serratia symbiotica*, a culturable, gut-associated bacterium isolated from the black bean aphid, dynamics of symbiont gut colonialization of multiple aphid species were studied and changes in aphid fitness were recorded. In addition, heterologous gene expression from engineered *S. symbiotica* in living aphids were induced (Elston et al. 2021). Lastly, selecting *Photorhabdus* bacterial symbionts for plant toxin resistance translated into

better infection potential of its nematode host against an herbivore that sequesters these toxins for self-protection (Machado et al. 2020). This work highlights the potential of engineering desired traits into bacterial symbionts to enhance the performance of their host in this largely untapped approach.

5. Aim of my PhD thesis

Bacterial symbionts play a crucial role in the biological control of root-feeding insects in agriculture by entomopathogenic nematodes. These nematodes, such as H*eterorhabditis bacteriophora*, carry pathogenic bacterial symbionts (*Photorhabdus* spp.) in their intestines and inject them into insect prey, utilizing the bacteria to kill and pre-digest the insect. While these nematodes are commonly used in sustainable agriculture, certain insect pests, like the WCR, have evolved mechanisms to resist nematode attacks, for example by exploiting the defence metabolites of host plants. In particular, WCR uses maize secondary metabolites to resist entomopathogenic nematodes, which likely limits the success of biocontrol programs (Robert et al. 2017). As this herbivore is rapidly becoming resistant to conventional pest control strategies, including insecticides, transgenic *Bt*-plants and simple crop rotations, improved biological control solutions are directly needed to control this pest in an environmentally friendly manner (Kuhlmann and van der Burgt 1998, Babendreier et al. 2006). As bacterial symbionts are crucial for the capacity of the nematodes to kill insects, and the benzoxazinoids that are sequestered by the WCR reduce the performance of these bacteria, I hypothesize that it should be possible to improve the killing efficacy of entomopathogenic nematodes towards the WCR by engineering benzoxazinoid-resistant symbionts through forward evolution, reintroducing the selected symbionts into commercially used nematodes and improving the control potential of the newly generated nematode-bacteria pairs against the WCR.

Here, I propose to use an interdisciplinary approach to improve symbiotic bacteria of entomopathogenic nematodes to create biocontrol agents that are able to overcome the plant-derived defences of the WCR. The project aims at testing these novel nematode-symbiont pairs under controlled field trials and estimating the capacity of engineered microbial symbionts for biological control and sustainable agriculture. If successful, this work would be directly applicable to combat one of the most damaging, invasive maize pests on the planet.

My thesis consists of five specific aims:

I. Generating benzoxazinoid-resistant *Photorhabdus* symbionts through forward-evolution

Forward evolution is a powerful approach to improve microbial traits in the absence of detailed mechanistic knowledge. Preliminary experiments by Machado *et al.* (2020) showed that exposure of *Photorhabdus* bacteria to MBOA, a benzoxazinoid sequestered by WCR larvae, can increase their resistance and infectivity in the *P. bodei* CN4 strain. However, this strain is not compatible with nematodes that perform well under European field conditions. I therefore will expand the forward evolution approach to include a broader range of *Photorhabdus* strains and selection regimes (Chapter 1).

II. Measuring the performance of the engineered symbiotic bacteria

Selection for xenobiotic resistance may have pleiotropic effects that may interfere with the usability of the engineered strains. To identify strains that are likely to be compatible with agricultural applications, I will test the selected bacterial strains for MBOA resistance and cross-resistance towards antibiotics as well as for possible trade-offs in bacterial growth caused by selection on MBOA (Chapter 1 and 3). In addition, to test whether the selected bacteria are still compatible with nematode hosts, I will re-establish symbiosis with two commercially used *Heterorhabditis* nematode strains (Dianem® and Meginem® Pro) *in vitro*. Lastly, I will determine the infectivity of the newly generated nematode-bacteria pairs towards the larvae of the WCR (Chapter 1).

III. Unravelling the genetic basis of benzoxazinoid resistance in *Photorhabdus*

Understanding the mechanisms that allow *Photorhabdus* bacteria resist benzoxazinoids is important for the exploitation and transferability of this trait in the context of biological control. I therefore will work towards the identification of the genetic basis of benzoxazinoid resistance in the strains generated through forward-evolution (Chapter 1). Depending on the type of mutation, further experiments targeting cross-resistance and collateral sensitivity will be conducted to gain a deeper functional understanding of the uncovered genetic changes (Chapter 3).

IV. Quantifying the potential of the engineered symbionts for biocontrol of WCR in the field

As field conditions differ significantly from the laboratory, it is essential to test enhanced biocontrol agents in an agriculturally relevant and realistic field setting. I will perform field experiments with the best performing nematode-symbiont pairs in terms of infectivity towards

larvae of the WCR to test our enhanced strains and to benchmark them with commercially mass-produced entomopathogenic nematodes. As experiments with the WCR are currently not possible in Swiss fields as this pest species is in the process of invading Switzerland and therefore still under quarantine, I will perform a series of field experiments over one field season in two different field locations near CABI's field laboratory in Hungary. Hungary has a diverse agriculture from small scale commercial and non-commercial farmers, via highly professional family farms to large scale agri-enterprises, allowing field experimentation comparable to a number of different agricultural systems across Europe, including Switzerland. In summary, this important step will provide a comprehensive, quantifiable assessment of the potential of the engineered *Photorhabdus* strains to improve the biological control of the WCR through entomopathogenic nematodes in the field. The field experiment will also provide crucial insights into the performance of the nematodes harbouring engineered symbionts under natural and variable field conditions (Chapter 2).

V. Examining cross-resistance and collateral sensitivity of the benzoxazinoid-resistant *Photorhabdus* strains

The evolution of resistance in bacteria to one antibiotic or drug is frequently associated with increased resistance to another antibiotic, termed cross-resistance. Conversely, increased resistance to one antibiotic can also often result in decreased resistance to another antibiotic, a phenomenon referred to as collateral sensitivity. By combining experimental evolution, whole-genome sequencing of laboratory-evolved bacteria and biochemical assays, I am outlining a map of cross-resistance and collateral sensitivity interactions in MBOA-selected *Photorhabdus* bacteria and provide insights into resistance patterns for a variety of environmental chemicals (Chapter 3).

The major aim of my PhD thesis is to improve the capacity to fight a major agricultural pest, the western corn rootworm, through biological control by engineering novel resistance-traits into bacterial symbionts of entomopathogenic nematodes. My research aims to conduct a comprehensive evaluation of how plant toxin resistance impacts performance, cross-resistance, collateral and potential for successful symbiosis of *Photorhabdus* bacteria. Moreover, I will conduct a field experiment to test our enhanced strains under realistic field conditions. I hope to be able to produce several enhanced nematode strains that have greater abilities to kill WCR larvae and contribute to an improved biological control solution.

References of General Introduction

Aragón P, Lobo JM (2012) Predicted effect of climate change on the invasibility and distribution of the Western corn rootworm. Agricultural and Forest Entomology 14: 13–18. https://doi.org/10.1111/j.1461-9563.2011.00532.x

Aragón P, Baselga A, Lobo JM (2010) Global estimation of invasion risk zones for the western corn rootworm *Diabrotica virgifera virgifera*: Integrating distribution models and physiological thresholds to assess climatic favourability. Journal of Applied Ecology 47: 1026–1035. https://doi.org/10.1111/j.1365-2664.2010.01847.x

Babendreier D, Keller S, Romeis J, Bigler F, Kuhlmann U, Toepfer S, Turlings T, Schaub L (2006) New approaches to combating corn rootworm. Agrarforschung 13: 80–82.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. Science 307: 1915–1920. https://doi.org/10.1126/science.1104816

Ball HJ, Weekman GT (1962) Insecticide resistance in the adult western corn rootworm in Nebraska. Journal of Economic Entomology 55: 439–441. https://doi.org/10.1093/jee/55.4.439

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annual Review of Microbiology 59: 155–189. https://doi.org/10.1146/annurev.micro.59.030804.121041

Bintrim SB, Ensign JC (1998) Insertional inactivation of genes encoding the crystalline inclusion proteins of *Photorhabdus luminescens* results in mutants with pleiotropic phenotypes. Journal of Bacteriology 180: 1261–1269. https://doi.org/10.1128/JB.180.5.1261-1269.1998

Blacquière T, Smagghe G, van Gestel CAM, Mommaerts V (2012) Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology 21: 973–992. https://doi.org/10.1007/s10646-012-0863-x

Blandino M, Ferracini C, Rigamonti I, Testa G, Saladini MA, Jucker C, Agosti M, Alma A, Reyneri A (2017) Control of western corn rootworm damage by application of soil insecticides at different maize planting times. Crop Protection 93: 19–27. https://doi.org/10.1016/j.cropro.2016.11.006

Bowen D, Blackburn M, Rocheleau T, Grutzmacher C, ffrench-Constant RH (2000) Secreted proteases from *Photorhabdus luminescens*: separation of the extracellular proteases from the insecticidal Tc toxin complexes. Insect Biochemistry and Molecular Biology 30: 69–74. https://doi.org/10.1016/S0965-1748(99)00098-3

Bowen D, Rocheleau TA, Blackburn M, Andreev O, Golubeva E, Bhartia R, ffrench-Constant RH (1998) Insecticidal toxins from the bacterium *Photorhabdus luminescens*. Science 280: 2129–2132. https://doi.org/10.1126/science.280.5372.2129

Braendle C, Miura T, Bickel R, Shingleton AW, Kambhampati S, Stern DL (2003) Developmental origin and evolution of bacteriocytes in the aphid–buchnera symbiosis. Benjamin Normark (Ed.). PLoS Biology 1: e21. https://doi.org/10.1371/journal.pbio.0000021

Branson T, Krysan J (1981) Feeding and oviposition behavior and life cycle strategies of *Diabrotica*: an evolutionary view with implications for pest management. Environmental Entomology 10: 826–831. https://doi.org/10.1093/ee/10.6.826

Branson TF, Ortman EE (1970) The host range of larvae of the western corn rootworm: further studies. Journal of Economic Entomology 63: 800–803. https://doi.org/10.1093/jee/63.3.800

Bravo A, Gómez I, Porta H, García-Gómez BI, Rodriguez-Almazan C, Pardo L, Soberón M (2013) Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity. Microbial Biotechnology 6: 17–26. https://doi.org/10.1111/j.1751-7915.2012.00342.x

Bruno P, Machado RAR, Glauser G, Köhler A, Campos-Herrera R, Bernal J, Toepfer S, Erb M, Robert CAM, Arce CCM, Turlings TCJ (2020) Entomopathogenic nematodes from Mexico that can overcome the resistance mechanisms of the western corn rootworm. Scientific Reports 10: 8257. https://doi.org/10.1038/s41598-020-64945-x

Chiang H (1973) Bionomics of the northern and western corn rootworms. Annual Review of Entomology 18: 47–72. https://doi.org/10.1146/annurev.en.18.010173.000403

Ciche T (2007) The biology and genome of *Heterorhabditis bacteriophora*. WormBook: The online review of *C. elegans* biology: 1–9. https://doi.org/10.1895/wormbook.1.135.1

Ciche TA, Ensign JC (2003) For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? Applied and Environmental Microbiology 69: 1890–1897. https://doi.org/10.1128/AEM.69.4.1890-1897.2003

Ciosi M, Miller NJ, Toepfer S, Estoup A, Guillemaud T (2011) Stratified dispersal and increasing genetic variation during the invasion of Central Europe by the western corn rootworm, *Diabrotica virgifera virgifera*: *D. v. virgifera* expansion in Central Europe. Evolutionary Applications 4: 54–70. https://doi.org/10.1111/j.1752-4571.2010.00133.x

Ciosi M, Toepfer S, Li H, Haye T, Kuhlmann U, Wang H, Siegfried B, Guillemaud T (2009) European populations of *Diabrotica virgifera virgifera* are resistant to aldrin, but not to methyl-parathion. Journal of Applied Entomology 133: 307–314. https://doi.org/10.1111/j.1439-0418.2008.01363.x

ffrench-Constant RH, Dowling A, Waterfield NR (2007) Insecticidal toxins from Photorhabdus bacteria and their potential use in agriculture. Toxicon 49: 436–451. https://doi.org/10.1016/j.toxicon.2006.11.019

Dillman AR, Guillermin ML, Lee JH, Kim B, Sternberg PW, Hallem EA (2012) Olfaction shapes host–parasite interactions in parasitic nematodes. Proceedings of the National Academy of Sciences 109. https://doi.org/10.1073/pnas.1211436109

Dix I, Burnell A, Griffin C, Joyce S, Nugent M, Downes M (1992) The identification of biological species in the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) by cross-breeding second-generation amphimictic adults. Parasitology 104: 509–518. https://doi.org/10.1017/S0031182000063770

Duchaud E, Rusniok C, Frangeul L, Buchrieser C, Givaudan A, Taourit S, Bocs S, Boursaux-Eude C, Chandler M, Charles J-F, Dassa E, Derose R, Derzelle S, Freyssinet G, Gaudriault S, Médigue C, Lanois A, Powell K, Siguier P, Vincent R, Wingate V, Zouine M, Glaser P, Boemare N, Danchin A, Kunst F (2003) The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. Nature Biotechnology 21: 1307–1313. https://doi.org/10.1038/nbt886

EC (2012) Survey results for the presence of *Diabrotica virgifera* Le Conte in the European Union in 2011. Report from the Health and Consumers Directorate-General of the European Commission.

Ehlers R (2003a) Biocontrol Nematodes. In: Hokkanen HMT, Hajek AE (Eds), Environmental Impacts of Microbial Insecticides: Need and Methods for Risk Assessment. Springer Netherlands, Dordrecht, 177–220. https://doi.org/10.1007/978-94-017-1441-9_10

Ehlers R (2003b) Entomopathogenic nematodes in the European biocontrol market. Communications in agricultural and applied biological sciences 68: 3–16.

Elston KM, Perreau J, Maeda GP, Moran NA, Barrick JE (2021) Engineering a culturable *Serratia symbiotica* strain for aphid paratransgenesis. Applied and Environmental Microbiology 87. https://doi.org/10.1128/AEM.02245-20

Fischer-LeSaux M, Viallardt V, Brunelt B, Normand' P (1999) Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. International Journal of Systematic Bacteriology 49: 1645–1656. https://doi.org/10.1099/00207713-49-4-1645

Foltin K, Robier J (2014) Halting the western corn rootworm by crop rotation–yes, but! Effects of oil pumpkin in Styria. Julius-Kühn-Archiv 444: 126. https://doi.org/10.5073/jka.2014.444.037

Glaeser SP, Tobias NJ, Thanwisai A, Chantratita N, Bode HB, Kämpfer P (2017) *Photorhabdus luminescens* subsp. *namnaonensis* subsp. nov., isolated from *Heterorhabditis baujardi* nematodes. International Journal of Systematic and Evolutionary Microbiology 67: 1046–1051. https://doi.org/10.1099/ijsem.0.001761

Gray ME, Felsot AS, Steffey KL, Levine E (1992) Planting time application of soil insecticides and western corn rootworm (Coleoptera: Chrysomelidae) emergence: Implications for long-term management programs. Journal of Economic Entomology 85: 544–553. https://doi.org/10.1093/jee/85.2.544

Gray ME, Sappington TW, Miller NJ, Moeser J, Bohn MO (2009) Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. Annual Review of Entomology 54: 303–321. https://doi.org/10.1146/annurev.ento.54.110807.090434

Grewal PS, Ehlers R-U, Shapiro-Ilan DI (Eds) (2005) Nematodes as biocontrol agents. CABI Pub, Wallingford, UK; Cambridge, MA, 505 pp.

Hazir S, Kaya HK, Stock SP, Keskin N (2003) Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests. Turkish Journal of Biology 27. https://journals.tubitak.gov.tr/biology/vol27/iss4/1.

Jackson JJ (1996) Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae). Journal of Economic Entomology 89: 366–372. https://doi.org/10.1093/jee/89.2.366

Johnigk S-A, Ehlers R-U (1999) Endotokia matricida in hermaphrodites of *Heterorhabditis* spp. and the effect of the food supply. Nematology 1: 717–726. https://doi.org/10.1163/156854199508748

Joyce SA, Clarke DJ (2003) A hexA homologue from *Photorhabdus* regulates pathogenicity, symbiosis and phenotypic variation: pathogenicity and symbiosis in *Photorhabdus*. Molecular Microbiology 47: 1445–1457. https://doi.org/10.1046/j.1365-2958.2003.03389.x

Kaya H, Gaugler R (1993) Entomopathogenic nematodes. Annual Review of Entomology 38: 181–206. https://doi.org/10.1146/annurev.en.38.010193.001145

Kenney E, Hawdon JM, O'Halloran D, Eleftherianos I (2019) *Heterorhabditis bacteriophora* excreted-secreted products enable infection by *Photorhabdus luminescens* through suppression of the Imd pathway. Frontiers in Immunology 10: 2372. https://doi.org/10.3389/fimmu.2019.02372

Khan A, Brooks W (1977) A chromogenic bioluminescent bacterium associated with the entomophilic nematode *Chromonema heliothidis*. Journal of Invertebrate Pathology 29: 253–261. https://doi.org/10.1016/S0022-2011(77)80030-X

Kiss J, Edwards CR, Berger HK, Cate P, Cean M, Cheek S, Derron J, Festić H, Furlan L, Igrc-Barčić J, Ivanova I, Lammers W, Omelyuta V, Princzinger G, Reynaud P, Sivcev I, Sivicek P, Urek G, Vahala O (2005) Monitoring of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) in Europe 1992-2003. In: Western corn rootworm: ecology and management. CABI Publishing, Wallingford, Oxfordshire; Cambridge, MA, 29–39. https://doi.org/10.1079/9780851998176.0029.

Koppenhöfer AM, Shapiro-Ilan DI, Hiltpold I (2020) Entomopathogenic nematodes in sustainable food production. Frontiers in Sustainable Food Systems 4: 125. https://doi.org/10.3389/fsufs.2020.00125

Koropatnick TA, Engle JT, Apicella MA, Stabb EV, Goldman WE, McFall-Ngai MJ (2004) Microbial factor-mediated development in a host-bacterial mutualism. Science 306: 1186–1188. https://doi.org/10.1126/science.1102218

Kriticos DJ, Reynaud P, Baker RHA, Eyre D (2012) Estimating the global area of potential establishment for the western corn rootworm (*Diabrotica virgifera virgifera*) under rain-fed and irrigated agriculture. EPPO Bulletin 42: 56–64. https://doi.org/10.1111/j.1365-2338.2012.02540.x

Krysan J, Miller T (1986) Methods for study of pest *Diabrotica*. Springer, New York. https://doi.org/10.1007/978-1-4612-4868-2.

Kuhlmann U, van der Burgt A (1998) Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe. Biocontrol News and Information 19: 59N-68N.

Kuhlmann U, Toepfer S, Zhang F (2005) Is classical biological control against western corn rootworm in Europe a potential sustainable management strategy? In: Western corn rootworm: ecology and management. CABI Pub, Wallingford, Oxfordshire; Cambridge, MA.

Kurtz B, Hiltpold I, Turlings T, Kuhlmann U, Toepfer S (2009) Comparative susceptibility of larval instars and pupae of the western corn rootworm to infection by three entomopathogenic nematodes. BioControl 54: 255–262. https://doi.org/10.1007/s10526-008-9156-y

Levine E, Oloumi-Sadeghi H (1991) Management of diabroticite rootworms in corn. Annual Review of Entomology 36: 229–255. https://doi.org/10.1146/annurev.en.36.010191.001305

Levine E, Spencer JL, Isard SA, Onstad DW, Gray ME (2002) Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. American Entomologist 48: 94–107. https://doi.org/10.1093/ae/48.2.94

Mabry TR, Spencer JL, Levine E, Isard SA (2004) Western corn rootworm (Coleoptera: Chrysomelidae) behavior is affected by alternating diets of corn and soybean. Environmental Entomology 33: 860–871. https://doi.org/10.1603/0046-225X-33.4.860

MacDonald TT, Monteleone G (2005) Immunity, Inflammation, and Allergy in the Gut. Science 307: 1920–1925. https://doi.org/10.1126/science.1106442

Machado RAR, Bruno P, Arce CCM, Liechti N, Köhler A, Bernal J, Bruggmann R, Turlings TCJ (2019) *Photorhabdus khanii* subsp. *guanajuatensis* subsp. nov., isolated from *Heterorhabditis atacamensis*, and *Photorhabdus luminescens* subsp. *mexicana* subsp. nov., isolated from *Heterorhabditis mexicana* entomopathogenic nematodes. International Journal of Systematic and Evolutionary Microbiology 69: 652–661. https://doi.org/10.1099/ijsem.0.003154

Machado RAR, Bhat AH, Abolafia J, Muller A, Bruno P, Fallet P, Arce CCM, Turlings TCJ, Bernal JS, Kajuga J, Waweru B, Toepfer S (2021) Multi-locus phylogenetic analyses uncover species boundaries and reveal the occurrence of two new entomopathogenic nematode species, *Heterorhabditis ruandica* n. sp. and *Heterorhabditis zacatecana* n. sp. Journal of Nematology 53: 1–42. https://doi.org/10.21307/jofnem-2021-089

Machado RAR, Wüthrich D, Kuhnert P, Arce CCM, Thönen L, Ruiz C, Zhang X, Robert CAM, Karimi J, Kamali S, Ma J, Bruggmann R, Erb M (2018) Whole-genome-based revisit of *Photorhabdus* phylogeny: proposal for the elevation of most *Photorhabdus* subspecies to the species level and description of one novel species *Photorhabdus bodei* sp. nov., and one novel subspecies *Photorhabdus laumondii* subsp. *clarkei* subsp. nov. International Journal of Systematic and Evolutionary Microbiology 68: 2664–2681. https://doi.org/10.1099/ijsem.0.002820

Machado RAR, Thönen L, Arce CCM, Theepan V, Prada F, Wüthrich D, Robert CAM, Vogiatzaki E, Shi Y-M, Schaeren OP, Notter M, Bruggmann R, Hapfelmeier S, Bode HB, Erb M (2020) Engineering bacterial symbionts of nematodes improves their biocontrol potential to counter the western corn rootworm. Nature Biotechnology 38: 600–608. https://doi.org/10.1038/s41587-020-0419-1

Meinke LJ, Sappington TW, Onstad DW, Guillemaud T, Miller NJ, Komáromi J, Levay N, Furlan L, Kiss J, Toth F (2009) Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) population dynamics. Agricultural and Forest Entomology 11: 29–46. https://doi.org/10.1111/j.1461-9563.2008.00419.x

Milstead JE (1979) *Heterorhabditis bacteriophora* as a vector for introducing its associated bacterium into the hemocoel of *Galleria mellonella* larvae. Journal of Invertebrate Pathology 33: 324–327. https://doi.org/10.1016/0022-2011(79)90033-8

Moeser J, Vidal S (2004) Do alternative host plants enhance the invasion of the maize pest *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae, Galerucinae) in Europe? Environmental Entomology 33: 1169–1177. https://doi.org/10.1603/0046-225X-33.5.1169

Moeser J, Hibbard BE (2005) A synopsis on the nutritional ecology of larvae and adults of *Diabrotica virgifera virgifera* in the New and Old World. In: Western corn rootworm: ecology and management. CABI Pub, Wallingford, Oxfordshire; Cambridge, MA.

Ode PJ (2006) Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. Annual Review of Entomology 51: 163–185. https://doi.org/10.1146/annurev.ento.51.110104.151110

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proceedings of the National Academy of Sciences 100: 1803–1807. https://doi.org/10.1073/pnas.0335320100

Pereira AE, Wang H, Zukoff SN, Meinke LJ, French BW, Siegfried BD (2015) Evidence of field-evolved resistance to bifenthrin in western corn rootworm (*Diabrotica virgifera virgifera* LeConte) populations in Western Nebraska and Kansas. Soberón M (Ed.). PLOS ONE 10: e0142299. https://doi.org/10.1371/journal.pone.0142299

Piel J (2002) A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. Proceedings of the National Academy of Sciences 99: 14002–14007. https://doi.org/10.1073/pnas.222481399

Poinar GO (1975) Description and biology of a new insect parasitic Rhabditoid, *Heterorhabditis bacteriophora* N. Gen., N. Sp. (Rhabditida; Heterorhabditidae N. Fam.). Nematologica 21: 463–470.

Poinar GO, Thomas GM, Hess R (1977) Characteristics of the specific bacterium associated with *Heterorhabditis bacteriophora* (Heterorhabditidae: Rhabditida). Nematologica 23: 97–102. https://doi.org/10.1163/187529277X00273

Rasmann S, Köllner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TCJ (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434: 732–737. https://doi.org/10.1038/nature03451

Richardson WH, Schmidt TM, Nealson KH (1988) Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. Applied and Environmental Microbiology 54: 1602–1605. https://doi.org/10.1128/aem.54.6.1602-1605.1988

Rivera MJ, Martini X, Khrimian A, Stelinski L (2017) A weevil sex pheromone serves as an attractant for its entomopathogenic nematode predators. Chemoecology 27: 199–206. https://doi.org/10.1007/s00049-017-0246-0

Robert CAM, Zhang X, Machado RAR, Schirmer S, Lori M, Mateo P, Erb M, Gershenzon J (2017) Sequestration and activation of plant toxins protect the western corn rootworm from enemies at multiple trophic levels. eLife 6: e29307. https://doi.org/10.7554/eLife.29307

Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnology Journal 9: 283–300. https://doi.org/10.1111/j.1467-7652.2011.00595.x

Scarborough CL, Ferrari J, Godfray HCJ (2005) Aphid protected from pathogen by endosymbiont. Science 310: 1781–1781. https://doi.org/10.1126/science.1120180

Siegfried BD, Meinke LJ, Scharf ME (1998) Resistance management concerns for areawide management programs. Journal of Agricultural and Urban Entomology 15: 359–369. http://www.scopus.com/inward/citedby.url?scp=0038595776&partnerID=8YFLogxK.

van der Sluijs JP, Simon-Delso N, Goulson D, Maxim L, Bonmatin J-M, Belzunces LP (2013) Neonicotinoids, bee disorders and the sustainability of pollinator services. Current Opinion in Environmental Sustainability 5: 293–305. https://doi.org/10.1016/j.cosust.2013.05.007

Smith RF (1966) Distributional patterns of selected Western North American insects: the distribution of diabroticites in Western North America. Bulletin of the Entomological Society of America 12: 108–110. https://doi.org/10.1093/besa/12.2.108

Sutter GR, Fisher JR, Elliott NC, Branson TF (1990) Effect of insecticide treatments on root lodging and yields of maize in controlled infestations of western corn rootworms (Coleoptera: Chrysomelidae). Journal of Economic Entomology 83: 2414–2420. https://doi.org/10.1093/jee/83.6.2414

Tailliez P, Laroui C, Ginibre N, Paule A, Pagès S, Boemare N (2010) Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X. vietnamensis* sp. nov., *P. luminescens* subsp. *caribbeanensis* subsp. nov., *P. luminescens* subsp. *hainanensis* subsp. nov., *P. temperata* subsp. *khanii* subsp. nov., *P. temperata* subsp. *tasmaniensis* subsp. nov., and the reclassification of *P. luminescens* subsp. *thracensis* as *P. temperata* subsp. *thracensis* comb. nov. International Journal of Systematic and Evolutionary Microbiology 60: 1921–1937. https://doi.org/10.1099/ijs.0.014308-0

Toepfer S, Kuhlmann U (2004) Survey for natural enemies of the invasive alien chrysomelid, *Diabrotica virgifera virgifera*, in Central Europe. BioControl 49: 385–395. https://doi.org/10.1023/B:BICO.0000034604.30847.44

Toepfer S, Gueldenzoph C, Ehlers R, Kuhlmann U (2005) Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. Bulletin of Entomological Research 95: 473–482. https://doi.org/10.1079/BER2005379

Toepfer S, Kuhlmann U (2006) Constructing life-tables for the invasive maize pest *Diabrotica virgifera virgifera* (Col.; Chrysomelidae) in Europe. Journal of Applied Entomology 130: 193–205. https://doi.org/10.1111/j.1439-0418.2006.01060.x

Toepfer S, Hatala-Zseller I, Ehlers R-U, Peters A, Kuhlmann U (2010) The effect of application techniques on field-scale efficacy: can the use of entomopathogenic nematodes reduce damage by western corn rootworm larvae? Agricultural and Forest Entomology 12: 389–402. https://doi.org/10.1111/j.1461-9563.2010.00487.x

van Tol RWHM, van der Sommen ATC, Boff MIC, van Bezooijen J, Sabelis MW, Smits PH (2001) Plants protect their roots by alerting the enemies of grubs. Ecology Letters 4: 292–294. https://doi.org/10.1046/j.1461-0248.2001.00227.x

Vachon V, Laprade R, Schwartz J-L (2012) Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. Journal of Invertebrate Pathology 111: 1–12. https://doi.org/10.1016/j.jip.2012.05.001

Van Rozen K, Ester A (2010) Chemical control of *Diabrotica virgifera virgifera* LeConte. Journal of Applied Entomology 134: 376–384. https://doi.org/10.1111/j.1439-0418.2009.01504.x

Waterfield NR, Ciche T, Clarke D (2009) *Photorhabdus* and a host of hosts. Annual Review of Microbiology 63: 557–574. https://doi.org/10.1146/annurev.micro.091208.073507

Wechsler S, Smith D (2018) Has resistance taken root in U.S. corn fields? Demand for insect control. American Journal of Agricultural Economics 100: 1136–1150. https://doi.org/10.1093/ajae/aay016

Wesseler J, Fall EH (2010) Potential damage costs of *Diabrotica virgifera virgifera* infestation in Europe - the 'no control' scenario: potential damage costs of *Dvv*. in Europe. Journal of Applied Entomology 134: 385–394. https://doi.org/10.1111/j.1439-0418.2010.01510.x

Wilcox JA (1972) Coleopterorum Catalogus Supplementa. In: Pars. 78. fasc. 2 (Editio Seconda). (Chrysomelidae: Luperini: Diabroticina). Junk, 431.

Wood TJ, Goulson D (2017) The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environmental Science and Pollution Research 24: 17285–17325. https://doi.org/10.1007/s11356-017-9240-x

Wright RJ, Scharf ME, Meinke LJ, Zhou X, Siegfried BD, Chandler LD (2000) Larval susceptibility of an insecticide-resistant western corn rootworm (Coleoptera: Chrysomelidae) population to soil insecticides: laboratory bioassays, assays of detoxification enzymes, and field performance. Journal of Economic Entomology 93: 7–13. https://doi.org/10.1603/0022-0493-93.1.7

Yang SH, Chen WH, Wang ET, Chen WF, Yan J, Han XZ, Tian CF, Sui XH, Singh RP, Jiang GM, Chen WX (2018) Rhizobial biogeography and inoculation application to soybean in four regions across China. Journal of Applied Microbiology 125: 853–866. https://doi.org/10.1111/jam.13897

Zhang J, Xu Y, Cao T, Chen J, Rosen BP, Zhao F-J (2017) Arsenic methylation by a genetically engineered *Rhizobium*-legume symbiont. Plant and Soil 416: 259–269. https://doi.org/10.1007/s11104-017-3207-z

Zhang X, van Doan C, Arce CCM, Hu L, Gruenig S, Parisod C, Hibbard BE, Hervé MR, Nielson C, Robert CAM, Machado RAR, Erb M (2019) Plant defense resistance in natural enemies of a specialist insect herbivore. Proceedings of the National Academy of Sciences 116: 23174–23181. https://doi.org/10.1073/pnas.1912599116

Zhou X, Kaya HK, Heungens K, Goodrich-Blair H (2002) Response of ants to a deterrent factor(s) produced by the symbiotic bacteria of entomopathogenic nematodes. Applied and Environmental Microbiology 68: 6202–6209. https://doi.org/10.1128/AEM.68.12.6202-6209.2002

Chapter I

Engineering bacterial symbionts of nematodes improves their biocontrol potential to counter the western corn rootworm

Unpublished work

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Abstract

Bacterial symbionts play a crucial role for entomopathogenic nematodes (EPNs) as they boost their ability to kill insects. Here we report that introducing engineered bacterial symbionts into two commercially used nematode strains results in high infectivity of those newly generated nematode–symbiont pairs to kill the larvae of the western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte, one of the most damaging maize pests worldwide. WCR larvae are known to sequester benzoxazinoid secondary metabolites that are produced by maize and use them to increase their resistance to the nematodes and their symbionts. For that reason, we isolated 27 *Photorhabdus* symbionts from different nematodes and increased their benzoxazinoid resistance through experimental evolution. Benzoxazinoid resistance evolved through multiple mechanisms, including a mutation in a multidrug efflux pump. We reintroduced benzoxazinoid-resistant *Photorhabdus* strains into two strains of *Heterorhabditis bacteriophora* EPNs and identified four nematode–symbiont pairs that were able to kill benzoxazinoid-sequestering WCR larvae as efficiently as the commercially available nematodes under laboratory conditions. Our results suggest that modification of bacterial symbionts provides a successful and time-efficient strategy to improve biocontrol of agricultural pests in the future.

KEY WORDS Bacterial engineering, symbiont exchange, benzoxazinoid resistance, *Diabrotica virgifera virgifera*, *Heterorhabditis bacteriophora*, entomopathogenic nematodes

Introduction

Symbiotic bacteria are ubiquitous in animal hosts. They affect development, nutrition, reproduction, speciation, defence against natural enemies and immunity (Piel 2002, Braendle et al. 2003, Oliver et al. 2003, Koropatnick et al. 2004, Baumann 2005, Bäckhed et al. 2005, MacDonald and Monteleone 2005, Scarborough et al. 2005). In these associations, bacteria form persistent infections within host individuals, and they have adapted such that they have little or no negative impact on the host's overall health; often, they are beneficial or even obligatory. Given this wide spectrum of associations and functions, symbiotic microorganisms constitute promising and mostly untapped sources for potential applications in medicine, bioremediation, industrial processes and agriculture. In addition, the symbiotic microorganisms could even be engineered to improve the functions on their own or the performance together with their hosts (Berasategui et al. 2016). The knowledge on bacterial symbiosis can provide novel paths for the control of agricultural pests and vectors of human diseases, through targeted manipulation of the symbionts or the host-symbiont associations. In most of the cases so far, bacterial symbionts have only been directly applied to biomedical and agricultural problems through targeted inoculation or transplantation. For instance, inoculation of soybean fields with nitrogen-fixing rhizobia has been shown to enhance symbiotic nitrogen fixation and positively correlates with the increase in soybean yield and seed protein content (Yang et al. 2018). Several *Photorhabdus* species that normally live in the gut of entomopathogenic nematodes (EPNs), produce a toxin which consists of a series of four native complexes encoded by toxin complex loci *tca, tcb, tcc* and *tcd*. Both *tca* and *tcd* encode complexes with strong oral toxicity to *Manduca sexta* and therefore represents a potential alternative to the insecticidal *Bacillus thuringiensis* (*Bt*) toxin for transgenic deployment (Bowen et al. 1998).

In contrast, the direct engineering of bacterial symbionts remains largely untapped and only a few direct examples are found in the current literature: Engineered rhizobia bacteria expressing an algal *arsM* gene can methylate and volatilize arsenic, providing a proof of concept for potential future use of legume-rhizobia symbionts for arsenic bioremediation (Zhang et al. 2017). In addition, selecting *Photorhabdus* bacterial symbionts for plant toxin resistance translate into better infection potential of its nematode host against an insect herbivore that sequesters these toxins for self-protection (Machado et al 2020). These studies

highlight the potential of engineering desired traits into bacterial symbionts to enhance the performance of their host in this largely untapped approach.

Photorhabdus bacteria are a member of the family Enterobacteriaceae that live in a mutualistic association with *Heterorhabditis* nematode worms. They share a complex life cycle and form a mutualistic pair that is highly pathogenic to soil-dwelling insects. The free-living stage of *Heterorhabditis* nematodes, called infective juveniles harbour *Photorhabdus* bacteria in their intestinal lumens (Ciche 2007). After locating an insect, *Heterorhabditis* infective juveniles enter the hosts body through natural apertures such as the mouth or anus, and release their bacterial symbionts into the insect hemocoel (Kenney et al. 2019). *Photorhabdus* bacteria multiply exponentially and produce toxins and immunosuppressors that lead to the death of the insect (Bowen et al. 2000). *Heterorhabditis* nematodes then feed on bacteria-digested insect tissues and reproduce for several generations. Upon nutrient depletion, *Photorhabdus* bacteria re-associate with *Heterorhabditis* nematodes and the new-born infective juveniles emerge from the insect cadaver and seek for a new host. Taken together, those aspects make EPNs biological agents of choice in crop pest management and several species of *Heterorhabditis* nematodes are nowadays commercialized for domestic or industrial uses as an alternative to pesticides (Kaya and Gaugler 1993, Koppenhöfer et al. 2020).

Insects, however, do not remain passive victims upon nematode attacks. They resist either through cellular or humoral innate immune responses (Castillo et al. 2011). An additional mechanism of defence is the sequestration of plant-derived allelochemicals as defence (Barbercheck et al. 1995, Kunkel et al. 2004, Erb and Robert 2016). This is the case for the western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte*,* one of the world's most devastating maize pests that causes more than $2 billion of direct economic damage in the United States every year (Wechsler and Smith 2018). All three larval stages of the WCR feed on maize roots, which can ultimately lead to plant lodging (Sutter et al. 1990). The species is commonly considered to originate from Mexico, where its original native host was probably *Tripsacum*, a close wild relative of maize (Smith 1966, Branson and Krysan 1981, Gray et al. 2009). Since its first introduction into Serbia in 1992, this invasive pest is rapidly spreading over Europe and is expected to be present in all maize cultivars all over Eurasia in the near future (Aragón et al. 2010, Ciosi et al. 2011, Aragón and Lobo 2012, Kriticos et al. 2012).

To control the WCR, the following strategies are common practice today: (1) crop rotation, (2) entomopathogenic nematodes, (3) transgenic maize and (4) synthetic insecticides (Levine and

Oloumi-Sadeghi 1991, Van Rozen and Ester 2010). However, in some regions of Europe, management options are limited since the use of transgenic maize producing *Bt*-toxins targeting rootworms are restricted or prohibited and the use of insecticides are highly regulated. Several studies have shown that the residues of insecticides such as systemic neonicotinoids are found in water, soil, sowing dust, pollen and nectar can have negative effects on non-target pollinating insects (Blacquière et al. 2012, van der Sluijs et al. 2013, Wood and Goulson 2017). The development of resistance to insecticides and issues related to human and environmental safety push the research to the development of new management practices (Levine and Oloumi-Sadeghi 1991). Improved biological control solutions are therefore urgently needed to control this pest in an environmentally friendly manner (Kuhlmann and van der Burgt 1998, Babendreier et al. 2006). For a pest like the WCR, that is most damaging below-ground at the larval stage, soil-dwelling entomopathogenic nematodes are considered strong candidates for biological control strategies (Toepfer et al. 2010). EPNs have a wide host range including larvae of the WCR (Jackson 1996, Toepfer et al. 2005). They actively search for a host and are safe to humans and the environment (Ehlers 2003). *Heterorhabditis bacteriophora* is a promising EPN species having the potential to successfully control the three larval stages of the WCR (Kurtz et al. 2009). One challenge to overcome is the partial resistance of the WCR to EPNs due to their capacity to exploit the defensive mechanisms of maize for their own benefit (Robert et al. 2017, Zhang et al. 2019, Machado et al. 2020). Metabolite analysis revealed that maize-fed WCR larvae accumulated significant amounts of benzoxazinoids in their body (Robert et al. 2017). The highest concentrations were found for glucosides HDMBOA-Glc (>100 mg/g fresh mass) and MBOA-Glc (>25 mg/g fresh mass). The resulting increased resistance was linked to these two chemicals and their two different modes of action (Robert et al. 2017). First, WCR larvae release 6-methoxy-2-benzoxazolinone *N*-glucoside (MBOA-Glc) via exudates and frass, which repels nematodes. Second, WCR larvae accumulate 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one *O*-glucoside (HDMBOA-Glc) and release a breakdown product, 6-methoxy-2-benzoxazolinone (MBOA), upon nematode infection that is toxic to nematodes as well as to the released symbiotic bacteria. WCR larvae that accumulate and sequester MBOA and HDMBOA-Glc therefore significantly reduce the survival and the infectivity efficiency of the nematode and its *Photorhabdus* bacterial symbionts by more than 50% compared to benzoxazinoid-deficient WCR larvae (Robert et al. 2017).

It is believed that herbivore natural enemies feeding on sequestering hosts will evolve the capacity to resist or tolerate plant defence metabolites in parallel with their hosts (Ode 2006). By exposing a benzoxazinoid-susceptible nematode strain to the western corn rootworm for 5 generations, a higher behavioural and metabolic resistance and benzoxazinoid-dependent infectivity toward the western corn rootworm was achieved (Zhang et al. 2019). Thus, herbivores that are exposed to a plant defence sequestering herbivore can evolve both behavioural and metabolic resistance to plant defence metabolites, traits that are associated with higher infectivity toward a defence sequestering herbivore. In addition, a previous study has shown that it is possible to improve the efficacy of EPNs toward WCR by engineering benzoxazinoid-resistant symbionts (Machado et al. 2020). Five *Photorhabdus* symbionts from different nematode strains were isolated and their MBOA resistance was increased by 40 up to 183 % through experimental evolution. By sequencing the whole genome of these evolved symbionts, it was proved that benzoxazinoid resistance evolved through multiple mechanisms, including a mutation in the aquaporin-like channel gene *aqpZ*. The benzoxazinoid-resistant *Photorhabdus* strains were then reintroduced into their original EPN hosts and one nematode carrying MBOA-selected symbionts killed more benzoxazinoid-containing WCR larvae compared to nematodes carrying their ancestral symbionts under lab conditions (Machado et al. 2020). However, the bacteria and nematode strain that showed increased efficacy originally comes from China and may therefore not be suitable as a European or American agriculture biocontrol strategy.

With this project we aim to target symbionts of entomopathogenic nematode strains in a same manner as Machado *et al*. (2020) but with nematodes and bacterial symbionts that are compatible with commercial-scale biological control in North America and Europe. We used directed evolution to generate benzoxazinoid-resistant *Photorhabdus* strains that are more likely to overcome the defence mechanisms of the WCR. We reintroduced MBOA resistant strains into two commercially used EPN hosts and evaluated their capacity to kill benzoxazinoid-sequestering WCR larvae to determine whether the efficacy of a biological control agent could be improved by targeted engineering of its bacterial symbiont.

Here, we propose to use an interdisciplinary approach to improve symbiotic bacteria of entomopathogenic nematodes to create biocontrol agents that are able to overcome the plant-derived defences of the western corn rootworm. If successful, this work would be directly applicable to combat one of the most damaging, invasive maize pests on the globe.

Materials and Methods

Bacterial and nematode strains

Photorhabdus bacterial strains were obtained by isolating from their original nematode hosts (see Fig. 1b for more details). 100 nematode infective juveniles were surface sterilized, resuspended in 100 µl of autoclaved water and ground up using a plastic pestle. The resulting solution was diluted several times and the different concentrations were plated onto LB agar (40 g/l) (Carl Roth, Switzerland). Pure cultures were obtained by replating *Photorhabdus*-like colonies. Bacterial taxonomic positions were confirmed by obtaining the 16S rRNA gene sequences. Nematodes were provided by e-nema (Raisdorf, Germany) and Andermatt Biocontrol (Grossdietwil, Switzerland). The nematodes were reared at 25 °C on last instars of the wax moth *Galleria mellonella* as described by Kaya and Stock (1997). The freshly emerged infective juveniles were stored in tap water in culture tissue flasks at 10 °C before use.

Experimental evolution

27 bacterial strains (EN01, DE2, DE6, PT1, IT6, IR2, HU2, CN4, IL9, MG6286, TT01, LJ, B, 0943, S5P8, S7, S8, S9, S10, S12, S14, S15, KC, MEX20, BIO, A and DIA) deriving from single cell colonies were cultured independently in pure LB (25 g/l) (Carl Roth, Switzerland) (control, C-) and in LB containing 200 µg/ml MBOA (Sigma-Aldrich, Switzerland) (MBOA selected, M-) at 28 °C with constant shaking (180 rpm) for 12 to 24 h. This concentration was chosen based on the results of the study by Machado *et al.* (2020), which aligns with the observed GI_{50} for these bacterial strains. One millilitre of the resulting bacterial cultures was transferred to 3 ml of fresh medium (either pure LB or LB containing 200 µg/ml MBOA) and cultured again for 12 to 24 h. This cycle was repeated for a total of 35 subcultures. Glycerol stocks were prepared after the first growth cycle to preserve the original genotypes (evolutionary ancestors), as well as every seven cycles and after the experimental evolution was completed (generation of LB and MBOA selected strains).

Evaluation of MBOA resistance and bacterial growth *in vitro*

To evaluate the growth of the 27 *Photorhabdus* strains coming from the experimental evolution experiment (27 strains LB-selected, 27 strains MBOA-selected), bacterial cultures were grown in different concentrations of MBOA and the optical density (OD_{600}) was measured in 384-well microtiter plates (Greiner Bio-One, Austria). Bacterial cultures grown for 16 hours at 28 °C in LB (25 g/l) (Carl Roth, Switzerland) were diluted to an $OD_{600} = 0.05$ and ten microliters of these bacterial solutions were inoculated into 70 µl of LB (12.5 g/l) containing MBOA at

concentrations that ranged from 0 to 400 µg/ml. Plates were incubated in a Synergy™ HT Multi-Detection Microplate Reader (BioTek, United States) at 28 °C with OD_{600} measurements carried out every 30 min for 48 hours. Plates were gently shaken orbitally (medium shaking speed and 5-s shaking cycles) before each measurement.

Whole-genome sequencing and variant calling analysis

Genomic DNA (gDNA) was extracted directly from fresh (less than one week old) colonies of *Photorhabdus* bacteria growing on LB-Agar plates using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, United States). In order to properly lyse the cells, the bacteria growing on solid LB media were inoculated in lysis buffer supplemented with proteinase K and heated at 55°C for 2 h. Afterwards, the manufacturer's instructions were followed. Genomic libraries were prepared using the TruSeq DNA PCR-Free LT Library Prep kit (FC-121-3003), and indexed libraries were pooled at equimolar concentrations and sequenced (2,150 bp) on an Illumina HiSeq 3000 instrument. Raw Illumina reads were quality trimmed by using Trimmomatic 0.36 (options: SLIDINGWINDOW:4:8 MINLEN:127) (Bolger et al. 2014). Assembly of the resulting reads was done using SPAdes 3.10.1 (k-mer sizes of 21, 33, 55, 77, 99 and 127 bp), and the obtained contigs were assembled to scaffolds using SSPACE 3.0 with default options (Bankevich et al. 2012). To fill the gaps, GapFiller 1.10 was used (Boetzer and Pirovano 2012). Scaffolds with a mean read depth of less than 20% of the median read depth for the longer scaffolds (≥ 5,000 bp) and scaffolds shorter than 200 bp were removed. The final assemblies were polished using Pilon 1.22 (ref. 70). To determine the genetic alterations associated with the observed changes in benzoxazinoid resistance following experimental evolution, genomic differences between the laboratory evolved strains and their evolutionary ancestors were evaluated by variant calling analyses using Snippy 4.6.0 (Victorian Bioinformatic Consortium, Australia, https://github.com/tseemann/snippy). Protein structure homology modelling was conducted using the SWISS-MODEL server (Kopp 2006, Bienert et al. 2017, Waterhouse et al. 2018).

In vitro nematode cultures and symbiont exchange

To transfer the newly selected bacteria into EPNs, we cultured nematodes in semisolid bacterial cultures. Overnight cultures of the bacteria in LB (25 g/l) were prepared and the resulting bacterial cultures (OD_{600} = 1) were centrifuged down. The supernatant was removed and the pellets were resuspended in the same volume of semisolid-phase nematode growth Gelrite (NGG) medium (0.5 g casein peptone, 1.5 g NaCl, 0.75 g Gelrite, 500 µl $CaCl_2 \cdot 2H_2O$ (147

g/l), 500 µl $MgSO_4 \cdot 7H_2O$ (246.6 g/l), 12.5 ml KH_2PO_4 (136 g/l) and 500 µl cholesterol (1 g/l in 99% ethanol) in 0.486 l of tap water) as the overnight cultures. 2.5 ml of semisolid NGG with bacteria were then spread onto the surface of a 5-mm solid-phase NGG layer (the same as semisolid NGG but containing 1.5 g of Gelrite instead) in a sterilized Petri dish (6 cm in diameter; Corning). After incubation at 24°C for 48 hours, 100 infective juveniles were released per Petri dish in the resulting semisolid bacterial cultures. Living infective juveniles were recorded three days later, and the number of developing adults and hermaphrodites were counted one week after incubation. Resulting nematode progeny was collected 2–3 weeks after incubation by washing second generation infective juveniles with sterile tap water from the plates and multiplying them in *G. mellonella* larvae. To confirm successful symbiont exchange, the guts of infected *G. mellonella* were streaked out on LB agar plates, *Photorhabdus*-like colonies were collected, gDNA extracted as previously described and was then followed by PCR and Sanger sequencing by designing primers for the strain specific mutated gene. Primers are listed in Supplementary Table 1.

Nematode infectivity assays

To evaluate infectivity of 21 newly generated nematode-bacteria pairs, 25 infective juveniles in 500 µl ml tap water were released in Solo cups (30 ml) (Frontier Scientific, United States) containing 2 g of autoclaved sand (Ø 1–4 mm) (Migros, Switzerland). Then, five benzoxazinoid-sequestering second- or third-instar WCR larvae, previously feeding on the roots of B73 maize plants, were added. Controls were treated with 500 µl of tap water. After incubating the cups at 24 ± 0.5 °C for 5 days, the percentage of nematode-infected larvae in each Solo cup was determined. The infection status of the larvae was assessed visually. Nematode-infected larvae show a characteristic red/orange colour, which was used as a marker of infection. Each Solo cup was treated as an independent replicate. Experiment was conducted three independent times with four to five replicates each time (n=14).

Statistical analysis

Datasets were analysed by ANOVA using Sigma Plot 15.1.1.26 (Smith 2019). Data of MBOA resistance and re-established symbiosis between nematodes and selected bacteria were analysed with a two-way ANOVA, data of insect infectivity was analysed using a one-way ANOVA. Basal bacterial growth in LB was analysed by using two-way repeated measurements ANOVA (RM-ANOVA). Normality and equality of variance were verified using Shapiro–Wilk test. Holm post hoc tests were used for multiple comparisons.

Results

Generate benzoxazinoid-resistant *Photorhabdus* symbionts through experimental evolution

When WCR larvae are infected by EPNs, they convert HDMBOA-Glc into MBOA (Robert et al. 2017). MBOA is toxic to both the nematode and the entomopathogenic bacteria by reducing the growth of *Photorhabdus* symbionts, which translates then into reduced lethality of their nematode host (Robert et al. 2017). As already Machado *et al.* (2020) observed an improvement in the efficacy of the EPN–bacterial partnership in killing WCR larvae by increasing the bacterial symbionts resistant to MBOA, we decided to go into the same direction, but with bacterial strains from a wider geographic range (Machado et al. 2020). First, we isolated *Photorhabdus* symbionts from 27 different *Heterorhabditis* strains (Fig. 1b). We then cultured those *Photorhabdus* strains independently in LB broth containing MBOA at a concentration of 200 µg/ml for 34 24-hour incubation cycles (Fig. 1a). As controls, the same bacterial strains were cultured in MBOA-free LB for 34 24-hour incubation cycles. At the end of the directed evolution process, we evaluated MBOA resistance of the different strains using 384-well microtiter culture plates and calculated the GI_{50} (half-maximal growth inhibition) for each strain.

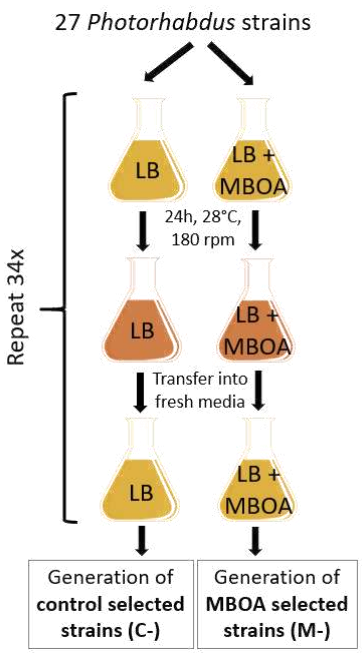
MBOA reduced the growth of all the tested strains in a dose-dependent and linear manner (Supplementary Fig. 1), but through the directed evolution, a significant increase in MBOA resistance could be observed ($F = 357,4$; $p < 0.001$, Fig. 1c). MBOA resistance was increased by up to 103 % in the MBOA-selected strains (Fig. 1c). The increase in MBOA resistance was greater for MBOA susceptible strains than for already MBOA-resistant strains. No significant differences in comparing the GI_{50} between LB- and MBOA-selected strains were observed for PT1, 0943, S8, KC. The most drastic increase in MBOA resistance was observed in S15, in which the GI_{50} increased from 191 to 386 µg/ml (Fig. 1c). This experiment showed that MBOA resistance can be improved by experimental evolution in *Photorhabdus* symbionts, but the degree of resistance is dependent on the strain.

Unravel the genetic basis of benzoxazinoid resistance in *Photorhabdus*

We sequenced the complete genomes of the 27 different *Photorhabdus* strains to evaluate how MBOA resistance evolved. By screening for mutations that were predicted to change gene or encoded protein function, we identified a variety of mutations, which were either altering DNA transcription, membrane architecture or membrane channels (Table 1). We identified 20

nonsynonymous point mutations, one insertion and one deletion. Surprisingly, there was only little overlap in the identified genes between strains, suggesting that MBOA resistance in *Photorhabdus* can be acquired by multiple mechanisms.

a



b

Bacteria strain	Bacteria species	Nematode strain	Nematode species	Origin
EN01	*P. laumondii* subsp. *laumondii*	EN 01	*H. bacteriophora*	Germany
DE2	*P. laumondii* subsp. *laumondii*	DE 2	*H. bacteriophora*	Germany
DE6	*P. laumondii* subsp. *laumondii*	DE 6	*H. bacteriophora*	Germany
PT1	*P. thracensis*	PT 1	*H. bacteriophora*	Portugal
IT6	*P. laumondii* subsp. *laumondii*	IT 6	*H. bacteriophora*	Italy
IR2	*P. kayaii*	IR 2	*H. bacteriophora*	Irland
HU2	*P. kayaii*	HU 2	*H. bacteriophora*	Hungary
CN4	*P. bodei*	CN 4	*H. beicherriana*	China
IL9	*P. laumondii* subsp. *laumondii*	IL 9	*H. bacteriophora*	Australia
MG6286	*P. laumondii* subsp. *laumondii*	MG 618b	*H. bacteriophora*	Switzerland
TT01	*P. laumondii* subsp. *laumondii*	M13e	*H. bacteriophora*	Trinidad and Tobago
LJ	*P. bodei*	LJ-24	*H. beicherriana*	China
B	*P. kleinii*	Hb b	*H. bacteriophora*	United States
0943	*P. thracensis*	0943	*H. bacteriophora*	Turkey
S5P8	*P. laumondii* subsp. *laumondii*	S5-P8	*H. bacteriophora*	United States
S7	*P. laumondii* subsp. *laumondii*	S7	*H. bacteriophora*	United States
S8	*P. kleinii*	S8	*H. georgiana*	United States
S9	*P. kleinii*	S9	*H. georgiana*	United States
S10	*P. kleinii*	S10	*H. georgiana*	United States
S12	*P. laumondii* subsp. *laumondii*	S12	*H. bacteriophora*	United States
S14	*P. laumondii* subsp. *laumondii*	S14	*H. bacteriophora*	United States
S15	*P. laumondii* subsp. *laumondii*	S15	*H. bacteriophora*	United States
KC	*P. laumondii* subsp. *laumondii*	Hb KC	*H. bacteriophora*	United States
MEX20	*P. khanii* subsp. *guanajuatensis*	MEX-20	*H. atacamensis*	Mexico
BIO	*P. laumondii* subsp. *laumondii*	Hb Bio	*H. bacteriophora*	United States
A	*P. laumondii* subsp. *laumondii*	Hb And	*H. bacteriophora*	Germany
DIA	*P. laumondii* subsp. *laumondii*	Dianem	*H. bacteriophora*	Germany

c

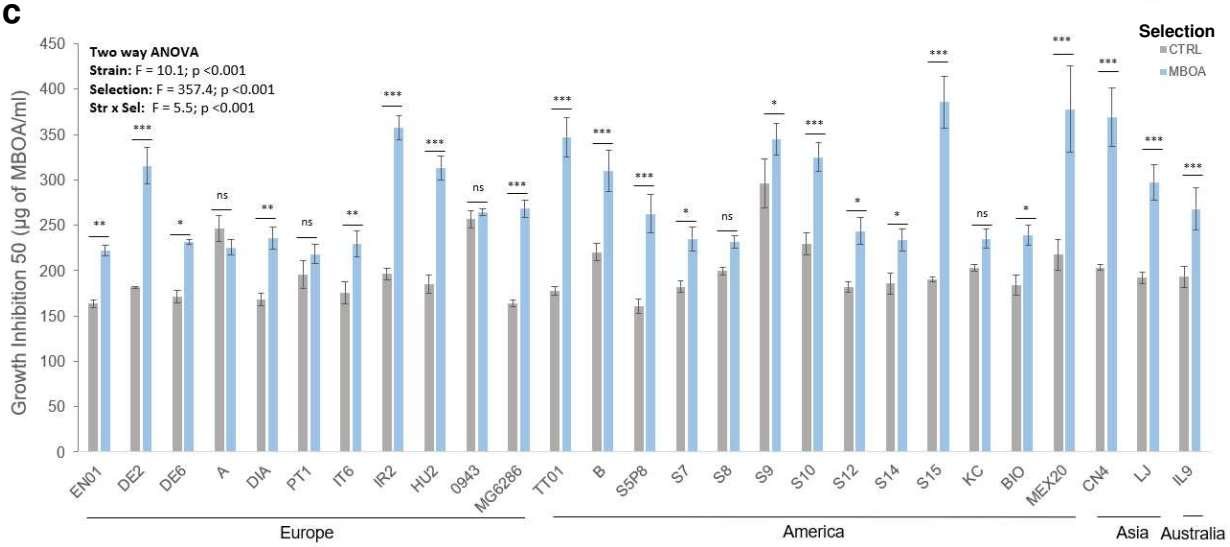


Figure 1: Selection on MBOA increases MBOA resistance of different *Photorhabdus* strains. (a) Schematic overview of the experimental evolution approach to select for MBOA resistance in *Photorhabdus*. Strains were either selected in MBOA-free LB control medium (Luria-Bertani broth) or in MBOA-containing LB medium (200 µg/ml). **(b)** Overview of the used bacterial strains. **(c)** Mean (± s.e.m.) MBOA concentrations that inhibit bacterial growth by 50% (Growth Inhibition 50, GI50) in the strains selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars). Bacterial species are grouped by their continental origin. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). Experiment was conducted four to six independent times with one replicate each time (n=4-6).

MBOA-selected EN01 (M-EN01, *P. laumondii subsp. laumondii*) had a single point mutation in the Na(+)/H(+) antiporter *nhaB* gene (c.1061C>T p.Pro354Leu) (Fig. 2a). This antiporter extrudes sodium in exchange for external protons. A single point mutation was observed in the *mppO* gene (c.842G>T p.Arg281Leu) (Fig. 2a), known to code for an oxidoreductase. Furthermore, in M-EN01, a single point mutation in the *ompR* gene was detected (c.614T>G p.Val205Gly) (Fig. 2a). This gene has been described as a transcriptional regulator of porins.
In M-IT6, we found a single point mutation in the osmolarity sensor protein *envZ* (c.1016G>A p.Arg339Gln), which is involved in medium osmoregulation (Fig. 2b). By controlling unsaturated fatty acids production, the HTH-type transcriptional repressor *fabR* regulates the physical properties of the membrane bilayer. MBOA selected IR2 bacteria (M-IR2, *P. kayaii*) have a single point mutation in this gene (c.116G>A p.Arg39His) (Fig. 2c). MBOA-selected IL9 (M-IL9, *P. laumondii subsp. laumondii*), has a single point mutation in the beta subunit of the DNA-directed RNA polymerase *rpoB* gene (c.1655C>T p.Pro552Leu) (Fig. 2d). A single point mutation in the gene *waaA* coding for 3-deoxy-D-manno-octulosonic acid transferase (c.668C>T p.Thr223Ile) was detected for the strain M-TT01 (*P. laumondii subsp. laumondii*) (Fig. 2e). This gene is involved in the biosynthesis of lipopolysaccharides. In addition, a stop gain was found in the same MBOA-selected strain in the O-antigen polymerase *wzy* (c.965G>A p.Trp322*), resulting in a shorter protein product (Fig. 2e). This MBOA-selected strains B, S5P8, S12 and MEX20 (M-B, *P. kleinii*; M-S5P8 and M-S12, *P. laumondii subsp. laumondii;* M-MEX20, *P. khaini* subsp. *guanajuatensis*) each had a single point mutation in different positions of the multidrug efflux pump subunit *acrB* gene (M-B: c.1390G>C p.Gly464Arg; M-S5P8: c.1421T>C p.Ile474Thr; M-S12: c.1208G>A p.Gly403Asp; M-MEX20: c.2924G>A p.Arg975His) (Fig. 2f,g,j,l). This drug efflux protein complex with broad substrate specificity uses a proton motive force to export a wide range of substrates. Furthermore, in M-S7 (*P. laumondii subsp. laumondii*), a single point mutation in the *ompR* gene was detected (c.659G>A p.Arg220His) (Fig. 2h) and in M-S10 (*P. kleinii*), a single point mutation was found for the *acrZ* gene which is encoding for a multidrug efflux pump accessory protein (c.7G>C p.Glu3Gln) (Fig. 2i). The *Photorhabdus* strain with the highest number of acquired mutations is MBOA-selected S15 (M-S15, *P. laumondii subsp. laumondii*) (Fig. 2k). Single point mutations were found in the multidrug efflux pump subunit *acrB* (c.2999G>A p.Gly1000Asp), in the inverse autotransporter beta domain-containing protein *ychP* (c.379A>G p.Thr127Ala), the RNA polymerase sigma factor *rpoD* (c.421G>A p.Glu141Lys) and in the XRE family transcriptional regulator *C6H65_03095* (c.52C>T

p.Arg18Cys) (Fig. 2k). An insertion resulting in a frameshift was detected in the sodium/pantothenate symporter *panF* (c.1167dupC p.Glu390fs). Insertion mutations were observed to shift stop codons which dramatically changes protein secondary and tertiary structure, similar to stop gains by single point mutations. Lastly for M-S15, a stop gain for the osmolarity sensor protein *envZ* (c.373G>T p.Glu125*) was detected, resulting in a shorter protein product compared to the wild type (Fig. 2k). MBOA-selected DIA (M-DIA, *P. laumondii* subsp. *laumondii*) had two single point mutation in different subunits of the DNA-directed RNA polymerase *rpo* gene (*rpoA*: c.821C>A p.Ala274Asp; *rpoC*: c.3223C>T p.Arg1075Cys) (Fig. 2m).

Table 1: Mutations in evolved MBOA-resistant strains.

Strain	Gene	Mutation type	Gene product name	Putative function
M-EN01	*nhaB*	SNP (c.1061C>T p.Pro354Leu)	Na(+)/H(+) antiporter	Extrudes sodium in exchange for external protons
	ompR	SNP (c.614T>G p.Val205Gly)	Transcriptional regulatory protein	Regulation of porins
	mppO	SNP (c.842G>T p.Arg281Leu)	Enduracididine beta-hydroxylase	Oxidoreductase
M-IT6	*envZ*	SNP (c.1016G>A p.Arg339Gln)	Osmolarity sensor protein	Osmoregulation
M-IR2	*fabR*	SNP (c.116G>A p.Arg39His)	HTH-type transcriptional regulator	Negative regulator of un-saturated fatty acid biosynthesis
M-IL9	*rpoB*	SNP (c.1655C>T p.Pro552Leu)	DNA-directed RNA polymerase subunit beta	Transcription of DNA to RNA
M-TT01	*waaA*	SNP (c.668C>T p.Thr223Ile)	3-deoxy-D-manno-octulosonic acid transferase	Lipopolysaccharide biosynthesis
	wzy	Stop gain (c.965G>A p.Trp322*)	O-antigen polymerase	Polymerization of O-antigens
M-B	*acrB*	SNP (c.1390G>C p.Gly464Arg)	Multidrug efflux pump subunit	Part of drug efflux protein complex
M-S5P8	*acrB*	SNP (c.1421T>C p.Ile474Thr)	Multidrug efflux pump subunit	
M-S7	*ompR*	SNP (c.659G>A p.Arg220His)	Transcriptional regulatory protein	Regulation of porins
M-S10	*acrZ*	SNP (c.7G>C p.Glu3Gln)	Multidrug efflux pump accessory protein	Part of drug efflux protein complex
M-S12	*acrB*	SNP (c.1208G>A p.Gly403Asp)	Multidrug efflux pump subunit	Part of drug efflux protein complex
M-S15	*acrB*	SNP (c.2999G>A p.Gly1000Asp)	Multidrug efflux pump subunit	
	ychP	SNP (c.379A>G p.Thr127Ala)	Inverse autotransporter beta domain-containing protein	Autotransporter
	panF	Frameshift (c.1167dupC p.Glu390fs)	Sodium/pantothenate symporter	Pantothenate transmembrane transport
	envZ	Stop gain (c.373G>T p.Glu125*)	Osmolarity sensor protein	Osmoregulation
	rpoD	SNP (c.421G>A p.Glu141Lys)	RNA polymerase sigma factor	Promotes the attachment of RNA polymerase
	C6H65_03095	SNP (c.52C>T p.Arg18Cys)	XRE family transcriptional regulator	DNA-binding transcription factor
M-MEX20	*acrB*	SNP (c.2924G>A p.Arg975His)	Multidrug efflux pump subunit	Part of drug efflux protein complex
M-DIA	*rpoA*	SNP (c.821C>A p.Ala274Asp)	DNA-directed RNA polymerase subunit alpha	Transcription of DNA to RNA
	rpoC	SNP (c.3223C>T p.Arg1075Cys)	DNA-directed RNA polymerase subunit beta'	

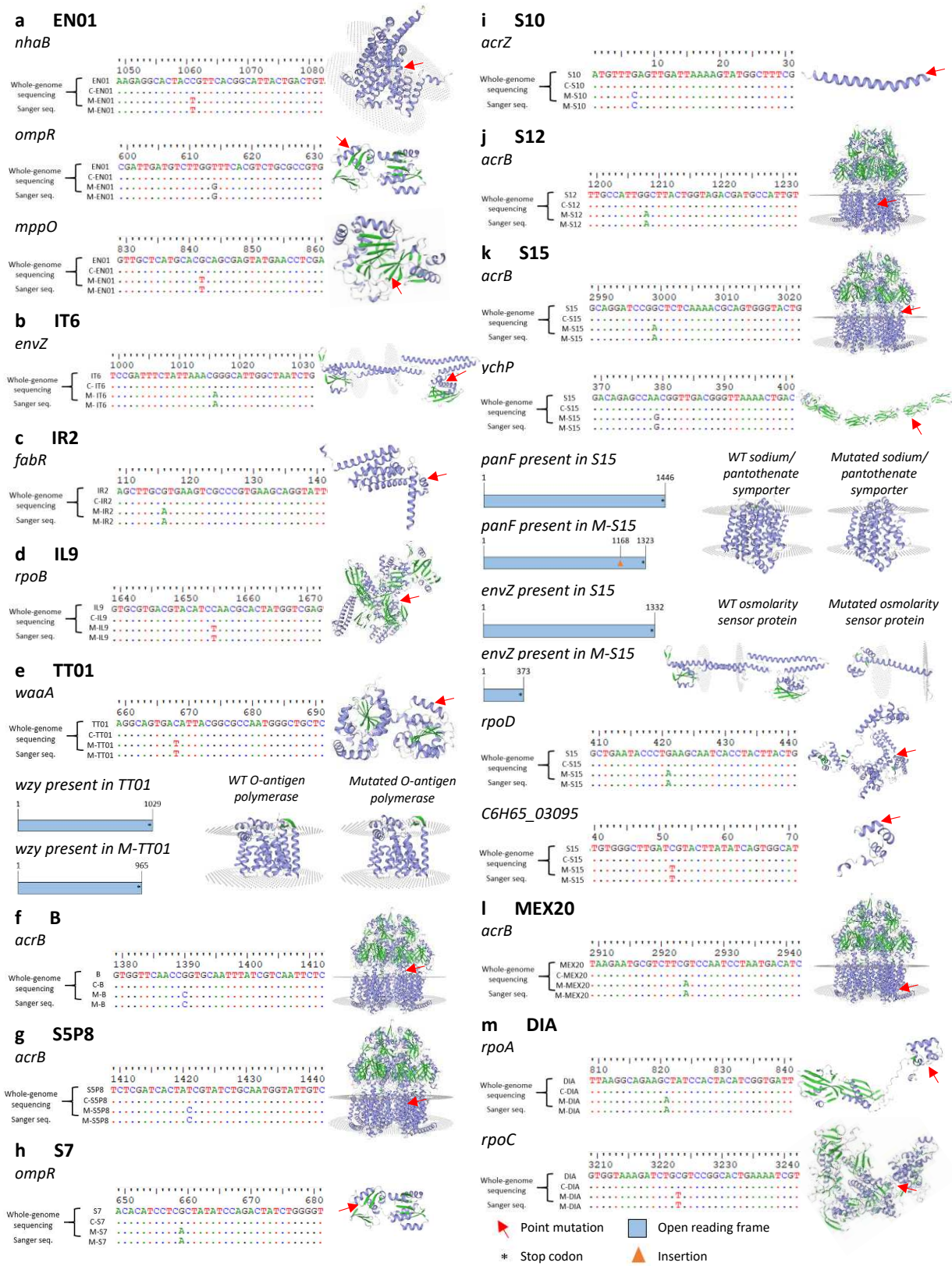


Figure 2. Increased MBOA resistance is associated with various mutations. Nonsynonymous gene mutations found in MBOA-selected strains (M-) relative to ancestral and LB-selected strains (C-) are depicted. Blue schemes represent open reading frames. Asterisks represent stop codons. If the protein structure does not change, only the model of the ancestral strain is shown, including a red arrow highlighting the place of a SNP. If the structure differs, protein structure models for the ancestral and MBOA-selected strains are shown.

Cost of MBOA resistance in *Photorhabdus*

To test for the potential cost of high MBOA resistance, we measured growth of MBOA- and LB-selected strains *in vitro* in the absence of MBOA. We only selected the 20 strains which showed highest increase in terms of MBOA resistance. For seven of these twenty strains, selection on MBOA did not influence their growth in LB (EN01, DE2; CN4, TT01, B, MEX20 and BIO) (Supplementary Fig. 2). For eight strains, selection on MBOA did negatively influence their growth in LB (DE6, IR2, HU2, IL9, S12, S14, S15 and DIA). On the other hand, five strains (IT6, MG6286, LJ, S5P8 and S10) showed faster and stronger growth when they were previously selected on MBOA. Overall, no direct link between MBOA selection and growth effect could be observed ($F = 3.6$; $p = 0.058$).

Measure the performance of the selected bacteria in the context of nematode symbiosis

To test whether the bacterial strains with increased MBOA resistance exhibit changes in their ability to support nematode growth or infective juvenile recovery, we cultured *H. bacteriophora* infective juvenile nematodes of the two commercially used strains Meginem® and Dianem® in vitro in artificial medium which was previously inoculated with the different bacterial strains and quantified the number of developing nematodes and adult hermaphrodites (Supplementary Fig. 3). Number of infective juveniles and hermaphrodites varied a lot between strains and selection regime of the bacterial symbionts. As for the number of recovered infective juveniles, no difference between the bacterial symbionts and the type of bacterial selection was observed for both nematode strains ($p > 0.078$). In contrast, the number of developed hermaphrodites was significantly affected by the bacterial identity (for both nematode strains $p < 0.001$) as well as their selection regime (for both nematode strains $p < 0.001$). Interestingly, MBOA-selected bacteria were more successful in re-establishing symbiosis with the two nematode strains than the LB-selected bacteria. We only observe no or very low numbers (<2) of developed hermaphrodites per Petri dish when Dianem® nematodes grew on medium containing LB-selected S9, S14, IT6, LJ or EN01 as well as MBOA-selected MEX20, LJ, S5P8 and DE6. For Meginem® nematodes, we observed no or very low numbers (<2) of developed hermaphrodites when nematodes grew on medium containing LB-selected IL9, LJ, S5P8, S9, MEX20 or EN01 as well as MBOA-selected DE6, MEX20 or B. High numbers (>25) of hermaphrodites per petri dish were recorded for MBOA-selected HU2 and DIA in Dianem® nematodes and for MBOA-selected DE2, HU2, S15, IL9 or DIA and for LB-selected S12 in Meginem® nematodes. Thus, selection on MBOA can affect the ability of *Photorhabdus* to

support nematode development in some, but not all, strains. Overall, twenty-nine bacteria-nematode pairs were initially collected from cultures growing on artificial media. Successful recolonization by the different bacterial symbionts was confirmed by re-isolating the symbionts, extracting gDNA and followed by PCR and Sanger sequencing with designed primers for the strain specific mutated gene. Primers are listed in Supplementary Table 1. Symbionts were not exchanged successfully in eight cases. The resulting twenty-one nematode colonies carrying different selected symbiotic bacteria have been successfully confirmed by symbiont-identification and were established in laboratory rearings (Table 2). Twelve of them consist of Dianem® nematodes, 8 of which (67%) carrying MBOA-selected bacteria; and nine of them consist of Meginem® nematodes, 6 of which (67%) carrying MBOA-selected bacteria.

Table 2: Established nematode colonies in laboratory rearings with confirmed exchange of symbiotic bacteria for LB (C) and MBOA (M) selected bacterial symbionts. Two commercial nematode strains were used: Dianem® TM (specifically developed to combat the western corn rootworm); and Meginem® Pro TM (generalist nematode strain developed to combat a broader spectrum of insect pests).

Bacterial strain		EN01		DE2		IT6		HU2		CN4		IL9		S10		S12		S15		BIO		DIA	
Selection regime		C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M
Nematode strain	*Dianem*		x		x		x	x	x				x	x		x	x		x	x			x
	Meginem		x					x			x		x	x		x	x		x				x

Infectivity of evolved symbiont–nematode pairs toward WCR

After selecting the bacteria for increased MBOA-resistance and establishing symbiosis with two commercially used nematode strains, we tested whether the evolved symbiont-nematode pairs can overcome the benzoxazinoid defences of WCR larvae. We infected benzoxazinoid sequestering WCR larvae with 25 infective juveniles per nematode-bacteria pair and checked insect mortality after 5 days (Fig. 3). Insect mortality was for most of the tested novel strains at least as high as the corresponding nematode strain with no exchanged symbionts (Dianem® nematodes with DIA bacteria and Meginem® nematodes with A bacteria). Selection of the bacterial symbionts did not have a direct effect on the infectivity against the WCR larvae ($p > 0.29$). By correlating MBOA resistance measured as the GI_{50} and insect mortality of LB-selected bacterial strains, we found no significant correlation (Supplementary Fig. 4). In contrast, a significant correlation between GI_{50} on MBOA and mortality of the WCR was observed for MBOA-selected strains ($p = 0.049$), suggesting that higher benzoxazinoid resistance leads to higher mortality in the WCR (Supplementary Fig. 4). In addition, exchanging symbionts of EPNs

with non-native bacterial strains may alter processes in the symbiosis and therefore overcome the benzoxazinoid defences of WCR larvae.

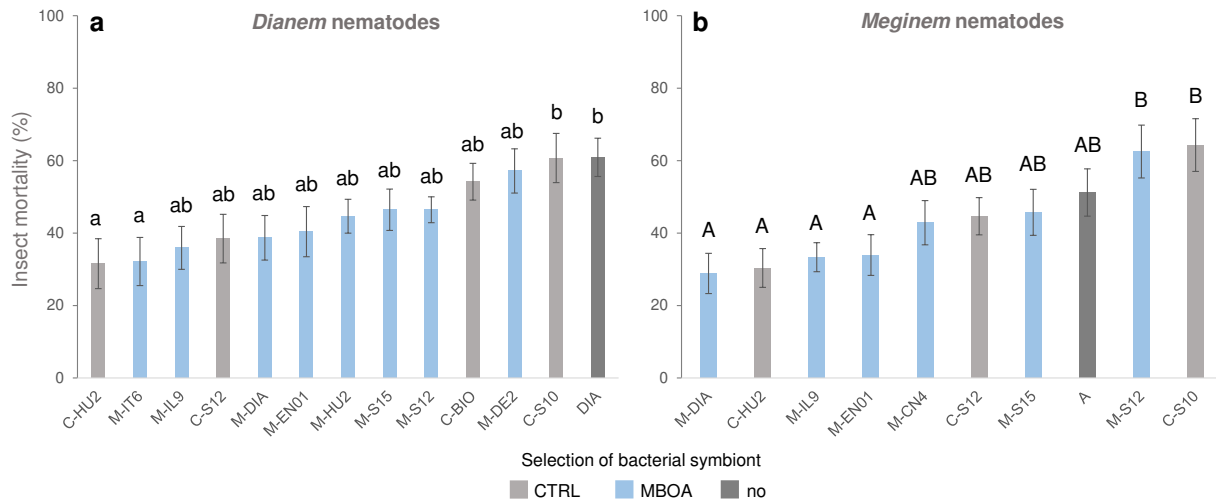


Figure 3: Nematodes harbouring selected symbionts show high biocontrol potential of benzoxazinoid-sequestering WCR larvae. Mean (± s.e.m.) percent of WCR larvae that died from EPN attack under laboratory conditions. Symbiotic bacteria were either LB-selected (C-, light grey bars), MBOA-selected (M-, blue bars) or were original symbionts of the respective nematode (dark grey bars). Strains are ordered by increasing insect mortality. Different letters indicate statistically significant differences between nematode-bacteria pairs within each used nematode strain ($P < 0.05$ by one-way ANOVA with Holm's multiple-comparisons test). Experiment was conducted three independent times with a total of fourteen evaluated arenas, and each arena consisted of 4 larvae (n=14).

Discussion

WCR is one of the most devastating insect pests of maize worldwide, which can cause extensive crop damage and consequent economic losses to farmers exceeding $2 billion annually if not controlled in the United States (Wechsler and Smith 2018). It also has become the main pest of continuous corn in Central and South-eastern Europe since its introduction near Belgrade in the beginning of this millennium, and it represents a major risk to Western Europe (Kuhlmann and van der Burgt 1998, Aragón et al. 2010). This challenges the aim to reduce the use of synthetic pesticides in sustainable agriculture. The use of herbivore natural enemies as biological control agents, particularly EPNs is a promising strategy in this context to combat root-feeding pests such as the larvae of the WCR (van Lenteren 2012). Nevertheless, improving the efficacy of nematode biocontrol is needed to improve the economic viability of this sustainable biocontrol approach. By exposing nematodes to the WCR for several generations, they can evolve both behavioural and metabolic resistance to plant defence metabolites, and these traits are associated with higher infectivity towards this defence sequestering herbivore (Zhang et al. 2019). In addition, a previous study has shown that it is also possible to improve the efficacy of EPNs toward WCR by engineering benzoxazinoid-resistant bacterial symbionts (Machado et al. 2020). Going into this same direction, we report a strategy to improve the biocontrol potential of natural control agents against the WCR, which accumulates plant secondary metabolites for their own defence. With a targeted experimental evolution, we increased benzoxazinoid resistance in a variety of *Photorhabdus* strains, one of the main compounds the WCR is sequestering. We then exchanged symbionts of two commercially used nematode strains and observed high efficacy in the novel generated pairs against larvae of the WCR. The approach of exchanging symbionts between nematode strains has been commonly used in the past, but the efficacy did not always increase with this mainly untargeted approach (Han et al. 1991, Gerritsen and Smits 1993). With our directed approach, we modified and exchanged bacterial symbionts and provided a basis for future selection programs as a successful and time-efficient strategy to improve biocontrol of a variety of agricultural pests in the future.

Natural evolutionary processes are affected by various types of constraints acting at the different levels of biological organization. Of particular importance are constraints where correlated changes occur in opposite directions, so called trade-offs. By selecting for a particular increase in benzoxazinoid resistance, we expected physiological or ecological trade-

offs in the selected bacteria. Antibiotic resistance, for example, is constrained by a growth-survival trade-off. When a bacterium is exposed to an antibiotic, it may develop antibiotic resistance to increase survival. This adaptation transfers resources from the growth to the survival component of fitness and, as a consequence, resistant genotypes are less fit than susceptible genotypes in the absence of antibiotic in terms of reduced growth, impaired competitive performance or metabolic imbalance (Lenski 1998, Wang et al. 2002, Andersson et al. 2007). Interestingly, we found that MBOA resistance was not directly related with substantial costs. The ability to support nematode development was even higher for MBOA-selected stains compared to MBOA-susceptible strains. If the symbiosis between nematodes and its symbiotic bacteria is impaired, it can be the result of direct nematicidal activity, reduced intestine colonization and/or reduced production of nematode growth factors by *Photorhabdus* (Han and Ehlers 1999, Joyce et al. 2008, Somvanshi et al. 2010, Easom and Clarke 2012, Tobias et al. 2017). To which extent the MBOA-selected strains could provide better symbiotic capabilities compared to the MBOA-susceptible strains is not known and needs further examination. It could be that MBOA-selected strains provide a better food signal production inducing the recovery of the nematodes (Strauch and Ehlers 1998). Overall, no significant trade-off between MBOA resistance and bacterial growth was observed. It is known that mutants defective in lipopolysaccharide biosynthesis often exhibit aberrant growth, but MBOA-selected TT01 bacteria did not exhibit reduced bacterial growth (Schnaitman and Klena 1993), contrary to the study of Machado et al (2020), where a significant growth reduction in the MBOA-selected TT01 strain was observed. Mutations in the *rpoC* genes are responsible to reduce maximal growth rates as well as affecting DNA replication, which could explain the reduced bacterial growth of MBOA-selected DIA bacteria (Petersen and Hansen 1991, Nandy et al. 2020). Similar, bacteria with mutations in the *rpoB* gene often exhibit aberrant growth, which could explain the reduced bacterial growth of MBOA-selected IL9 bacteria (Jin and Gross 1989, Lai et al. 2002, Qi et al. 2014). In addition, various studies showed that *rpoB* mutations influence the symbiotic association between the symbionts and their entomopathogenic nematode hosts (Qiu et al. 2012). Although we did not observe reduced symbiotic ability of MBOA-selected IL9, infectivity against the WCR together with its nematode host was among the lowest of the tested strains. Nematode growth, development and reproduction of nematodes depend on successful interaction between the symbiotic bacteria and its nematode host, with the bacteria producing growth factors that stimulate hermaphrodite development

(Strauch and Ehlers 1998). Overall, swapping experiments proved difficult, as many symbioses between nematodes and non-native bacterial symbionts failed to establish because of incompatibilities between nematode host and selected and non-native bacteria. Nevertheless, we could produce twenty-one bacteria-nematode pairs with confirmed symbiotic exchange and the majority of those showed high biocontrol potential against the WCR.

Our study highlighted the importance of using diverse bacterial starting material. We could not only prove that not all selected strains show negative effects of MBOA resistance but we also found evidence that each strain likely evolved different mechanisms to overcome MBOA toxicity. MBOA resistance was associated with a variety of mutations falling into three broad categories: DNA transcription, membrane architecture and membrane channels. We found that a mutation in *fabR,* an HTH-type transcriptional regulator that is negatively regulating unsaturated fatty acid biosynthesis resulted in an 82% increase of MBOA resistance in the selected strain IR2. MBOA-selected TT01 exhibits increased MBOA resistance by more than 95%. Two mutations may be possible for this increase: Firstly, a point mutation in the *waaA* gene, coding for 3-deoxy-D-mannooctulosonic acid transferase responsible for lipopolysaccharide biosynthesis. Secondly, a point mutation was found in M-TT01 in the *wzy* gene resulting in a stop gain and a shorter, protein product, which is likely non-functional. The final protein product, the O-antigen polymerase plays an important role in catalysing the addition of newly synthesized O-antigen repeating units to a glycolipid. Highest increase of MBOA resistance was observed for the strain M-S15 with more than 102%. This strain exhibits the most mutations among the analysed strains. It includes a single nucleotide polymorphism in the *acrB* gene coding for a subunit of a multidrug efflux pump that may lead to altered substrate specificity and therefore promoting the efflux of this toxin from the cytoplasm. These identified loci represent promising targets for improving the resistance of bacterial symbionts to environmental toxins.

With our work, we highlighted the potential and the possibilities of directly engineering bacterial symbionts for desired traits such as benzoxazinoid resistance to enhance the performance of their host against the WCR that sequesters benzoxazinoids for self-protection to the level of already existing and commercially used nematode products. Our results encourage to use a similar experimental approach to improve other biological control agents and contribute to a more sustainable and pesticide-free crop production.

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Competing interests

The authors declare no competing interests.

References of Chapter I

Andersson DI, Patin SM, Nilsson AI, Kugelberg E (2007) The biological cost of antibiotic resistance. Enzyme-Mediated Resistance to Antibiotics: Mechanisms, Dissemination, and Prospects for Inhibition: 339–348. https://doi.org/10.1128/9781555815615.ch21

Aragón P, Lobo JM (2012) Predicted effect of climate change on the invasibility and distribution of the Western corn rootworm. Agricultural and Forest Entomology 14: 13–18. https://doi.org/10.1111/j.1461-9563.2011.00532.x

Aragón P, Baselga A, Lobo JM (2010) Global estimation of invasion risk zones for the western corn rootworm *Diabrotica virgifera virgifera*: Integrating distribution models and physiological thresholds to assess climatic favourability. Journal of Applied Ecology 47: 1026–1035. https://doi.org/10.1111/j.1365-2664.2010.01847.x

Babendreier D, Keller S, Romeis J, Bigler F, Kuhlmann U, Toepfer S, Turlings T, Schaub L, others (2006) New approaches to combating corn rootworm. Agrarforschung 13: 80–82.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. Science 307: 1915–1920. https://doi.org/10.1126/science.1104816

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA (2012) SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. Journal of Computational Biology 19: 455–477. https://doi.org/10.1089/cmb.2012.0021

Barbercheck ME, Wang J, Hirsh IS (1995) Host plant effects on entomopathogenic nematodes. Journal of Invertebrate Pathology 66: 169–177. https://doi.org/10.1006/jipa.1995.1080

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annual Review of Microbiology 59: 155–189. https://doi.org/10.1146/annurev.micro.59.030804.121041

Berasategui A, Shukla S, Salem H, Kaltenpoth M (2016) Potential applications of insect symbionts in biotechnology. Applied Microbiology and Biotechnology 100: 1567–1577. https://doi.org/10.1007/s00253-015-7186-9

Bienert S, Waterhouse A, de Beer TAP, Tauriello G, Studer G, Bordoli L, Schwede T (2017) The SWISS-MODEL Repository - new features and functionality. Nucleic Acids Research 45: D313–D319. https://doi.org/10.1093/nar/gkw1132

Blacquière T, Smagghe G, van Gestel CAM, Mommaerts V (2012) Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology 21: 973–992. https://doi.org/10.1007/s10646-012-0863-x

Boetzer M, Pirovano W (2012) Toward almost closed genomes with GapFiller. Genome Biology 13: R56. https://doi.org/10.1186/gb-2012-13-6-r56

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bowen D, Blackburn M, Rocheleau T, Grutzmacher C, ffrench-Constant RH (2000) Secreted proteases from *Photorhabdus luminescens*: separation of the extracellular proteases from the insecticidal Tc toxin complexes. Insect Biochemistry and Molecular Biology 30: 69–74. https://doi.org/10.1016/S0965-1748(99)00098-3

Bowen D, Rocheleau TA, Blackburn M, Andreev O, Golubeva E, Bhartia R, ffrench-Constant RH (1998) Insecticidal toxins from the bacterium *Photorhabdus luminescens*. Science 280: 2129–2132. https://doi.org/10.1126/science.280.5372.2129

Braendle C, Miura T, Bickel R, Shingleton AW, Kambhampati S, Stern DL (2003) Developmental origin and evolution of bacteriocytes in the aphid–buchnera symbiosis. Benjamin Normark (Ed.). PLoS Biology 1: e21. https://doi.org/10.1371/journal.pbio.0000021

Branson T, Krysan J (1981) Feeding and oviposition behavior and life cycle strategies of *Diabrotica*: an evolutionary view with implications for pest management. Environmental Entomology 10: 826–831. https://doi.org/10.1093/ee/10.6.826

Castillo JC, Reynolds SE, Eleftherianos I (2011) Insect immune responses to nematode parasites. Trends in Parasitology 27: 537–547. https://doi.org/10.1016/j.pt.2011.09.001

Ciche T (2007) The biology and genome of *Heterorhabditis bacteriophora*. WormBook: The online review of *C. elegans* biology: 1–9. https://doi.org/10.1895/wormbook.1.135.1

Ciosi M, Miller NJ, Toepfer S, Estoup A, Guillemaud T (2011) Stratified dispersal and increasing genetic variation during the invasion of Central Europe by the western corn rootworm, *Diabrotica virgifera virgifera*: *D. v. virgifera* expansion in Central Europe. Evolutionary Applications 4: 54–70. https://doi.org/10.1111/j.1752-4571.2010.00133.x

Easom CA, Clarke DJ (2012) HdfR is a regulator in *Photorhabdus luminescens* that modulates metabolism and symbiosis with the nematode *Heterorhabditis*. Environmental Microbiology 14: 953–966. https://doi.org/10.1111/j.1462-2920.2011.02669.x

Ehlers R (2003) Biocontrol Nematodes. In: Hokkanen HMT, Hajek AE (Eds), Environmental Impacts of Microbial Insecticides: Need and Methods for Risk Assessment. Springer Netherlands, Dordrecht, 177–220. https://doi.org/10.1007/978-94-017-1441-9_10

Erb M, Robert CA (2016) Sequestration of plant secondary metabolites by insect herbivores: molecular mechanisms and ecological consequences. Current Opinion in Insect Science 14: 8–11. https://doi.org/10.1016/j.cois.2015.11.005

Gerritsen LJM, Smits PH (1993) Variation in pathogenicity of recombinations of *Heterorhabditis* and *Xenarhabdus luminescens* strains. Fundamental and Applied Nematology 16: 367–373.

Gray ME, Sappington TW, Miller NJ, Moeser J, Bohn MO (2009) Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. Annual Review of Entomology 54: 303–321. https://doi.org/10.1146/annurev.ento.54.110807.090434

Han R, Ehlers R-U (1999) Trans-specific nematicidal activity of *Photorhabdus luminescens*. Nematology 1: 687–693. https://doi.org/10.1163/156854199508711

Han R, Wouts W, Li L (1991) Development and virulence of *Heterorhabditis* spp. strains associated with different *Xenorhabdus luminescens* isolates. Journal of Invertebrate Pathology 58: 27–32. https://doi.org/10.1016/0022-2011(91)90158-M

Jackson JJ (1996) Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae). Journal of Economic Entomology 89: 366–372. https://doi.org/10.1093/jee/89.2.366

Jin DJ, Gross CA (1989) Characterization of the pleiotropic phenotypes of rifampin-resistant *rpoB* mutants of *Escherichia coli*. Journal of Bacteriology 171: 5229–5231. https://doi.org/10.1128/jb.171.9.5229-5231.1989

Joyce SA, Brachmann AO, Glazer I, Lango L, Schwär G, Clarke DJ, Bode HB (2008) Bacterial biosynthesis of a multipotent stilbene. Angewandte Chemie International Edition 47: 1942–1945. https://doi.org/10.1002/anie.200705148

Kaya H, Gaugler R (1993) Entomopathogenic nematodes. Annual Review of Entomology 38: 181–206. https://doi.org/10.1146/annurev.en.38.010193.001145

Kaya HK, Stock SP (1997) Techniques in insect nematology. In: Manual of techniques in insect pathology. Elsevier, 281–324. https://doi.org/10.1016/B978-012432555-5/50016-6.

Kenney E, Hawdon JM, O'Halloran D, Eleftherianos I (2019) *Heterorhabditis bacteriophora* excreted-secreted products enable infection by *Photorhabdus luminescens* through suppression of the Imd pathway. Frontiers in Immunology 10: 2372. https://doi.org/10.3389/fimmu.2019.02372

Kopp J (2006) The SWISS-MODEL Repository: new features and functionalities. Nucleic Acids Research 34: D315–D318. https://doi.org/10.1093/nar/gkj056

Koppenhöfer AM, Shapiro-Ilan DI, Hiltpold I (2020) Entomopathogenic nematodes in sustainable food production. Frontiers in Sustainable Food Systems 4: 125. https://doi.org/10.3389/fsufs.2020.00125

Koropatnick TA, Engle JT, Apicella MA, Stabb EV, Goldman WE, McFall-Ngai MJ (2004) Microbial factor-mediated development in a host-bacterial mutualism. Science 306: 1186–1188. https://doi.org/10.1126/science.1102218

Kriticos DJ, Reynaud P, Baker RHA, Eyre D (2012) Estimating the global area of potential establishment for the western corn rootworm (*Diabrotica virgifera virgifera*) under rain-fed and irrigated agriculture. EPPO Bulletin 42: 56–64. https://doi.org/10.1111/j.1365-2338.2012.02540.x

Kuhlmann U, van der Burgt A (1998) Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe. Biocontrol News and Information 19: 59N-68N.

Kunkel BA, Grewal PS, Quigley MF (2004) A mechanism of acquired resistance against an entomopathogenic nematode by *Agrotis ipsilon* feeding on perennial ryegrass harboring a fungal endophyte. Biological Control 29: 100–108. https://doi.org/10.1016/S1049-9644(03)00119-1

Kurtz B, Hiltpold I, Turlings T, Kuhlmann U, Toepfer S (2009) Comparative susceptibility of larval instars and pupae of the western corn rootworm to infection by three entomopathogenic nematodes. BioControl 54: 255–262. https://doi.org/10.1007/s10526-008-9156-y

Lai C, Xu J, Tozawa Y, Okamoto-Hosoya Y, Yao X, Ochi K (2002) Genetic and physiological characterization of *rpoB* mutations that activate antibiotic production in *Streptomyces lividans*. Microbiology 148: 3365–3373. https://doi.org/10.1099/00221287-148-11-3365

Lenski RE (1998) Bacterial evolution and the cost of antibiotic resistance. Int Microbiol 1: 265–270.

van Lenteren JC (2012) The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. BioControl 57: 1–20. https://doi.org/10.1007/s10526-011-9395-1

Levine E, Oloumi-Sadeghi H (1991) Management of diabroticite rootworms in corn. Annual Review of Entomology 36: 229–255. https://doi.org/10.1146/annurev.en.36.010191.001305

MacDonald TT, Monteleone G (2005) Immunity, Inflammation, and Allergy in the Gut. Science 307: 1920–1925. https://doi.org/10.1126/science.1106442

Machado RAR, Thönen L, Arce CCM, Theepan V, Prada F, Wüthrich D, Robert CAM, Vogiatzaki E, Shi Y-M, Schaeren OP, Notter M, Bruggmann R, Hapfelmeier S, Bode HB, Erb M (2020) Engineering bacterial symbionts of nematodes improves their biocontrol potential to counter the western corn rootworm. Nature Biotechnology 38: 600–608. https://doi.org/10.1038/s41587-020-0419-1

Nandy P, Chib S, Seshasayee A (2020) A mutant RNA polymerase activates the general stress response, enabling *Escherichia coli* adaptation to late prolonged stationary phase. Ellermeier CD (Ed.). mSphere 5: e00092-20. https://doi.org/10.1128/mSphere.00092-20

Ode PJ (2006) Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. Annual Review of Entomology 51: 163–185. https://doi.org/10.1146/annurev.ento.51.110104.151110

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proceedings of the National Academy of Sciences 100: 1803–1807. https://doi.org/10.1073/pnas.0335320100

Petersen SK, Hansen FG (1991) A missense mutation in the *rpoC* gene affects chromosomal replication control in *Escherichia coli*. Journal of Bacteriology 173: 5200–5206. https://doi.org/10.1128/jb.173.16.5200-5206.1991

Piel J (2002) A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. Proceedings of the National Academy of Sciences 99: 14002–14007. https://doi.org/10.1073/pnas.222481399

Qi Q, Preston GM, MacLean RC (2014) Linking system-wide impacts of RNA polymerase mutations to the fitness cost of rifampin resistance in *Pseudomonas aeruginosa*. Lenski R (Ed.). mBio 5: e01562-14. https://doi.org/10.1128/mBio.01562-14

Qiu X, Yan X, Liu M, Han R (2012) Genetic and proteomic characterization of *rpoB* mutations and their effect on nematicidal activity in *Photorhabdus luminescens* LN2. Uversky VN (Ed.). PLoS ONE 7: e43114. https://doi.org/10.1371/journal.pone.0043114

Robert CAM, Zhang X, Machado RAR, Schirmer S, Lori M, Mateo P, Erb M, Gershenzon J (2017) Sequestration and activation of plant toxins protect the western corn rootworm from enemies at multiple trophic levels. eLife 6: e29307. https://doi.org/10.7554/eLife.29307

Scarborough CL, Ferrari J, Godfray HCJ (2005) Aphid protected from pathogen by endosymbiont. Science 310: 1781–1781. https://doi.org/10.1126/science.1120180

Schnaitman CA, Klena JD (1993) Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiological Reviews 57: 655–682. https://doi.org/10.1128/mr.57.3.655-682.1993

van der Sluijs JP, Simon-Delso N, Goulson D, Maxim L, Bonmatin J-M, Belzunces LP (2013) Neonicotinoids, bee disorders and the sustainability of pollinator services. Current Opinion in Environmental Sustainability 5: 293–305. https://doi.org/10.1016/j.cosust.2013.05.007

Smith J (2019) SigmaPlot (Version 15). Systat Software Inc., San Jose CA.

Smith RF (1966) Distributional patterns of selected Western North American insects: the distribution of diabroticites in Western North America. Bulletin of the Entomological Society of America 12: 108–110. https://doi.org/10.1093/besa/12.2.108

Somvanshi VS, Kaufmann-Daszczuk B, Kim K, Mallon S, Ciche TA (2010) *Photorhabdus* phase variants express a novel fimbrial locus, *mad* , essential for symbiosis. Molecular Microbiology 77: 1021–1038. https://doi.org/10.1111/j.1365-2958.2010.07270.x

Strauch O, Ehlers R-U (1998) Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. Applied Microbiology and Biotechnology 50: 369–374. https://doi.org/10.1007/s002530051306

Sutter GR, Fisher JR, Elliott NC, Branson TF (1990) Effect of insecticide treatments on root lodging and yields of maize in controlled infestations of western corn rootworms (Coleoptera: Chrysomelidae). Journal of Economic Entomology 83: 2414–2420. https://doi.org/10.1093/jee/83.6.2414

Tobias NJ, Heinrich AK, Eresmann H, Wright PR, Neubacher N, Backofen R, Bode HB (2017) *Photorhabdus*-nematode symbiosis is dependent on hfq-mediated regulation of secondary metabolites. Environmental microbiology 19: 119–129. https://doi.org/10.1111/1462-2920.13502

Toepfer S, Gueldenzoph C, Ehlers R, Kuhlmann U (2005) Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. Bulletin of Entomological Research 95: 473–482. https://doi.org/10.1079/BER2005379

Toepfer S, Hatala-Zseller I, Ehlers R-U, Peters A, Kuhlmann U (2010) The effect of application techniques on field-scale efficacy: can the use of entomopathogenic nematodes reduce damage by western corn rootworm larvae? Agricultural and Forest Entomology 12: 389–402. https://doi.org/10.1111/j.1461-9563.2010.00487.x

Van Rozen K, Ester A (2010) Chemical control of *Diabrotica virgifera virgifera* LeConte. Journal of Applied Entomology 134: 376–384. https://doi.org/10.1111/j.1439-0418.2009.01504.x

Wang X, Minasov G, Shoichet BK (2002) Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. Journal of Molecular Biology 320: 85–95. https://doi.org/10.1016/S0022-2836(02)00400-X

Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TAP, Rempfer C, Bordoli L, Lepore R, Schwede T (2018) SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Research 46: W296–W303. https://doi.org/10.1093/nar/gky427

Wechsler S, Smith D (2018) Has resistance taken root in U.S. corn fields? Demand for insect control. American Journal of Agricultural Economics 100: 1136–1150. https://doi.org/10.1093/ajae/aay016

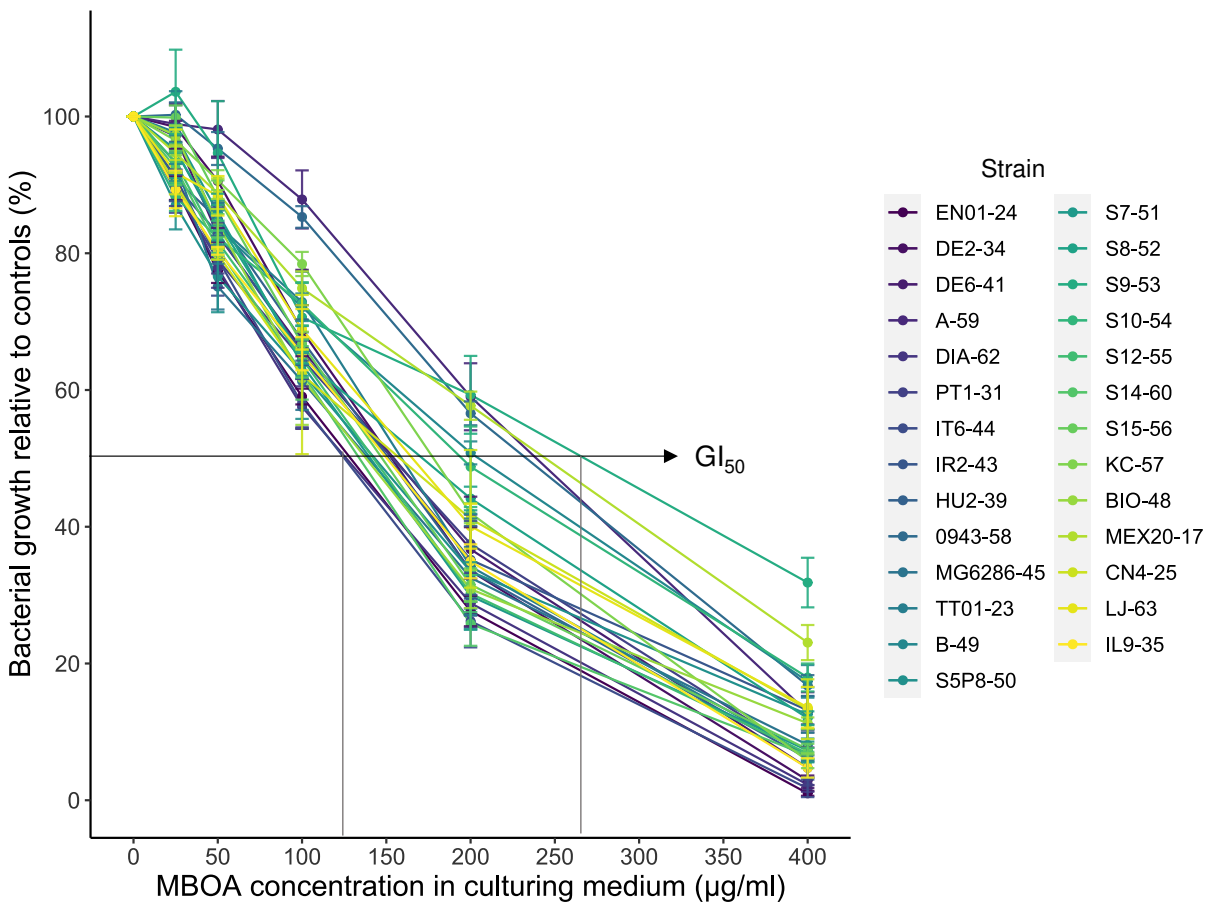
Wood TJ, Goulson D (2017) The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environmental Science and Pollution Research 24: 17285–17325. https://doi.org/10.1007/s11356-017-9240-x

Yang SH, Chen WH, Wang ET, Chen WF, Yan J, Han XZ, Tian CF, Sui XH, Singh RP, Jiang GM, Chen WX (2018) Rhizobial biogeography and inoculation application to soybean in four regions across China. Journal of Applied Microbiology 125: 853–866. https://doi.org/10.1111/jam.13897

Zhang J, Xu Y, Cao T, Chen J, Rosen BP, Zhao F-J (2017) Arsenic methylation by a genetically engineered *Rhizobium*-legume symbiont. Plant and Soil 416: 259–269. https://doi.org/10.1007/s11104-017-3207-z

Zhang X, van Doan C, Arce CCM, Hu L, Gruenig S, Parisod C, Hibbard BE, Hervé MR, Nielson C, Robert CAM, Machado RAR, Erb M (2019) Plant defense resistance in natural enemies of a specialist insect herbivore. Proceedings of the National Academy of Sciences 116: 23174–23181. https://doi.org/10.1073/pnas.1912599116

Supplementary Information Chapter I



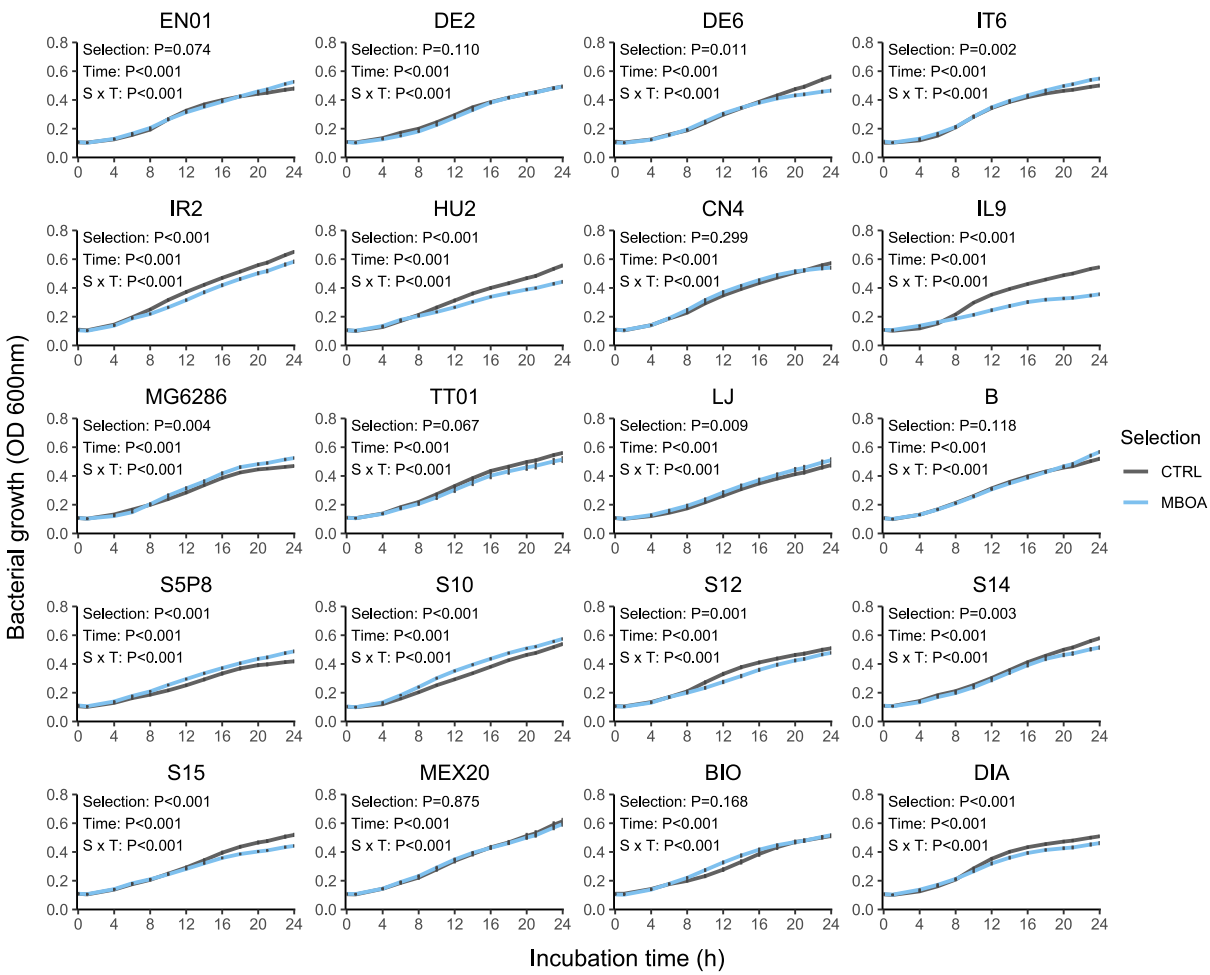
Supplementary Figure 1

MBOA reduces the growth of all the tested strains in a dose-dependent and linear manner. Mean (± s.e.m.) bacterial growth of twenty-seven *Photorhabdus* strains in Luria-Bertani liquid medium containing different concentrations of MBOA relative to controls. Data of dose-dependent reduction was used to calculate growth inhibition 50 values (half-maximal growth, GI_{50}). Bacterial growth was measured four to five independent times (n=4-5).

Strain	Selection	Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
EN01	M	*nhaB*	TCCGACAGGTATTGGAAGA	AAACCGTACCGACAAATACG
DE2	M	*nhaB*	TTCTTTTCTATCGTTGCCGT	ACACACAGCAAACCGAC
IT6	M	*envZ*	ATTGAAGAGTGTAACGCCAT	CCAGACCTAACCCTGTTCC
HU2	C	*rpoA*	GGTTGAGATTGATGGTGTACTG	ACGCGCTGCTTCAACATT
HU2	M	*fabR*	ACGTCAGTTAATGAGGCAAG	GTTCATTTCTTTGCGATACCA
CN4	M	*cpxA*	TATGGCCGCTCTGAAATG	AGAGGCGTGCGTAGTT
IL9	M	*rpoB*	ACACTGATGCCTCAGGATATG	CCTGTGCGATAACGAAGT
S10	C	*acrZ*	GTTTGAGTTGATTAAAAGTATGGC	CTTTTCAGAACGACCAATACG
S12	C	-	CACTGAATCGCAATACACC	ATACCTTGTGTCACGCTGT
S12	M	-	CACTGAATCGCAATACACC	ATACCTTGTGTCACGCTGT
S15	M	-	CACTGAATCGCAATACACC	ATACCTTGTGTCACGCTGT
BIO	C	*rpoC*	CCATTAGATGGTGGCGATG	CTTTGGTGATACCCAACAGGT
DIA	M	*rpoC*	GATGGCGAAACCGTTA	AACACGTGGCAAACCA

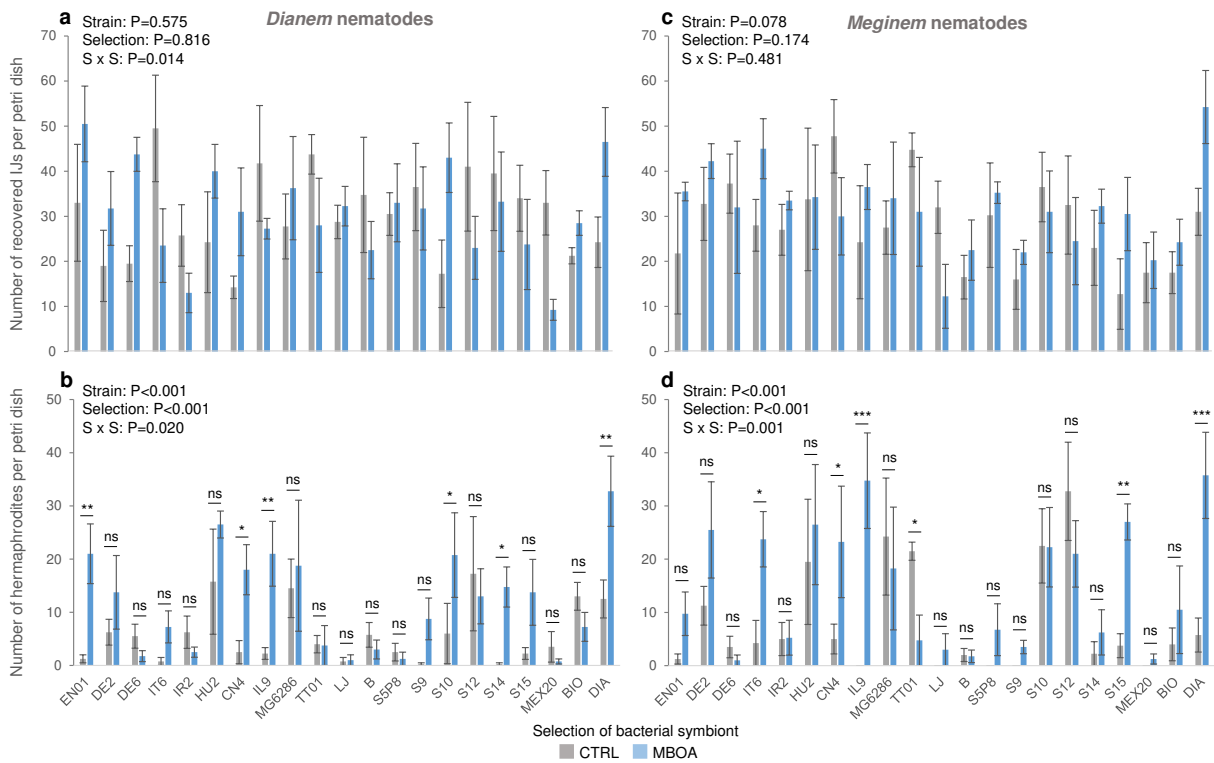
Supplementary Table 1

Primer sequences used to detect point mutations in the selected bacterial strains to confirm symbiont exchange of nematodes.



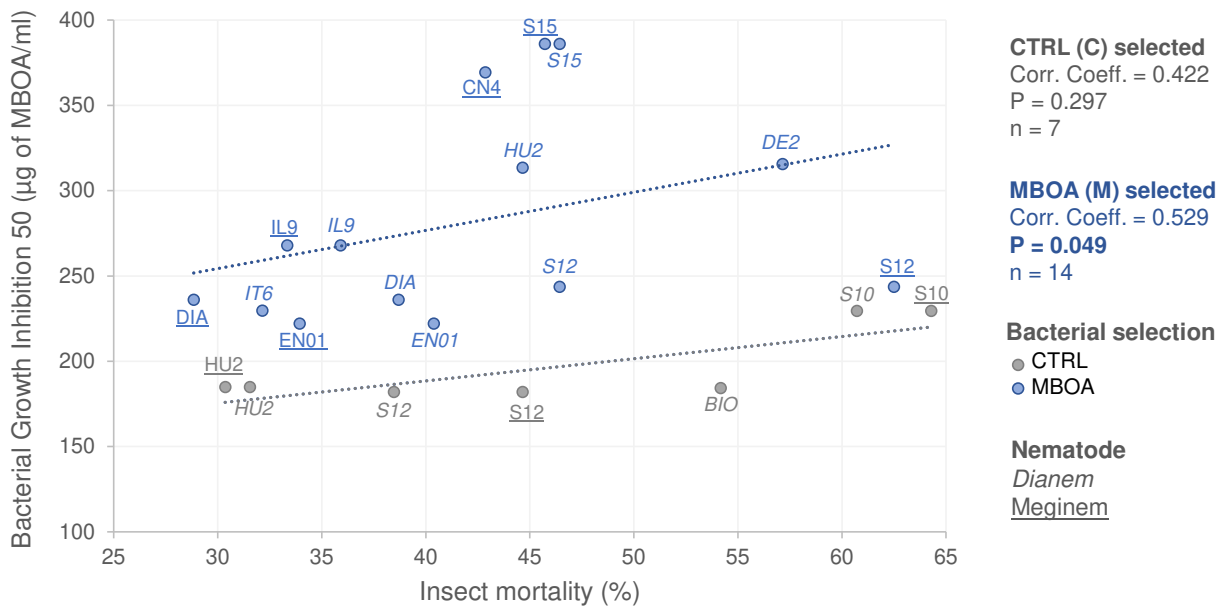
Supplementary Figure 2

Selection on MBOA impacts basal growth of a subset of bacterial strains. Mean (± s.e.m.) bacterial growth in LB for control- (CTRL, grey line) and MBOA-selected (MBOA, blue line) strains. Differences between selection regime and timepoints were determined using two-way repeated measurements ANOVA with Holm's multiple comparisons test. The experiment was repeated eight independent times (n=8).



Supplementary Figure 3

Bacterial identity and selection on MBOA alter symbiotic capability of a subset of bacterial strains. Mean (± s.e.m.) number of early-instar nematodes (a and c) and hermaphrodite females (b and d) developing from Dianem® and Meginem® infective juvenile nematodes incubated in bacteria previously selected on LB (C, grey bars) or MBOA (M, blue bars) in semi-solid bacterial cultures. Two commercial nematode strains were used: Dianem® TM (specifically developed to combat the western corn rootworm); and Meginem® Pro TM (generalist nematode strain developed to combat a broader spectrum of insect pests). Higher numbers of hermaphroditic females indicate stronger symbiotic compatibility between the nematodes and the bacteria. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). Nematode development was evaluated three independent times (n=3).



Supplementary Figure 4

Correlation of insect mortality and GI_{50} for control- and MBOA-selected bacterial symbionts. Grey data points represent control-(LB) selected bacterial symbionts (C, CTRL), blue data points represent MBOA-selected (M, MBOA) bacteria. For insect mortality: Nematode identity is labelled in italic for Dianem® nematodes and underlined for Meginem® nematodes. Results of Spearman Correlation tests are depicted on the right side. GI_{50} data consist out of four to six independent replicates (n=4-6), insect mortality data was repeated three independent times (n=3).

Chapter II

Field testing of improved symbiotic bacteria in *Heterorhabditis* strains against the invasive alien maize pest western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)

Unpublished work

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Abstract

Maize plants synthesize a variety of secondary metabolites which play important roles in a plant's life cycle, including plant-insect interactions. A particular secondary type of metabolites of maize are benzoxazinoids that act as allelochemicals and natural insecticides. However, the maize specialist *Diabrotica virgifera virgifera* is able to sequester benzoxazinoids and to use these plant toxins to defend themselves against entomopathogenic nematodes and their bacterial symbionts. This may limit their biocontrol potential. By engineering bacterial symbionts for increased resistance to the benzoxazinoid 6-methoxy-2-benzoxazolinone (MBOA), we designed new pairs of symbiont bacteria and nematodes which are able to kill benzoxazinoid-sequestering *D. v. virgifera* larvae efficiently under lab conditions. Tested in two field trials, these pairs similarly controlled the pest as did commercial nematode strains. This suggests that the selection processes did not lead to any major trade off, but also that other pathogenicity factors (rather than benzoxazinoid resistance) may be more relevant for successful biocontrol under field conditions. Our results suggest that modification of bacterial symbionts and targeting candidate genes to engineer better biocontrol agents provides a successful and time-efficient strategy to improve the pathogenicity of entomopathogenic nematodes against other agricultural pests.

KEY WORDS Biological control, field trial, entomopathogenic nematode, bacterial symbiont exchange, 6-methoxy-2-benzoxazolinone (MBOA) resistance, biological control agents

Introduction

The western corn rootworm *Diabrotica virgifera* ssp. *virgifera* LeConte (Coleoptera: Chrysomelidae) is a widespread and serious pest of maize, *Zea mays* (L.). Annual costs of damage due to yield loss and management practices are estimated to exceed $2 billion in the United States of America (Wechsler and Smith 2018). If no control measures were implemented, yield losses are estimated to be around 472 million euros in Europe (Wesseler and Fall 2010). *D. v. virgifera* is hypothesized to originate from Mexico, where several pestiferous *Diabrotica* species occur (Krysan and Smith 1987). With the expansion of maize cultivation in the twentieth century, it quickly spread throughout the maize growing regions of the United States and Canada (Krysan and Smith 1987, Gray et al. 2009). Since its first introduction into Serbia in 1992, it has spread across the European continent, resulting in well-established populations in approximately nineteen European countries (Kiss et al. 2005, Gray et al. 2009, Meinke et al. 2009, EC 2012) (http://extension.entm.purdue.edu/wcr/). It is very likely that in the future, *D. v. virgifera* will be present in all maize cultivars all over Eurasia (Kuhlmann and van der Burgt 1998, Aragón et al. 2010, Ciosi et al. 2011).

The majority of yield loss attributed to this univoltine pest species is caused by larvae feeding on the roots of maize, which may ultimately result in plant lodging (Krysan and Miller 1986). Adults emerge between mid-June and early August and can occasionally reduce yields through intensive silk feeding (Chiang 1973). To control this pest, the following strategies are common practice today: (1) crop rotation, (2) entomopathogenic nematodes, (3) transgenic maize and (4) synthetic insecticides (Levine and Oloumi-Sadeghi 1991, Van Rozen and Ester 2010). However, in some regions of Europe, management options are limited as the use of transgenic maize producing *Bt* (*Bacillus thuringiensis*) targeting rootworms is restricted and limitations have been placed on the use of insecticides. Several studies showed that the residuals of certain insecticides are found in water, soil, sowing dust, pollen and nectar and can have negative effects on non-targets including beneficial insects such as honeybees (Heimbach et al. 2008, Pistorious and Bischoff 2008). The use of chemical pesticides in maize cultivation can interfere with effective integrated pest management and biological control programmes that have been implemented for other European maize pests (Babendreier et al. 2006, Lamichhane 2020). Thus, more sustainable biological control options against *D. v. virgifera* are vitally important (Kuhlmann and van der Burgt 1998, Babendreier et al. 2006).

Among biological control approaches, the use of entomopathogenic nematodes (EPNs) has been quite successful in sustainable agriculture (Toepfer et al. 2010a). EPNs have a relatively wide host range including larvae of *D. v. virgifera* (Jackson 1996; Toepfer et al. 2005; Grewal, Ehlers, and Shapiro-Ilan 2005) and infective juveniles (IJs) actively search for a host. EPNs can be mass produced, both *in vivo* and *in vitro*. EPNs have the potential for application in integrated pest management programs and are safe to humans and the environment (Ehlers 2003). *Heterorhabditis bacteriophora* Poinar (Nematoda: Rhabditida) is one of the most common biological control agents against *D. v. virgifera* in Europe (Toepfer et al. 2005). IJs attack all three larval stages of *D. v. virgifera* as well as to some extent the pupal stage (Jackson 1996, Kurtz et al. 2009).

The life cycle of EPNs is characterized by an egg stage, four juvenile stages, and an adult stage. They live in close symbiosis with *Photorhabdus* bacteria and form a mutualistic relationship specialised in infecting soil-dwelling insects. The free-living larval stage of *Heterorhabditis* nematodes, the IJs, can live up to several weeks in the soil, seeking for a suitable host by sensing cues emitted by conspecific nematodes or by insects such as sex pheromones, respiratory CO_2 or by perceiving herbivore-induce volatiles produced by damaged plants (van Tol et al. 2001, Rasmann et al. 2005, Dillman et al. 2012, Rivera et al. 2017, Machado and Von Reuss 2022). IJs of the genus of *Heterorhabditis* harbour *Photorhabdus* bacteria in their intestinal lumens (Ciche 2007). After entering the insect's body, the nematodes release the bacteria into the insect hemocoel. *Photorhabdus* bacteria grow exponentially in the insect reaching cell densities of up to 10^9 cfu/insect within 48 hours. *Photorhabdus* secrets toxins and other molecules that damage host tissues and reduce the effectiveness of the normally highly potent insect innate immune system (Watson et al. 2005, Goodrich-Blair and Clarke 2007). The death of the insect usually coincides with the entry of the bacteria into the post-exponential growth phase and at this stage, all of the internal organs and tissues of the insect are converted into bacteria-associated biomass (Bowen et al. 2000). The nematodes then feed on bacteria-digested insect tissues and reproduce for several generations. Upon nutrient depletion, *Photorhabdus* bacteria re-associate with the nematode hosts and the new-born IJs emerge from the insect cadaver and seek for new hosts.

D. v. virgifera is very resistant to EPNs due to its capacity to exploit the defensive mechanisms of maize for its own benefit (Robert et al. 2017). On one hand, *D. v. virgifera* larvae release 6-methoxy-2-benzoxazolinone N-glucoside (MBOA-Glc), which acts repellent against nematodes.

On the other hand, *D. v. virgifera* larvae accumulate 2-hydroxy- 4,7-dimethoxy-1,4-benzoxazin-3-one O-glucoside (HDMBOA-Glc) and release a breakdown product, 6-methoxy-2-benzoxazolinone (MBOA), upon nematode attack. Both HDMBOA-Glc and MBOA reduce the survival of the nematode and its *Photorhabdus* bacterial symbionts (Robert et al. 2017, Zhang et al. 2019, Machado et al. 2020). *D. v. virgifera* larvae that accumulate benzoxazinoids can reduce the infectiveness of *H. bacteriophora* by more than 50 % compared to benzoxazinoid-deficient *D. v. virgifera* larvae (Robert et al. 2017). Herbivore natural enemies that are exposed to a plant defence sequestering herbivore can evolve both behavioural and metabolic resistance to plant defence metabolites, and these traits are associated with higher infectivity toward a defence sequestering herbivore. The exposure of a benzoxazinoid-susceptible nematode strain to *D. v. virgifera* for 5 generations results in higher behavioural and metabolic resistance and benzoxazinoid-dependent infectivity towards *D. v. virgifera* (Zhang et al. 2019). Similarly, selecting *Photorhabdus* symbionts of EPNs for MBOA resistance translate into better infection potential of its nematode host against *D. v. virgifera* that sequesters these toxins for self-protection (Machado et al. 2020). This work highlighted the potential of engineering desired traits into bacterial symbionts to enhance the performance of their host. To continue this work, we conducted these experiments in a same manner as Machado *et al.* (2020) but increased the number of *Photorhabdus* species and strains used. We proved again that MBOA resistance can be increased by experimental evolution in Photorhabdus symbionts with most of the strains exhibiting an increase of MBOA resistance of more than 35 % compared to their unselected ancestors. The majority of these selected strains were then successfully reintroduced into two commercially used strains of *Heterorhabditis* nematodes (Dianem® TM, Meginem® Pro TM) and 4 nematode-bacteria pairs have shown promising virulence against *D. v. virgifera* larvae with mortality of *D. v. virgifera* larvae ranging from 45 to 60 % under lab conditions (mortality of *D. v. virgifera* larvae after 5 days incubation of 25 IJs and 5 *D. v. virgifera* larvae). As a next and more realistic step, the four *H. bacteriophora* strains with selected and exchanged symbiotic *Photorhabdus* bacteria are tested for their control effects on *D. v. virgifera* larvae compared to two commercial *H. bacteriophora* nematode products (Dianem® TM, Meginem® Pro TM) and a standard soil insecticide under field conditions in Hungary. We assessed pest suppression (adult emergence counts), nematode persistence by baited soil samples and differences in plant parameters such as height, leaf numbers and root damage among the different control treatments. This first field trail with engineered bacterial

symbionts of nematodes will support the development of a biological control product for use against *D. v. virgifera* larvae in the future.

Materials and Methods

Biocontrol potential of selected bacteria- nematodes combinations under field conditions

To test whether EPN carrying benzoxazinoid-resistant bacterial symbionts can improve their efficacy in controlling *D. v. virgifera* in the field, we first generated MBOA-resistant *Photorhabdus* bacteria through experimental evolution (Refer to Chapter 1). MBOA resistance increased for most of the selected strains by more than 40 %. Both selected and unselected bacterial strains were then introduced into two commercially used *Heterorhabditis bacteriophora* stains (Meginem®-Pro, Andermatt Biocontrol Switzerland, and Dianem® e-nema GmbH, Germany) (Table 1). This procedure was carried out following the methods described in Chapter 1 (Section "Measure the performance of the selected bacteria in the context of nematode symbiosis", page 42). After testing the pathogenicity of twenty-one newly-generated nematode–symbiont pairs against *D. v. virgifera* larvae under laboratory conditions, we identified four pairs that were able to kill benzoxazinoid-sequestering *D. v. virgifera* larvae more efficiently. Then, we tested their efficacy under field conditions as described below.

Field sites

The efficacy of novel bacteria-EPN pairs against *D. v. virgifera* larvae was evaluated using artificially infested maize plants in a field experiment. The study was carried out on two fields in the CABI location in Hódmezővásárhely in southern Hungary (46°26'00.9"N 20°20'06.1"E). Fields had been ploughed, tilled and harrowed in early April prior to maize sowing. Maize seeds of the hybrid GK Szegedi 386 (middle late silage/grain maize, UFA Semences, Budapest, Hungary) were treated with a fungicide (XL TM 035 FS, Syngenta, Hungary) according to the manufacturer's protocol and sown on the 13th of April. Individual maize seeds were sown 190 mm apart from each other in rows separated by 750 mm. Both fields were treated once with 0.16 l herbicide (75 % Izoxaflutol, Merlin TM SC, Bayer Crop Science) per hectare at the 2–5 maize leaf stage. Mechanical weeding was carried out in June 2022. The mean, maximum and minimum average daily temperatures were 10.8, 17.5 and 4.6 °C in April 18.7, 25.7, 11.9 °C in May, 23.9, 31.6, 16.2 °C in June, and 25.1, 33, 17.8 °C in July 2022, respectively (Davis Instruments Corp., Hayward, CA, USA). Rainfall was 32 mm in April, 28 mm in May, 19 mm in June and 13 mm July, being a much drier June and July than average.

Fields had no topographic relieve drift and therefore considered homogenous. To determine soil moisture and soil bulk density, six soil cores per field were taken randomly at the beginning and in the middle of the experiment as described by Toepfer *et al.* (2010b). The soil analysis

revealed a 1.10 and 1.09 g/cm^3 soil bulk density and 19.9 and 19.3 % soil moisture (w% = grav. %) for field 1 and field 2 three weeks after sowing (6th of May). Soil moisture (w% = grav. %) decreased in the middle of the experiment (10 weeks after sowing, 22nd of June) with 11.3 % for field 1 and 11.4 % for field 2 with a soil bulk density of 0.95 and 1.16 g/cm^3, respectively.

Diabrotica v. virgifera infestation

To simulate well-established and homogenously distributed pest populations, we artificially infested plants with *D. v. virgifera* eggs. Artificial infestations can lead to similar larval development and adult emergence as natural populations (Fisher 1984). *D. v. virgifera* eggs were obtained from a laboratory culture established from field-collected beetles in 2021 in southern Hungary (for rearing procedures, refer to Singh and Moore 1985). *Diabrotica v. virgifera* eggs were overwintered at 6–8 °C in moist sand. Diapause was broken in mid-April 2022 by transferring the eggs to 20 °C. For egg recovery, the sand was sieved through a 250-µm mesh, and recovered eggs were mixed into a solution of 0.15 % aqueous agar. Maize plants of both fields were then infested with around 300 viable and ready-to-hatch eggs per plant when the plants were at the first to third leaf stage in the first week of May. The eggs were applied with a standard pipette (5ml, Eppendorf AG, Hamburg, Germany) in half-portions (in about 1 to 2 ml water–agar each) into two 100–140 mm-deep holes at a distance of 50–80 mm from both sides of the maize plants. In addition, a batch of the eggs was incubated at 25° C in the laboratory to monitor time of first hatch as well as hatching rate of the larvae. In the laboratory, *D. v. virgifera* larvae started to hatch around 1 week after egg application date and hatching lasted until mid of May. An average hatching rate of 88 ± 10.2 % (n=6) was determined, thus the eggs were of good quality. In the field, larvae are expected to emerge between the middle and end of May and second-instar larvae are expected early June (Toepfer and Kuhlmann 2006).

Treatments

Nine different treatments were applied in the first week of May including four novel nematode-symbiont pairs, 2 original commercial nematodes, a standard insecticide as positive control, and no treatment with and without *D. v. virgifera* as negative controls (Table 1). Per field, 4 sets of 8 plants (plots) were established for each of the nine treatments, including 4 buffer plants between the individual plots. An untreated buffer row of maize was established between each treatment row (see Fig. 1).

Table 1: Description of the different treatments evaluated in this study.

	Treatment code	Bacteria Strain	Bacteria Species	Selection of symbiont	Nematode strain	Nematode-symbiont pair	Dose	Plants / plot (set)	Plots	Fields
1	**Hb_Meg (commercial)**	A-59	*P. laumondii. laumondii*	no	Meginem	*H. bacteriophora* strain Meg with *Photorhabdus* A-59	25000 IJs	6 ** (8)	4	2
2	**Hb_Dia (commercial)**	DIA-62	*P. laumondii. laumondii*	no	Dianem	*H. bacteriophora* strain Dia with *Photorhabdus* DIA-62	25000 IJs	6 ** (8)	4	2
3	**Hb_C_Meg**	S10-54	*P. kleinii*	Control	Meginem	*H. bacteriophora* strain Meg with *Photorhabdus* S10-54	25000 IJs	6 ** (8)	4	2
4	**Hb_C_Dia**	S10-54	*P. kleinii*	Control	Dianem	*H. bacteriophora* strain Dia with *Photorhabdus* S10-54	25000 IJs	6 ** (6)	4	2
5	**Hb_M_Meg**	S12-55	*P. laumondii. laumondii*	MBOA	Meginem	*H. bacteriophora* strain Meg with *Photorhabdus* S12-55	25000 IJs	6 ** (8)	4	2
6	**Hb_M_Dia**	S12-55	*P. laumondii. laumondii*	MBOA	Dianem	*H. bacteriophora* strain Dia with *Photorhabdus* S12-55	25000 IJs	6 ** (8)	4	2
7	**C+**					**Untreated control** with *D. v. virgifera*	-	6 ** (8)	4	2
8	**C-**					**Untreated control** without *D. v. virgifera*	-	6 ** (8)	4	2
9	**Tefluthrin**					**Tefluthrin** (granule Force 1.5G, 15 g a.i./ kg) (Pyrethroid)	1.3 mg a.i.	6 ** (8)	4	2

** 10 plant planted, 8 treated and infested, 6 middle ones assessed, except for treatment Hb_C_Dia where only 6 plants were treated

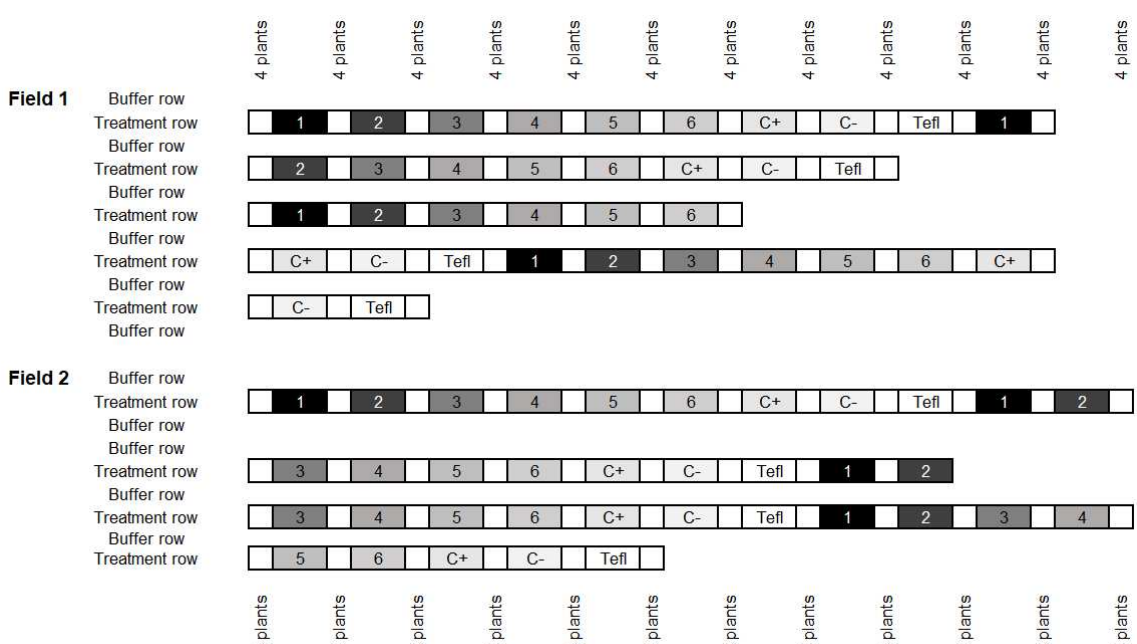

Figure 1: Experimental set-up of the two fields to assess the biocontrol potential of newly generated symbiont-nematode pairs against the larvae of *Diabrotica virgifera virgifera*. Per field, 4 sets of 8 plants (plots) were established per each of the nine treatments, including 4 buffer plants between the individual plots. An untreated buffer row of maize was established between the treatment rows. Numbers 1 to 6 represent different nematode treatments. For more treatment details, see Table 1.

Nematode-symbiont pairs

Two commercial *Heterorhabditis bacteriophora* products: Meginem® Pro (Andermatt Biocontrol AG, Switzerland) and Dianem® (e-nema GmbH, Germany), and four newly developed nematode-bacterial symbiont pairs were used (Table 1). Meginem® Pro consists of *H. bacteriophora* strain Meginem® carrying wild type *P. laumondii* subsp. *laumondii* A-59 symbionts. Dianem® consists of *H. bacteriophora* strain Dianem® carrying wild type *P. laumondii* subsp. *laumondii* DIA-62 symbionts. The four newly developed nematode strains consisted of: (1) *H. bacteriophora* strain Meginem® carrying control-selected *P. kleinii* S10-54 symbionts, (2) *H. bacteriophora* strain Dianem® carrying control-selected *P. kleinii* S10-54, (3) *H. bacteriophora* strain Meginem® carrying MBOA-selected *P. laumondii* subsp. *laumondii* S12-55 and (4) *H. bacteriophora* strain Dianem® carrying MBOA-selected *P. laumondii* subsp. *laumondii* S12-55 (see Chapter 1 for more details).

Nematodes were reared in last instar *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae according to Kaya and Stock (1997). All nematodes were stored in sterile tap water in the dark at a constant temperature of 10 °C until used for experiments. About 2–3 h before application, liquid cultures of EPNs were diluted with sterile tap water to the required concentration. Using a pipette, 2.5 ml tap water containing 12,500 IJs were injected twice 100 mm deep into the soil at distances of 150 mm from the plant, totalling 25000 juveniles per plant (2 billion IJ/ha). These injections simulated the commonly practised application of nematodes as a fluid into soil during sowing of maize (Toepfer et al. 2008, 2010a).

To evaluate the quality of each of the nematodes prior and after field application, living and dead IJs from the application bottles were counted under the stereomicroscope to assess if conditions were suitable for the nematodes during application of the six strains in the field (Kaya and Stock 1997). The percentage of alive IJs was for all strains higher than 96 %, with no significant decrease of living nematodes caused during the application process in the field (paired t-test, $p = 0.06$).

After application to the field, subsamples of all nematodes per treatment and field were tested with laboratory quality control bioassays with larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) to determine the quality of nematodes applied into the soil (Toepfer et al. 2008). Mortality and colouration of *Tenebrio* larvae were assessed after one and two weeks of incubation at 22 °C in the dark. Mortality of 60–73 % (nematodes applied in field 1) and 40-73 % (nematodes applied in field 2) of *T. molitor* was found for all nematode treatments. The

quality of applied nematodes in field 1 was considered of sufficient level of virulence for subsequent applications and analyses according to the nematode producer e-nema, which requires at least 50 % mortality. For the field 2, quality of two nematode strains was lower (M-S12 Meginem® 40 %; C-S10 Dianem® 45 %). As we worked with two separate batches of nematodes for the two fields, the reduction of quality could be explained by the transport from Switzerland to Hungary and the handling of nematodes applied in field 2.

Nematode persistence in the soil was assessed through the in-field baited-soil sample method at the middle and the end of the field experiment, e. g. after 6 and 12 weeks after the EPN treatment. 50ml Falcon tubes (with a sawed-off base that was replaced with gauze) were filled with 35 ml of soil (experimental soil: garden soil: sand; 2:2.1), one germinated maize seedling and 10 or 6 *Tenebrio* larvae (for nematode persistence 6 and 12 weeks after treatment, respectively). Lids of the Falcon tubes were pierced to ensure sufficient air flow. Per plot, 3 tubes were used, resulting in 12 tubes per field. The tubes were placed into the soil inside of the soil cores. All tubes were recollected 7 days after placing the tubes in the fields and dead and infected *Tenebrio* larvae were recorded. All tubes were kept at 20 °C and one week later, the counting procedure was repeated. Establishment was defined by the percentage of samples that were positively tested for EPN infection. In order to standardize the rate of positively tested soil samples among fields, proportions of EPN infections in the treated plots compared with the control plots were calculated for each field.

Standard insecticide

As a positive control, the standard soil insecticide tefluthrin (Force™ 1.5 G, Syngenta, Budapest, Hungary, Formulation: Fine granule, FG of GIFAP code) was used (Table 1). This is the pyrethroid with the active substance 2,3,5,6-Tetrafluoro-4-methylbenzyl(Z)-(1RS,3RS)-3-(2-chloro-3,3,3-trifluoro-1-propenyl-2,2 dimethylcyclopropane-carboxylate. Granules were pre-weighted into 1.5 ml reaction tubes (0.65 mg per tube). Two tubes of insecticide were then applied 100 mm deep into the soil at distances of 80 mm from the plant, totalling of 1.3 mg Force™ per plant, which is the equivalent of 13.3 kg per ha.

Untreated control

Two types of negative controls were used: (1) untreated *D. v. virgifera* egg-infested plots (C+) to estimate damage and (2) untreated uninfested plots to estimate the growth potential of maize plants without damage (C-).

Assessing the biocontrol effect of nematodes

To evaluate the control potential of the nematode strains, three plant parameters were assessed throughout the season, these are root damage, plant height and number of leaves. In the beginning of June 2022 (nine weeks after maize sowing and 6 weeks after *Diabrotica* and nematode treatments), plant height and leaf number (fully developed leaves) per maize plant were determined. Height was determined by measuring the plant height form the ground to the tip of the youngest, fully developed leaf.

The root systems of maize plants (in ca. 250·250 mm and 200 mm depth) were dug out in the beginning of August 2022 from the experimental plots to measure root damage. Soil adhering to the roots was carefully removed by gently shaking the plants and any remaining soil was then removed by using a high-pressure water spray. Root damage was rated using two scales: (i) the 1.0–6.0 Iowa scale (Hills and Peters 1971), which has traditionally been the most commonly used scale even though it may overestimate the importance of small levels of damage, such as feeding scars; and (ii) the relatively new 0.00–3.00 node injury scale (Oleson et al. 2005) which is, compared with the 1.0–6.0 Iowa scale, closer to a linear and decimal scale measuring only the roots or nodes that are completely destroyed. The latter scale assesses the heavy damage to the roots whilst the first assesses more the general damage including minor damage. The economic threshold level in conventional grain maize is reached at a rating of 3 on the Iowa scale (Journey and Ostlie 2000) and 0.25 on the node injury scale (Oleson et al. 2005). To avoid subjective bias, the expert blindly conducted the rating without knowing whether roots were from treated or untreated blocks.

Data analysis

The data for all experiments were analysed with linear models (LM) or linear mixed-effects models (LMER) using R statistical software (R version 4.3.1, The R Foundation for Statistical Computing, Austria), package lme4 (Bates et al. 2015). For all categorical factors, contrasts were set to orthogonal.

Root damage data were analysed by LMER including the fixed factors control treatment (T) and field (F). “Block” was included as a random factor in all models. Plant height and number of leaves data were standardized for both fields by calculating % differences to the untreated and *D. v. virgifera* infected plants (C+). Subsequently, data was analysed with linear models (LM) including the fixed factor control treatment (T). For plant height and number of leaves, only

data of field 1 were analysed because plants grew overall very badly on field 2. Plant height and number of leaves of field 2 are presented in Supplementary Figure 1.

Effects of factors and interactions for all models were determined from ANOVA tables with Type III sum of squares (car package, Fox and Weisberg 2019) with α = 0.05. Significant differences between individual prey treatments were determined with Tukey's tests (emmeans package, Lenth 2023), except for the persistence data where treatment differences were determined with Games Howell post hoc multiple comparison tests.

Results

EPNs reduce root damage inflicted by *D. v. virgifera* larvae

All tested EPN-bacteria pairs significantly reduced heavy root damage (measured by the node injury scale) caused by *D. v. virgifera* between 42.2 and 61.7 % compared to the untreated (C+) control, where only larvae but no control management were applied ($p < 0.001$; Fig. 2A). Benzoxazinoid resistance of the bacteria did not improve the efficacy of EPNs against heavy root damage, as no significant differences were observed among the different applied bacteria-nematode pairs (pooled field data, $p > 0.4$). In some cases, however, the bacteria-nematodes pairs were as effective as the insecticide tefluthrin. This was the case for the treatments using *H. bacteriophora* Dianem® ($p > 0.14$) but not *H. bacteriophora* Meginem® ($p < 0.004$).

All tested EPN species significantly reduced general root damage (measured by the Iowa scale) caused by *D. v. virgifera* between 11.2 and 18.3 % compared to the untreated (C+) control, ($p < 0.024$; Fig. 2B). Benzoxazinoid resistance of the bacteria did not improve the efficacy of EPN against general root damage, as no significant differences were observed among the different applied bacteria-nematode pairs (pooled field data, $p > 0.4$). Nematode treatments could not reduce general root damage to the same level as insecticide application did ($p < 0.002$).

EPNs reduce the negative impact of *D. v. virgifera* larvae on plant growth

Uninfested plants (C-) were on average 74.8 ± 11.8 % taller than *D. v. virgifera* - infested plants (C+). The different control strategies in general significantly reduce the observed negative impact of *D. v. virgifera* on plant growth (Fig. 3A). The only significant difference in the increase of plant height within the nematode treatments was observed between plants treated with the nematode treatment Dianem® DIA-62 and the nematode treatment Dianem® M-S12, with plants being treated with the latter growing shorter ($p = 0.04$).

Uninfested plants (C-) had on average 35.7 ± 4.7 % more leaves compared to *D. v. virgifera* infested plants (C+) (Fig 3B). No significant differences in the number of leaves were observed when plants were treated with the different bacteria-nematode pairs ($p > 0.34$). Nematode application did reduce the observed negative impact of *D. v. virgifera* on number of leaves to the same level as insecticide application did (Fig 3B).

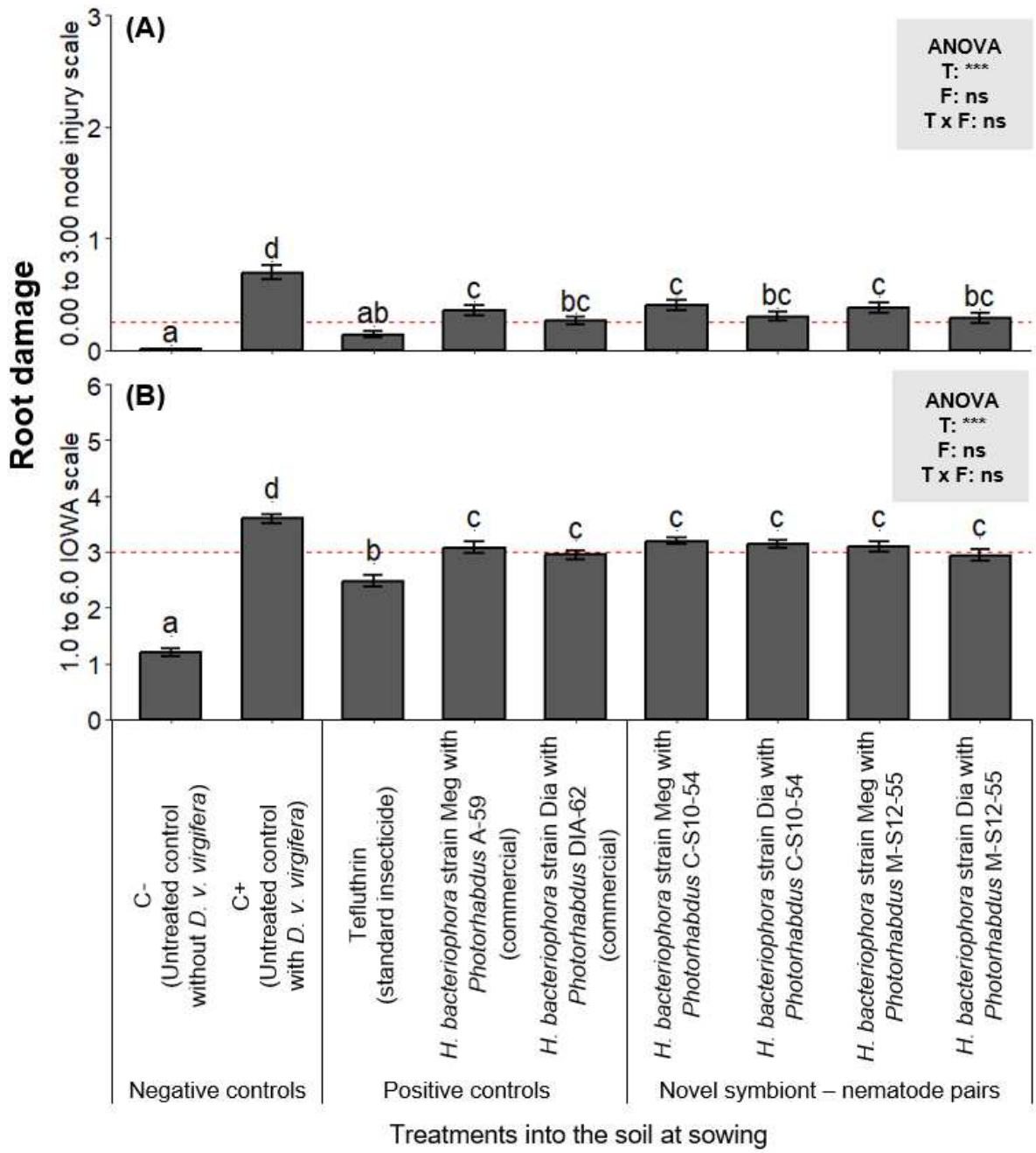

Figure 2: Root damage caused by *Diabrotica v. virgifera* larvae in artificially infested maize fields. Root damage was assessed using the 0.00 to 3.00 Oleson's node-injury scale (A) and the 1.0 to 6.0 modified Iowa scale (B). Dashed red lines indicate economic threshold levels in conventional grain maize (3 on the Iowa scale and 0.25 on the node injury scale). Treatments consists of two commercial *Heterorhabditis bacteriophora* products: Meginem® Pro carrying A-59 bacteria and Dianem® with carrying Dia-62 bacteria, four newly developed nematode-bacterial symbiont pairs where the bacteria were either resistant (M) or susceptible (C) to benzoxazinoids, intact plants (C-), plants infested with *D. v. virgifera* without nematode treatments (C+) and plants infested with *D. v. virgifera* and treated with an insecticide (Tefluthrin) served as controls. Error bars = SEM. Results of ANOVA with fixed factors control treatment (T) and field (F) are presented in grey boxes. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Different letters on bars indicate the significant difference between treatments as per post hoc Tukey HSD test at $p < 0.05$ following ANOVA.

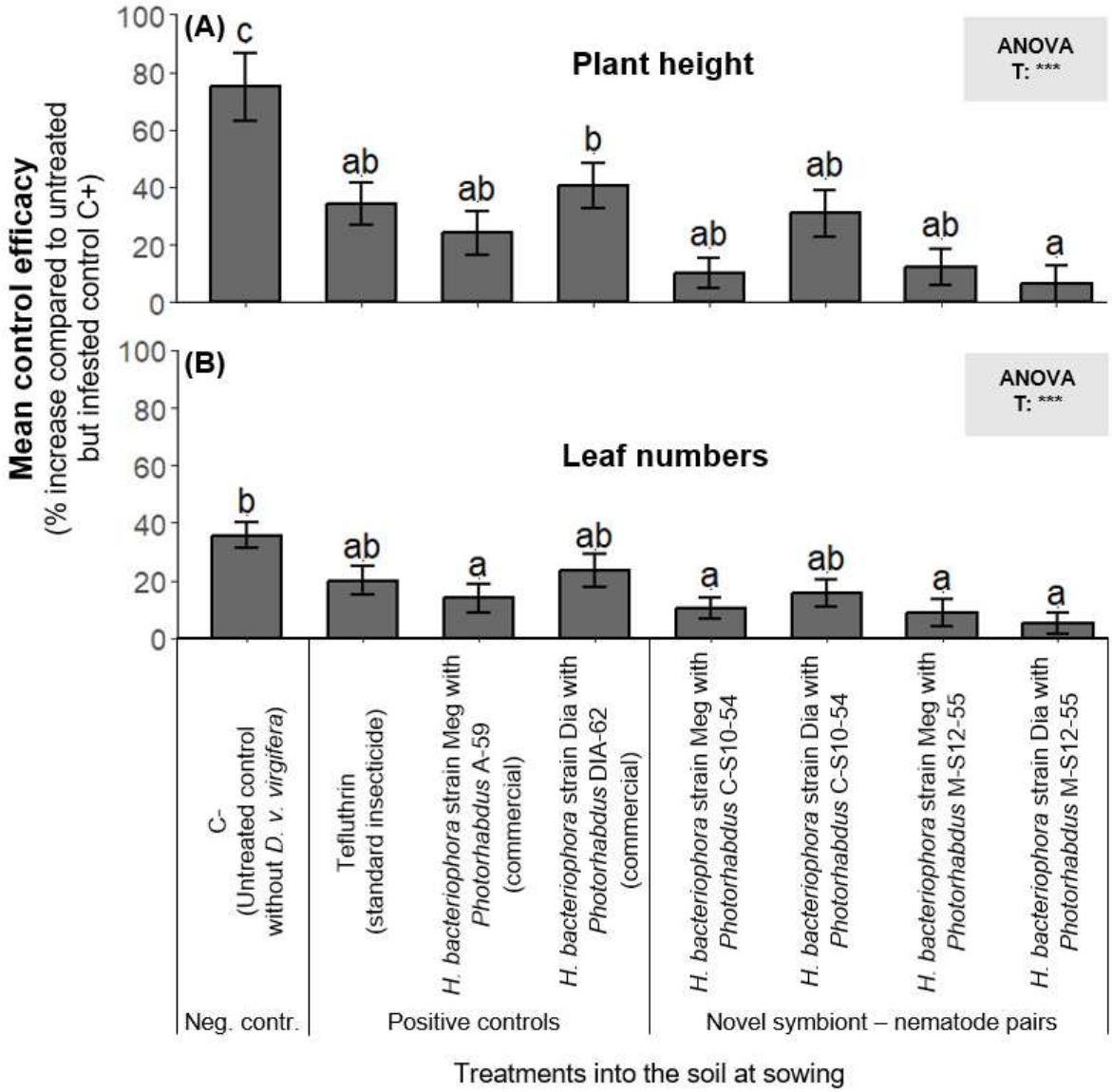

Figure 3: Efficacy of different treatments in reducing effects of *Diabrotica v. virgifera* larvae on plant height (A) and leaf numbers (B) compared to the untreated but infested control. Treatments consists of two commercial *Heterorhabditis bacteriophora* products: Meginem® Pro carrying A-59 bacteria and Dianem® with carrying DIA-62 bacteria, four newly developed nematode-bacterial symbiont pairs where the bacteria were either resistant (M) or susceptible (C) to benzoxazinoids, intact plants (C-) and plants infested with *D. v. virgifera* and treated with an insecticide (Tefluthrin). Only data of field 1 is presented. Error bars = SEM. Results of ANOVA with fixed factor control treatment (T) is presented in grey boxes. Asterisks indicate significant differences (*p < 0.05, **p < 0.01, ***p < 0.001). Different letters on bars indicate the significant difference between treatments as per post hoc Tukey HSD test at p < 0.05 following ANOVA.

EPNs successfully established in the soil after field application

All novel as well as commercial bacteria-nematode pairs applied into the soil established (Fig. 4). All pairs persisted in the soil for at least 12 weeks, suggesting they likely propagated in insect larvae. There were no major differences in the persistence of the different bacteria-nematodes pairs (6 weeks post treatment: GLM, $F_{5;143} = 1.97$, $p = 0.087$, $R^2 = 0.03$; 12 weeks post treatment: $F_{5;143} = 1.15$, $p = 0.335$, $R^2 = 0.005$).

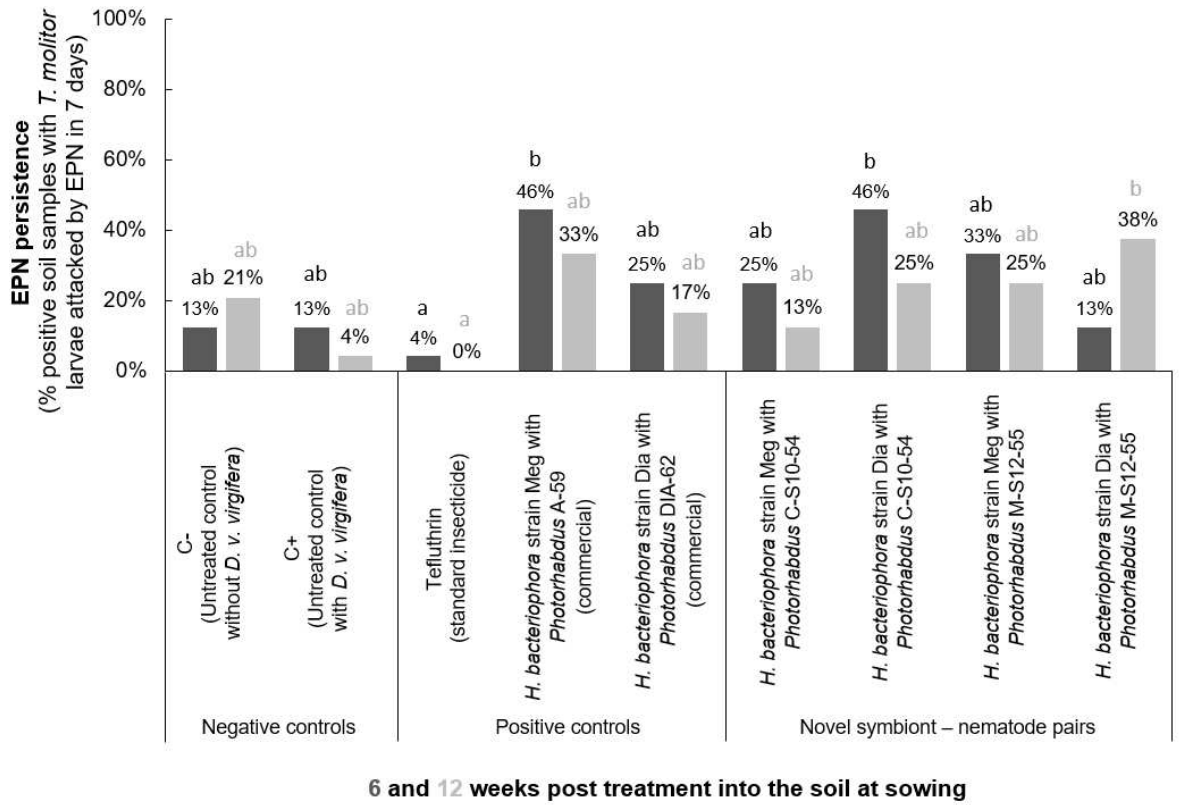

Figure 4: Persistence of different pairs of entomopathogenic nematode strains of *Heterorhabditis bacteriophora* and their symbiont bacteria applied into-soil treatments against the larvae *Diabrotica v. virgifera* in artificially infested maize fields measured as % positive soil samples with *T. molitor* larvae attacked by EPN in 7 days. Persistence assessed through in-field baiting of nematodes with *Tenebrio molitor* larvae at 6- and 12-weeks post treatment. Treatments consists of two commercial *Heterorhabditis bacteriophora* products: Meginem® Pro carrying A-59 bacteria and Dianem® with carrying Dia-62 bacteria, four newly developed nematode-bacterial symbiont pairs where the bacteria were either resistant (M) or susceptible (C) to benzoxazinoids, intact plants (C-), plants infested with *D. v. virgifera* without nematode treatments (C+) and plants infested with *D. v. virgifera* and treated with an insecticide (Tefluthrin) served as controls. Different letters above bars indicate significant differences according to Games Howell post hoc multiple comparison tests.

Discussion

The specialised insect herbivore *D. v. virgifera* is among the most damaging maize pests and can cause major yield losses. It is currently invading Europe and it is likely that *D. v. virgifera* will be present in all maize growing regions of Eurasia in the future (Kuhlmann and van der Burgt 1998, Aragón et al. 2010, Ciosi et al. 2011). This challenges the aim to reduce the use of synthetic pesticides in sustainable agriculture. The use of herbivore natural enemies as biological control agents is a promising strategy in this context (van Lenteren 2012). EPNs are a promising strategy to combat root-feeding pests such as the larvae of *D. v. virgifera* without the need for synthetic pesticides. However, *D. v. virgifera* is able to sequester benzoxazinoids and use these plant toxins to defend themselves against entomopathogenic nematodes and their bacterial symbionts, limiting their biocontrol potential (Robert et al. 2017). By improving the efficacy of nematode biocontrol, we hope to provide an economic and sustainable approach for the future to control this pest species. Here, we report the first approach of testing engineered bacterial symbionts of nematodes (for more detail see Chapter 1) that are resistant to the chemical defences of the target herbivore under field conditions.

In the light of the previously published results, it is known that some species of the genus *Heterorhabditis* are symbiotically associated with various species and subspecies of the genus *Photorhabdus* and also exchanging of symbionts is possible (Kazimierczak et al. 2017, Machado et al. 2020). Therefore, it seemed likely, that exchanging original bacterial symbionts with better performing bacteria in terms of *D. v. virgifera* infectivity might be beneficial for EPNs. Our results revealed that engineering and exchanging bacterial symbionts of EPNs do not negatively impact their performance under field conditions. However, no clear improvements of the novel bacteria – EPN pairs were found compared with readily available commercial EPNs. As often the case with biological control agents their efficacy is sometimes variable, and a better understanding of the factors that determine their success is thus important to improve their use. Efficacy of EPN application against a targeted pest species depends among other biotic and abiotic factors on the ability of the biocontrol agent to persist in the pest's environment, at least until emergence of the first larvae. Persistence of EPNs in the soil can vary depending on the type of habitat and the EPN species or strain (Susurluk 2005). It is as well largely depending on environmental factors, including, to some extent, soil parameters (Toepfer et al. 2009a, 2009b, 2010b). Soil is a complex of different physical, chemical and

biological components (Poinar 1990, Hominick et al. 1996). Temperature and humidity in the soil are among the most important factors sometimes limiting the success of EPNs, directly influencing their host searching activity, pathogenicity and survival (Kaya 1990, Radová and Trnková 2010). Thus, moisture may affect persistence of EPNs in the soil (Klein 1990). It must be considered that the high temperatures and lack of significant precipitation in our field trials during summer 2022 was probably not favourable for the survival and persistence of the EPNs in the soil because of low soil moisture. Such optimal sub-conditions cannot be expected every year, but it is possible that in the future we have to deal with more extreme weather conditions (Lubchenco and Karl 2012). Repetition of the experiment throughout several seasons would be needed to make more precise predictions about persistence of our strains in the field.

Preventing root damage in *D. v. virgifera* infested maize field is one of the key requirements for a successful pest control strategy that is actively used by farmers. The economic threshold level in conventional grain maize is suggested to be reached at a rating of 3.0 on the Iowa scale (Journey and Ostlie 2000) and 0.25 on the node injury scale (Oleson et al. 2005). These values were developed in the USA and are currently also used in some European countries. In our experiment, the resulting general root damage measured by the 1.0 to 3.0 Iowa scale in all the tested nematode strain treatments was pushed down to the threshold value of 3, suggesting an economic valuable control potential of all our developed bacteria-nematode pairs. For heavy root damage, only the Dianem® nematodes with and without exchanged bacterial symbionts were able to reduce root damage to an economically acceptable threshold around 0.25 on the node injury scale. All Dianem® nematodes were as efficient in preventing heavy root damage as the used soil insecticide. Similar results were also observed in a field study in 2016 in Slovenia, where *H. bacteriophora* nematodes (Dianem® strain) were used to control the western corn rootworm by decreasing emerging beetle numbers (Modic et al. 2018). The highest number of *D. v. virgifera* beetles was caught in the untreated negative control, followed by the granular soil insecticides thiacloprid and tefluthrin, and then by the nematode strain Dianem®. The evaluation of emerging beetles showed a significant reduction of 56 % by Dianem® (2.33 emerging beetles per plant or 28.0 per m^2) against the untreated control (4.5 emerging beetles per plant or 54 per m^2) (Modic et al. 2018). In fact, several studies could highlight the potential of entomopathogenic nematodes of the strain *H. bacteriophora* Dianem® to control the larvae of the *D. v. virgifera* in maize fields (Kurtz et al. 2007, Pilz et al.

2014, Kahrer et al. 2015, Toepfer et al. 2009b). Our results underline that this nematode strain represents a successful candidate in controlling *D. v. virgifera* larvae, even with exchanged symbionts.

Reducing the use of synthetic pesticides is an important aim in sustainable agriculture. The use of herbivore natural enemies as biological control agents is a promising strategy in this context (van Lenteren 2012). However, the efficacy of biocontrol agents is often limited, and a better understanding of the factors that determine their success is thus important to improve their use (Lommen et al. 2017). Given that engineering and exchanging bacterial symbionts of EPNs do not negatively impact their performance, we could show as one of the first studies the possible applications and further opportunities working with exchanged *Photorhabdus* symbionts in *Heterorhabditis* nematodes. Although we could not directly highlight an improvement of our newly generated symbiont-nematode pairs in controlling *D. v. virgifera* larvae in the field compared to already commercially available nematode products, we are confident that our experimental approach can be implemented as a time-efficient and successful strategy to both improve the lethality of EPNs against other agricultural pests and to improve other biocontrol-relevant traits.

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Competing interests

The authors declare no competing interests.

References of Chapter II

Aragón P, Baselga A, Lobo JM (2010) Global estimation of invasion risk zones for the western corn rootworm *Diabrotica virgifera virgifera*: Integrating distribution models and physiological thresholds to assess climatic favourability. Journal of Applied Ecology 47: 1026–1035. https://doi.org/10.1111/j.1365-2664.2010.01847.x

Babendreier D, Keller S, Romeis J, Bigler F, Kuhlmann U, Toepfer S, Turlings T, Schaub L, others (2006) New approaches to combating corn rootworm. Agrarforschung 13: 80–82.

Bates D, Mächler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models Using **lme4**. Journal of Statistical Software 67. https://doi.org/10.18637/jss.v067.i01

Bowen D, Blackburn M, Rocheleau T, Grutzmacher C, ffrench-Constant RH (2000) Secreted proteases from *Photorhabdus luminescens*: separation of the extracellular proteases from the insecticidal Tc toxin complexes. Insect Biochemistry and Molecular Biology 30: 69–74. https://doi.org/10.1016/S0965-1748(99)00098-3

Chiang H (1973) Bionomics of the northern and western corn rootworms. Annual Review of Entomology 18: 47–72. https://doi.org/10.1146/annurev.en.18.010173.000403

Ciche T (2007) The biology and genome of *Heterorhabditis bacteriophora*. WormBook: The online review of *C. elegans* biology: 1–9. https://doi.org/10.1895/wormbook.1.135.1

Ciosi M, Miller NJ, Toepfer S, Estoup A, Guillemaud T (2011) Stratified dispersal and increasing genetic variation during the invasion of Central Europe by the western corn rootworm, *Diabrotica virgifera virgifera*: *D. v. virgifera* expansion in Central Europe. Evolutionary Applications 4: 54–70. https://doi.org/10.1111/j.1752-4571.2010.00133.x

Dillman AR, Guillermin ML, Lee JH, Kim B, Sternberg PW, Hallem EA (2012) Olfaction shapes host–parasite interactions in parasitic nematodes. Proceedings of the National Academy of Sciences 109. https://doi.org/10.1073/pnas.1211436109

EC (2012) Survey results for the presence of *Diabrotica virgifera* Le Conte in the European Union in 2011. Report from the Health and Consumers Directorate-General of the European Commission.

Ehlers R (2003) Biocontrol Nematodes. In: Hokkanen HMT, Hajek AE (Eds), Environmental Impacts of Microbial Insecticides: Need and Methods for Risk Assessment. Springer Netherlands, Dordrecht, 177–220. https://doi.org/10.1007/978-94-017-1441-9_10

Fisher J (1984) Comparison of emergence of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) from cut and uncut corn plants in artificial and natural infestations. Journal of the Kansas Entomological Society 57: 405–408. https://www.jstor.org/stable/25084537.

Fox J, Weisberg S (2019) An R companion to applied regression. Third. Sage, Thousand Oaks CA. https://socialsciences.mcmaster.ca/jfox/Books/Companion/.

Goodrich-Blair H, Clarke DJ (2007) Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination: Bacteria-host interactions. Molecular Microbiology 64: 260–268. https://doi.org/10.1111/j.1365-2958.2007.05671.x

Gray ME, Sappington TW, Miller NJ, Moeser J, Bohn MO (2009) Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. Annual Review of Entomology 54: 303–321. https://doi.org/10.1146/annurev.ento.54.110807.090434

Grewal PS, Ehlers R-U, Shapiro-Ilan DI (Eds) (2005) Nematodes as biocontrol agents. CABI Pub, Wallingford, UK; Cambridge, MA, 505 pp.

Heimbach U, Rautmann D, Ganzelmeier H (2008) Influence of sowing technique of maize on drift of dust. IOBC IWGO—NEWSLETTER 29.

Hills TM, Peters DC (1971) A method of evaluating postplanting insecticide treatments for control of western corn rootworm larvae. Journal of Economic Entomology 64: 764–765. https://doi.org/10.1093/jee/64.3.764

Hominick WM, Reid AP, Bohan DA, Briscoe BR (1996) Entomopathogenic nematodes: biodiversity, geographical distribution and the convention on biological diversity. Biocontrol Science and Technology 6: 317–332. https://doi.org/10.1080/09583159631307

Jackson JJ (1996) Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae). Journal of Economic Entomology 89: 366–372. https://doi.org/10.1093/jee/89.2.366

Journey AM, Ostlie KR (2000) Biological control of the western corn rootworm (Coleoptera: Chrysomelidae) using the entomopathogenic nematode, *Steinernema carpocapsae*. Environmental Entomology 29: 822–831. https://doi.org/10.1603/0046-225X-29.4.822

Kahrer A, Pilz C, Heimbach U, Wechselberger K, Grabenweger G (2016) Five years of experience in biocontrol of the western corn rootworm, *Diabrotica virgifera virgifera* by entomoparasitic nematodes. IOBC/WPRS Bulletin 2016 113: 81–85.

Kaya H (1990) Soil ecology. In: Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, FL, 281–324. https://doi.org/10.1201/9781351071741.

Kaya HK, Stock SP (1997) Techniques in insect nematology. In: Manual of techniques in insect pathology. Elsevier, 281–324. https://doi.org/10.1016/B978-012432555-5/50016-6.

Kazimierczak W, Skrzypek H, Sajnaga E, Skowronek M, Waśko A, Kreft A (2017) Strains of *Photorhabdus* spp. associated with polish *Heterorhabditis* isolates: their molecular and phenotypic characterization and symbiont exchange. Archives of Microbiology 199: 979–989. https://doi.org/10.1007/s00203-017-1368-z

Kiss J, Edwards CR, Berger HK, Cate P, Cean M, Cheek S, Derron J, Festić H, Furlan L, Igrc-Barčić J, Ivanova I, Lammers W, Omelyuta V, Princzinger G, Reynaud P, Sivcev I, Sivicek P, Urek G, Vahala O (2005) Monitoring of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) in Europe 1992-2003. In: Western corn rootworm: ecology and management. CABI Publishing, Wallingford, Oxfordshire; Cambridge, MA, 29–39. https://doi.org/10.1079/9780851998176.0029.

Klein M (1990) Efficacy against soil-inhabiting insect pests. In: Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, FL. https://doi.org/10.1201/9781351071741.

Krysan J, Miller T (1986) Methods for study of pest *Diabrotica*. Springer, New York. https://doi.org/10.1007/978-1-4612-4868-2.

Krysan J, Smith R (1987) Systematics of the *virgifera* species group of *Diabrotica* (Coleoptera: Chrysomelidae: Galerucinae). Entomography 5: 375–484.

Kuhlmann U, van der Burgt A (1998) Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe. Biocontrol News and Information 19: 59N-68N.

Kurtz B, Toepfer S, Ehlers R-U, Kuhlmann U (2007) Assessment of establishment and persistence of entomopathogenic nematodes for biological control of western corn rootworm. Journal of Applied Entomology 131: 420–425. https://doi.org/10.1111/j.1439-0418.2007.01202.x

Kurtz B, Hiltpold I, Turlings T, Kuhlmann U, Toepfer S (2009) Comparative susceptibility of larval instars and pupae of the western corn rootworm to infection by three entomopathogenic nematodes. BioControl 54: 255–262. https://doi.org/10.1007/s10526-008-9156-y

Lamichhane JR (2020) Parsimonious use of pesticide-treated seeds: an integrated pest management framework. Trends in Plant Science 25: 1070–1073. https://doi.org/10.1016/j.tplants.2020.08.002

van Lenteren JC (2012) The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. BioControl 57: 1–20. https://doi.org/10.1007/s10526-011-9395-1

Lenth RV (2023) emmeans: estimated marginal means, aka least-squares means. https://CRAN.R-project.org/package=emmeans.

Levine E, Oloumi-Sadeghi H (1991) Management of diabroticite rootworms in corn. Annual Review of Entomology 36: 229–255. https://doi.org/10.1146/annurev.en.36.010191.001305

Lommen STE, de Jong PW, Pannebakker BA (2017) It is time to bridge the gap between exploring and exploiting: prospects for utilizing intraspecific genetic variation to optimize arthropods for augmentative pest control - a review. Entomologia Experimentalis et Applicata 162: 108–123. https://doi.org/10.1111/eea.12510

Lubchenco J, Karl TR (2012) Predicting and managing extreme weather events. Physics Today 65: 31–37. https://doi.org/10.1063/PT.3.1475

Machado RAR, Von Reuss SH (2022) Chemical ecology of nematodes. CHIMIA 76: 945. https://doi.org/10.2533/chimia.2022.945

Machado RAR, Thönen L, Arce CCM, Theepan V, Prada F, Wüthrich D, Robert CAM, Vogiatzaki E, Shi Y-M, Schaeren OP, Notter M, Bruggmann R, Hapfelmeier S, Bode HB, Erb M (2020) Engineering bacterial symbionts of nematodes improves their biocontrol potential to counter the western corn rootworm. Nature Biotechnology 38: 600–608. https://doi.org/10.1038/s41587-020-0419-1

Meinke LJ, Sappington TW, Onstad DW, Guillemaud T, Miller NJ, Komáromi J, Levay N, Furlan L, Kiss J, Toth F (2009) Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) population dynamics. Agricultural and Forest Entomology 11: 29–46. https://doi.org/10.1111/j.1461-9563.2008.00419.x

Modic Š, Žigon P, Kolmanič A, Godeša T, Razinger J (2018) Effectiveness of different control measures against western corn rootworm larvae *Diabrotica virgifera virgifera* LeConte, 1868. Acta agriculturae Slovenica 111. https://doi.org/10.14720/aas.2018.111.1.15

Oleson JD, Park Y-L, Nowatzki TM, Tollefson JJ (2005) Node-injury scale to evaluate root injury by corn rootworms (Coleoptera: Chrysomelidae). Journal of Economic Entomology 98: 1–8. https://doi.org/10.1093/jee/98.1.1

Pilz C, Toepfer S, Knuth P, Strimitzer T, Heimbach U, Grabenweger G (2014) Persistence of the entomoparasitic nematode *Heterorhabditis bacteriophora* in maize fields. Journal of Applied Entomology 138: 202–212. https://doi.org/10.1111/j.1439-0418.2012.01743.x

Pistorious J, Bischoff G (2008) Insecticidal seed treatments and honeybee poisoning incidents in Germany 2008 - background and facts. IOBC IWGO-NEWSLETTER 29.

Poinar G (1990) Taxonomy and biology of Steinernematidae and Heterorhabditidae. In: Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, FL, 23–61.

Radová Š, Trnková Z (2010) Effect of soil temperature and moisture on the pathogenicity of two species of entomopathogenic nematodes (Rhabditida: Steinernematidae). Journal of Agrobiology 27: 1–7. https://doi.org/10.2478/s10146-009-0001-4

Rasmann S, Köllner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TCJ (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434: 732–737. https://doi.org/10.1038/nature03451

Rivera MJ, Martini X, Khrimian A, Stelinski L (2017) A weevil sex pheromone serves as an attractant for its entomopathogenic nematode predators. Chemoecology 27: 199–206. https://doi.org/10.1007/s00049-017-0246-0

Robert CAM, Zhang X, Machado RAR, Schirmer S, Lori M, Mateo P, Erb M, Gershenzon J (2017) Sequestration and activation of plant toxins protect the western corn rootworm from enemies at multiple trophic levels. eLife 6: e29307. https://doi.org/10.7554/eLife.29307

Singh P, Moore R (1985) Handbook of insect rearing. Elsevier Science Publishers, Amsterdam. 1985. http://worldveg.tind.io/record/1416.

Susurluk I (2005) Establishment and persistence of the entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis bacteriophora*. PhD Thesis. Christian Albrechts University

Toepfer S, Gueldenzoph C, Ehlers R, Kuhlmann U (2005) Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. Bulletin of Entomological Research 95: 473–482. https://doi.org/10.1079/BER2005379

Toepfer S, Kuhlmann U (2006) Constructing life-tables for the invasive maize pest *Diabrotica virgifera virgifera* (Col.; Chrysomelidae) in Europe. Journal of Applied Entomology 130: 193–205. https://doi.org/10.1111/j.1439-0418.2006.01060.x

Toepfer S, Peters A, Ehlers R-U, Kuhlmann U (2008) Comparative assessment of the efficacy of entomopathogenic nematode species at reducing western corn rootworm larvae and root damage in maize. Journal of Applied Entomology 132: 337–348. https://doi.org/10.1111/j.1439-0418.2008.01274.x

Toepfer S, Haye T, Erlandson M, Goettel M, Lundgren JG, Kleespies RG, Weber DC, Walsh GC, Peters A, Ehlers R-U, Strasser H, Moore D, Keller S, Vidal S, Kuhlmann U (2009a) A review of the natural enemies of beetles in the subtribe Diabroticina (Coleoptera: Chrysomelidae): implications for sustainable pest management. Biocontrol Science and Technology 19: 1–65. https://doi.org/10.1080/09583150802524727

Toepfer S, Burger R, Ehlers R-U, Peters A, Kuhlmann U (2009b) Controlling western corn rootworm larvae with entomopathogenic nematodes: effect of application techniques on plant-scale efficacy: Techniques to control *Diabrotica v. virgifera* with nematodes. Journal of Applied Entomology 134: 467–480. https://doi.org/10.1111/j.1439-0418.2009.01469.x

Toepfer S, Hatala-Zseller I, Ehlers R-U, Peters A, Kuhlmann U (2010a) The effect of application techniques on field-scale efficacy: can the use of entomopathogenic nematodes reduce damage by western corn rootworm larvae? Agricultural and Forest Entomology 12: 389–402. https://doi.org/10.1111/j.1461-9563.2010.00487.x

Toepfer S, Kurtz B, Kuhlmann U (2010b) Influence of soil on the efficacy of entomopathogenic nematodes in reducing *Diabrotica virgifera virgifera* in maize. Journal of Pest Science 83: 257–264. https://doi.org/10.1007/s10340-010-0293-6

van Tol RWHM, van der Sommen ATC, Boff MIC, van Bezooijen J, Sabelis MW, Smits PH (2001) Plants protect their roots by alerting the enemies of grubs. Ecology Letters 4: 292–294. https://doi.org/10.1046/j.1461-0248.2001.00227.x

Van Rozen K, Ester A (2010) Chemical control of *Diabrotica virgifera virgifera* LeConte. Journal of Applied Entomology 134: 376–384. https://doi.org/10.1111/j.1439-0418.2009.01504.x

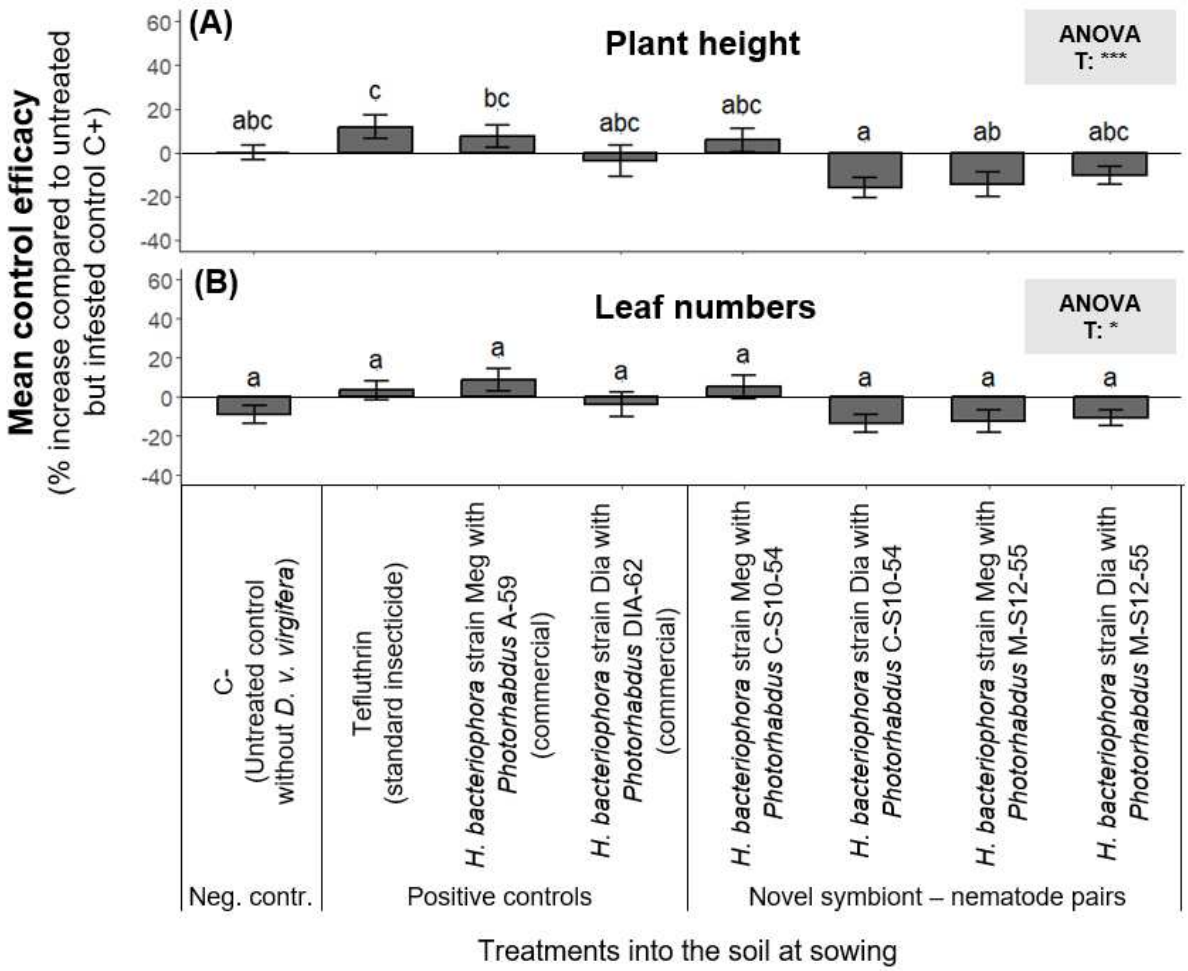
Watson RJ, Joyce SA, Spencer GV, Clarke DJ (2005) The *exbD* gene of *Photorhabdus temperata* is required for full virulence in insects and symbiosis with the nematode *Heterorhabditis*: Iron acquisition in *Photorhabdus*. Molecular Microbiology 56: 763–773. https://doi.org/10.1111/j.1365-2958.2005.04574.x

Wechsler S, Smith D (2018) Has resistance taken root in U.S. corn fields? Demand for insect control. American Journal of Agricultural Economics 100: 1136–1150. https://doi.org/10.1093/ajae/aay016

Wesseler J, Fall EH (2010) Potential damage costs of *Diabrotica virgifera virgifera* infestation in Europe - the 'no control' scenario: potential damage costs of *Dvv*. in Europe. Journal of Applied Entomology 134: 385–394. https://doi.org/10.1111/j.1439-0418.2010.01510.x

Zhang X, van Doan C, Arce CCM, Hu L, Gruenig S, Parisod C, Hibbard BE, Hervé MR, Nielson C, Robert CAM, Machado RAR, Erb M (2019) Plant defense resistance in natural enemies of a specialist insect herbivore. Proceedings of the National Academy of Sciences 116: 23174–23181. https://doi.org/10.1073/pnas.1912599116

Supplementary Information Chapter II



Supplementary Figure 1

Efficacy of different treatments in reducing effects of *Diabrotica v. virgifera* larvae on plant height (A) and leaf numbers (B) compared to the untreated but infested control in field 2. Treatments consists of two commercial *Heterorhabditis bacteriophora* products: Meginem® Pro carrying A-59 bacteria and Dianem® with carrying DIA-62 bacteria, four newly developed nematode-bacterial symbiont pairs where the bacteria were either resistant (M) or susceptible (C) to benzoxazinoids, intact plants (C-) and plants infested with *D. v. virgifera* and treated with an insecticide (Tefluthrin). Only data of field 2 is presented. Error bars = SEM. Results of ANOVA with fixed factor control treatment (T) is presented in grey boxes. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Different letters on bars indicate the significant difference between treatments as per post hoc Tukey HSD test at $p < 0.05$ following ANOVA.

Chapter III

Cross-resistance and collateral sensitivity to environmental chemicals after directed evolution of *Photorhabdus* to benzoxazinoids

Unpublished work

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Abstract

Microorganisms have evolved various mechanisms that enable them to tolerate lethal concentrations of toxic compounds. By altering physical properties of the cellular membrane, regulating the levels of DNA transcription, or by using efflux pumps, microorganisms protect themselves against certain environmental chemicals. In addition, an increased tolerance to certain toxins can also increase (cross-resistance) or decrease (collateral sensitivity) the tolerance to other toxins. Here we tested if selecting *Photorhabdus* bacteria for increased tolerance to 6-methoxy-2-benzoxazolinone (MBOA) alters their tolerance to other chemicals such as other benzoxazinoids, insecticides, pesticides, secondary plant metabolites and antibiotics. Our results show that MBOA-selected *Photorhabdus* strains are more tolerant to the structurally similar compounds 2-benzoxalinone (BOA) and 6-chloroacetyl-2-benzoxazolinone as well as to caffeine, nicotine and sinigrin. On the other hand, MBOA tolerant strains are more susceptible to the antibiotics ampicillin and kanamycin. Our results highlight the importance of cross-resistance and collateral sensitivity assessment after directed evolution for an environmental toxin in *Photorhabdus* bacteria.

KEY WORDS Transport mechanisms, hyper-sensibility, efflux pumps, 6-methoxy-2-benzoxazolinone, antibiotic resistance

Introduction

Microorganisms are constantly exposed to deleterious chemicals that are naturally occurring in the environment. They must be able to tolerate or resist natural antibiotics produced by other microorganism, or toxins produced by natural enemies or hosts (Peschel and Sahl 2006, Koprivnjak and Peschel 2011, Ruiz et al. 2013, Ullah et al. 2015). Common targets of toxic compounds are the cell wall, the cytoplasmic membrane by affecting the structure of biomembranes or the biosynthetic pathway of protein and nucleic acid synthesis that is essential for microbial growth. Nevertheless, bacteria show excellent mechanisms to resist toxic compounds and to protect cells from continuous exposure to harmful chemicals. Although the mechanisms of resistance vary from compound to compound, they typically involve one or more of the following mechanisms: alteration of the drug target in the bacterial cell, enzymatic modification or destruction of the drug itself, changes in membrane permeability or limitation of drug accumulation as a result of drug exclusion or active drug efflux (Bryan 1988, Dever and Dermody 1991, Davies 1994, Kumar and Schweizer 2005, Pagès et al. 2008).

One powerful strategy for self-protection against toxic compounds is to build an external permeability barrier. This mechanism is observed in gram-negative bacteria that form an outer membrane, and also in the mycobacteria-nocardia-corynebacteria group, a class of gram-positive bacteria, that form a mycolate-containing cell wall (Nikaido and Vaara 1985). These permeability barriers, however, can only slow down the influx of toxic compounds. Therefore, bacteria have additional mechanisms to cope with harmful substances that can still reach the cytoplasm. For example, bacteria metabolize antimicrobial substances into non-toxic compounds, they can modify the target site of action and by the presence of broadly-specific efflux pumps, they reduce the concentration of antimicrobials in the cytoplasm to sub-toxic levels (Abril et al. 1989, Duetz et al. 1998, Van Bambeke et al. 2000, Nikaido 2001, Poole 2002, Ramos et al. 2002, Trieber and Taylor 2002). There are currently numerous examples of efflux systems that provide resistance to a broad range of structurally unrelated antimicrobial substances – so-called multidrug efflux systems (Paulsen et al. 1996, Nikaido 1998). These efflux pumps play a major role in solvent tolerance in bacteria. Often, the protective mechanisms overlap with a wide range of structurally diverse chemicals and unfortunately, such drug extrusion mechanisms are a major cause of intrinsic bacterial resistance to antibiotics. Its relevance has only recently been acknowledged, and therefore, most recent

research work in this field has focused on these antibiotic efflux pumps (Poole 2000, 2001, Van Bambeke et al. 2000, Ryan et al. 2001). They are involved in outward transport of a surprisingly large variety of drugs in almost all cell types, from prokaryotes and archaebacteria through fungi and higher eukaryotes. Drug efflux decreases the load on enzyme-mediated detoxification systems by avoiding their saturation, while chemical modifications by the enzyme-based systems provide drug pumps with better substrates (Ishikawa et al. 1997, Suzuki and Sugiyama 1998). Most drug efflux pumps have a broad substrate specificity and, therefore, may deal with a wide range of drugs of completely unrelated pharmacological classes. This opens interesting perspectives for new and possibly beneficial resistances for other chemicals, but on the other hand, cross-resistance must be handled with caution as it may lead to severe antibiotic resistances. With respect to gram-negatives, there are numerous reports of biocide–antibiotic cross-resistance. That includes pine oil- and triclosan- resistant *Escherichia coli* displaying the multiple antibiotic resistant (mar) phenotype, chlorhexidine-resistant *Pseudomonas stutzeri* displaying cross-resistance to multiple antibiotics, and triclosan-resistant *Ps. aeruginosa* showing elevated resistance to several antibiotics (Moken et al. 1997, McMurry et al. 1998, Russell et al. 1998, Tattawasart et al. 1999, Chuanchuen et al. 2001). The latter arise from mutational up-regulation of endogenous multidrug efflux systems that accommodate both antibiotics and biocides (Moken et al. 1997, McMurry et al. 1998, Chuanchuen et al. 2001). On the other hand, increased resistance to one antibiotic can also often result in increased susceptibility to another antibiotic, a phenomenon referred to as collateral sensitivity antibiotic (Obolski et al. 2015, Pál et al. 2015). A prominent example of collateral sensitivity is the >16-fold lower minimal inhibitory concentration (MIC) of tigecycline-resistant *E. coli* for nitrofurantoin where resistance to tigecycline and sensitivity to nitrofurantoin are caused by a mutation of the *lon* gene (Roemhild et al. 2020). Collateral sensitivity is considered as a negative cross-resistance to antibiotics and is a common pleiotropic consequence of resistance mutations and resistance genes (Lázár et al. 2013, Barbosa et al. 2017, Nichol et al. 2019, Rosenkilde et al. 2019). This occurs predominantly between antibiotics that have different killing mechanisms and mode of actions. However, we still have little understanding of the molecular mechanisms that lead to collateral sensitivity.

Evolution of resistance to environmental toxins can result from modification of an anti-bacterial target or from functional bypassing of that target, or it can be contingent on impermeability, efflux, or enzymatic inactivation. As DNA is replicated, uncorrected base

substitutions occur randomly with a frequency of $\sim 10^{-9}$ to 10^{-10} per gene (Bridges 2001). Copying errors may lead to the partial or complete deletion of individual genes (Bridges 2001). In addition, bacteria acquire resistance genes by different mechanisms such as integrons and transposons (Kapil 2005). These processes lead to altered gene products or changed levels of transcription, which may lead to beneficial effects for the bacteria by increasing their fitness. In experimental evolution, selection can act on any and all traits and genes relevant to fitness under the environmental regimes of interest.

Experimental evolution is an alternative research framework that offers the opportunity to study such evolutionary processes experimentally in real time. Many evolution experiments seek to understand how populations adapt to particular environmental conditions and what their possible advantages could be, usually defined in terms of a particular environmental factor, such as temperature, nutrition, other environmental stressors or competition (Bennett and Lenski 1993, Santos et al. 1997, Kolss et al. 2009, Dhar et al. 2011, terHorst 2011). Most of these studies rely on natural (i.e., uncontrolled) genetic variation sampled from a base population or generated *de novo* by random mutations. Over the last decade, a fast growth of studies covering this topic was observed, mainly because the traditional focus on phenotypic aspects of adaptation has been increasingly combined with genomic data, facilitated by technological advances.

We used an experimental evolution approach to select for benzoxazinoid (BX) resistance in *Photorhabdus* bacteria. Through the directed evolution, a significant effect in the increase in MBOA resistance for most of the tested bacterial strains was observed (Chapter 1). We found that MBOA resistance in *Photorhabdus* was associated with genetic alterations at different loci across the different strains. The detected genomic changes fell into three broad categories: DNA transcription, membrane architecture and membrane channels. As a further step, we aim in investigating possible side effects (cross-resistance or collateral sensitivity) caused by this directed selection. We hypothesize that selected *Photorhabdus* bacteria exhibit higher resistance towards structural similar molecules such as other benzoxazinoids, so called cross-resistance. Through general mutations affecting import and export in the pre-selected strains, an increase of resistance for other chemical compounds such as insecticides, pesticides and secondary plant metabolites, that are sequestered by a variety of insects, may also be possible. Lastly, costs and collateral sensitivity as a result of increased MBOA resistance are addressed by investigating growth trade-offs.

Materials and Methods

Bacterial strains

Photorhabdus bacteria were isolated from their original nematode hosts with two methods: For the first method, 100 nematode infective juveniles (IJ) were surface sterilized, resuspended in 100 µl of autoclaved and distilled water and ground up using a plastic pestle. The resulting solution was diluted several times and the different concentrations were plated onto LB agar plates (1.5 % agar, 40 g/l) (Carl Roth, Switzerland). The second method was to isolate *Photorhabdus* from infected *Galleria mellonella* parasitized by different strains of nematodes. In this method, last instar wax moth larvae *G. mellonella* were placed into a Petri dish padded with moist filter paper with approximately 100 IJs per insect. After about 48 hours, the cadavers were dissected with a needle and a drop of the haemolymph was streaked on LB agar plates with a sterile loop. Pure cultures were obtained by replating *Photorhabdus*-like colonies. Bacterial identification was carried out based on 16S rRNA gene sequences.

Directed evolution for MBOA resistance

To increase MBOA tolerance in these different *Photorhabdus* strains, a directed evolution approach was used as described in Chapter 1. Briefly, twenty-seven bacterial strains (EN01, DE2, DE6, PT1, IT6, IR2, HU2, CN4, IL9, MG6286, TT01, LJ, B, 0943, S5P8, S7, S8, S9, S10, S12, S14, S15, KC, MEX20, BIO, A and DIA) were cultured independently in LB containing 200 µg/ml MBOA (MBOA-selected, M-) or pure LB (control-selected, C-) at 28 °C with constant shaking (180 rpm) for 24 h (Table 1). This MBOA concentration was chosen based on the observed Growth Inhibition 50 (half-maximal growth inhibition, GI_{50}) from previous experiments (Machado et al. 2020). To ensure genetic homogeneity of the starting bacterial strains before the selection regime, starting cultures for experimental evolution were obtained from a single colony. After 24 h, one millilitre of the resulting bacterial cultures was transferred to 3 ml of fresh medium and cultured again. Subculturing cycles were repeated every 24 h for a total of 37 times. In addition, an aliquot of the MBOA-free bacterial culture from the first growth cycle was used to prepare glycerol stocks to preserve the original genotypes (evolutionary ancestors). The 20 best strains in term of increase in MBOA resistance (EN01, DE2, DE6, IT6, IR2, HU2, CN4, IL9, MG6286, TT01, LJ, B, S5P8, S10, S12, S14, S15, MEX20, BIO and DIA) (for details see Chapter 1) were selected and used for the following experiment (both control- and MBOA- selected strains, so 40 strains in total).

Whole-genome sequencing and variant calling analysis

To identify genetic mutations that could explain the increased MBOA tolerance as well as collateral sensitivity to environmental chemicals after directed evolution of *Photorhabdus* to benzoxazinoids, the procedure described in Chapter 1 was followed (Section "Whole-genome sequencing and variant calling analysis", page 35). Shortly, gDNA was extracted and complete genomes were sequenced. To determine the genetic alterations associated with the observed changes in benzoxazinoid resistance following experimental evolution, genomic differences between the laboratory evolved strains and their evolutionary ancestors were evaluated by variant calling analyses.

Evaluation of cross resistance and bacterial growth *in vitro*

To evaluate the influence of acquired MBOA-resistance by *Photorhabdus* bacteria on the ability to grow on other chemical substances, bacterial growth was estimated by measuring the optical density (OD_{600}) of bacterial cultures in 384-well microtiter plates (Greiner Bio-One, Austria) (Wiegand et al. 2008, Robert et al. 2017). Bacterial cultures grown for 20 h at 28 °C were diluted to an OD_{600} = 0.05. Ten microliters of these bacterial solutions were inoculated into 70 µl of LB (12.5 g/l) (Carl Roth) containing either 2-benzoxazolinone (BOA), caffeine, (-)-nicotine, digitoxin, rutin trihydrate, (−)-sinigrin hydrate, 6-chloroacetyl-2-benzoxazolinone, 2-mercaptopyridine N-oxide sodium salt (pyrithione), fenitrothion or the antibiotics ampicillin and kanamycin (Table 1).

Due to the high number of plates, three different plate readers were used. Resistance to each chemical class was tested in the same instrument (Table 2). The following settings were used: For the Synergy™ HT Multi-Detection Microplate Reader (BioTek, United States), OD_{600} measurements were carried out every 30 min for 48 h. Plates were gently shaken orbitally (medium shaking speed and 5-s shaking cycles) before each measurement. For the VarioScan Flash microplate reader (Thermo Scientific, United States), OD measurements were carried out every 60 min for 48 h. Plates were shaken orbitally (1-mm amplitude, 600 rpm and 5-s shaking cycles) before each measurement. Lastly, for the SpectraMax i3 plate reader (Molecular Devices, Korea), OD measurements were carried out every 30 min for 48 h. Plates were shaken orbitally (1.7-mm amplitude, 422 rpm, 5-s shaking cycles) before each measurement. All three plate readers were set to 28°C for the whole experimental duration.

Table 1: List of used chemical compounds. Name of chemical compound, the concentration range, CAS number and the seller, the biological function of the chemical, the used plate reader to observe growth rates and the number of plates (replicates) are presented. PSM = Plant secondary metabolite.

Chemical	Concentration range	CAS number	Seller	Biological function	Plate reader	n
2-Benzoxazolinone (BOA)	0 - 1000 µg/ml	59-49-4	Sigma-Aldrich	PSM: Benzoxazinoid produced by Poaceae	VarioScan	3
Caffeine	0 - 2000 µg/ml	58-08-2	Sigma-Aldrich	PSM: Pseudo-alkaloids with insecticidal properties	VarioScan	3
Nicotine	0 - 6,25 µl/ml	54-11-5	Sigma-Aldrich	PSM: Alkaloid accumulated in tobacco (*Nicotiana tabacum*) and sequestered by *Manduca sexta*	SpectraMax	3
Digitoxin	0 - 1600 µg/ml	71-63-6	Sigma-Aldrich	PSM: Cardiac glycoside produced by the genus of *Digitalis*	SpectraMax	3
Rutin	0 - 1000 µg/ml	250249-75-3	Sigma-Aldrich	PSM: Flavonoid glycoside found in many plants	VarioScan	3
Sinigrin	0 - 2000 µg/ml	3952-98-5	Sigma-Aldrich	PSM: Aliphatic glucosinolate present in plants of the Brassicaceae family	SpectraMax	3
6-Chloroacetyl-2-benzoxazolinone	0 - 40 µg/ml	54903-10-5	Sigma-Aldrich	Precursor of herbicide, insecticide, and fungicide synthesis	Synergy™	3
2-Mercaptopyridin-N-oxid sodium salt (Pyrithione)	0 - 10 µg/ml	3811-73-2	Sigma-Aldrich	Biological activity as anti-fungal or anti-bacterial agent	SpectraMax	2
Fenitrothion	0 - 5 µl/ml	122-14-5	Sigma-Aldrich	Phosphorothioate (organophosphate) insecticide	SpectraMax	3
Ampicillin	0 - 100 µg/ml *	69-52-3	Sigma-Aldrich	Penicillin antibiotic	Synergy™	3
Kanamycin	0 - 12,5 µg/ml	25389-94-0	Sigma-Aldrich	Aminoglycoside antibiotic	SpectraMax	3

*: for the two naturally resistant strains S10 and MEX20 up to 800 µg/ml

Statistical analysis

Differences in GI_{50} values for chemical compounds which reduced bacterial growth in a dose-dependent and linear manner (MBOA, BOA, nicotine, caffeine, 6-chloroacetyl-2-benzoxazolinone, digitoxin, fenitrothion, ampicillin and kanamycin) were analysed by two-way ANOVA with strain and selection as fixed factors using Sigma Plot 15.1.1.26 (Smith 2019). For pyrithione and sinigrin, bacterial growth reduction was analysed by a two-way ANOVA with the fixed factors strain and selection. In addition, a two-tailed t-test was conducted between the different concentrations. For rutin, which increased bacterial growth, growth increase was analysed by a two-way ANOVA with the fixed factors strain and selection. Normality and equality of variance were verified using Shapiro–Wilk test. Correlation tests for the eight chemicals with a linear and dose-dependent reduction (cor.test) were performed in R studio (Best and Roberts 1975, Hollander et al. 2013, RStudio Team 2020).

Results

Photorhabdus rapidly evolve resistance to MBOA in vitro

MBOA reduced the growth of all the tested strains in a dose-dependent and linear manner (Supplementary Figure 1, S1), but through directed evolution, a significant effect in the increase in MBOA resistance was observed ($F = 357.4$; $p < 0.001$, Figure S3). MBOA resistance was increased by up to 102.5 % in the MBOA-selected strains (Figure S3, Table 2). The increase in MBOA resistance was greater for MBOA susceptible strains compared to already MBOA-resistant strains. This experiment shows that not selected *Photorhabdus* strains were susceptible to MBOA, but susceptibility varied among strains. MBOA resistance can be improved by experimental evolution in *Photorhabdus* symbionts, but the degree of resistance is dependent on the strain and the selection regime.

Evolution of MBOA resistance increases cross-resistance and collateral sensitivity to other environmental toxins

As a next step, we selected the 20 most MBOA resistant strains and evaluated their growth in the presence of a variety of other chemicals. 2-benzoxazolinone (BOA), 6-chloroacetyl-2-benzoxazolinone, nicotine, caffeine, fenitrothion, digitoxin, ampicillin and kanamycin affected bacterial growth in a dose-dependent and linear manner (Figure S2), but the direct selection on MBOA altered the resistance patterns of some of the selected strains (Figures S4 to S9, S11 and S12, Table 2). Sinigrin did not affect bacterial growth at even the highest concentration of 1000 µg/ml (Table S1). In contrast, pyrithione at a dose of 5 µg/ml (Table S2) was highly toxic and none of the bacterial strains were able to grow. Lastly, rutin trihydrate at a concentration of 1000 µg/ml increased bacterial growth by 35 % in average (Figure S10). Results for all chemical compounds with all tested doses are summarized in a heatmap (Figure 1).

Resistance to BOA. The directed evolution on MBOA had a significant effect on the bacteria's ability to grow in the presence of 2-benzoxazolinone (BOA), a structurally similar benzoxazinoid to MBOA ($F = 86.4$; $p < 0.001$, Figure S4). BOA resistance was increased by up to 74.7 % in the MBOA-selected strains, the average GI_{50} increase accounts to +25.2 % (Table 2). Non-significant differences between the GI_{50} of selected and non-selected strains were observed for DE2, DE6, IT6, IR2, TT01, S12, S14, BIO, CN4 and LJ ($p > 0.16$). A positive correlation between the GI_{50} of MBOA and BOA was observed ($R = 0.67$, $p < 0.001$), indicating that MBOA resistance leads to BOA resistance (Figure S13).

Resistance to nicotine. The directed evolution on MBOA had a significant and positive effect on the bacteria's ability to grow in the presence of nicotine ($F = 496.8$; $p < 0.001$, Figure S5). The increase of the GI_{50} on nicotine was on average +41.3 % higher for the MBOA selected strains compared to the control selected strains (Table 2). Greatest increase of resistance by up to 203.4 % was observed for the strain S5P8 (Table 2). Non-significant differences between the GI_{50} of selected and non-selected strains were observed for HU2, B, S12, BIO, CN4 and LJ ($p > 0.051$). A positive correlation between the GI_{50} of MBOA and nicotine was observed ($R = 0.74$, $p < 0.001$), indicating that MBOA resistance leads to nicotine resistance (Figure S13).

Resistance to caffeine. MBOA selection has little influence on caffeine resistance as only few bacterial strains were more resistant to both compounds ($F = 35.4$; $p < 0.001$, Figure S6). A significant increase in the GI_{50} was observed for the MBOA-selected strains DE2, DE6, S5P8, S12, S15 and IL9 ($p \leq 0.02$). The increase of the GI_{50} on caffeine was on average +17 % higher for the MBOA selected strains compared to the control selected strains (greatest increase of the resistance by up to 122.3 % for the strain DE6, Table 2). For all other strains, no significant increase in the GI_{50} was observed after selection on MBOA ($p > 0.11$). No significant correlation was found between the GI_{50} of MBOA and caffeine ($R = 0.2$, $p = 0.22$, Figure S13).

Resistance to 6-chloroacetyl-2-benzoxazolinone. MBOA selection has little influence on 6-chloroacetyl-2-benzoxazolinone resistance as only few bacterial strains were more resistance to both compounds ($F = 12.5$; $p < 0.001$, Figure S7). A significant increase in the GI_{50} was observed for the MBOA-selected strains DIA, B, S10 and MEX20 ($p \leq 0.04$). The increase of the GI_{50} on 6-chloroacetyl-2-benzoxazolinone was on average +26.1 % higher for the MBOA selected strains compared to the control selected strains (greatest increase of the resistance by up to 137.4 % for the strain MEX20, Table 2). Interestingly, increased MBOA resistance resulted in 6-chloroacetyl-2-benzoxazolinone susceptibility for IR2 ($p = 0.03$). A positive correlation between the GI_{50} of MBOA and 6-chloroacetyl-2-benzoxazolinone was observed ($R = 0.34$, $p = 0.032$), indicating that MBOA resistance leads to 6-chloroacetyl-2-benzoxazolinone resistance (Figure S13).

Resistance to digitoxin. The directed evolution on MBOA had little effect on the bacteria's ability to grow in the presence of digitoxin ($F = 3.3$; $p = 0.07$, Figure S8). Overall, a decrease of -3.1 % for the GI_{50} on digitoxin was observed for the MBOA selected strains compared to the control selected strains (Table 2). Interestingly, the GI_{50} on digitoxin of the strain IL9-35

decreased when the strain was previously selected on MBOA ($p = 0.002$). A positive correlation between the GI_{50} of MBOA and digitoxin was observed ($R = -0.35$, $p = 0.025$), indicating that MBOA resistance leads to digitoxin resistance (Figure S13).

Resistance to fenitrothion. The directed evolution on MBOA had little effect on the bacteria's ability to grow in the presence of fenitrothion, a phosphorothioate (organophosphate) insecticide. Overall, a decrease of -0.6 % for the GI_{50} on fenitrothion was observed for the MBOA selected strains compared to the control selected strains (Table 2). A higher GI_{50} for MBOA-selected strains was observed for the strain S15 ($p < 0.001$). On the other hand, the GI_{50} on fenitrothion of the strains IR2, HU2 and S10 decreased when the strains were previously selected on MBOA ($p \leq 0.005$). Although differences between the individual strains were visible ($F = 5.3$; $p < 0.001$, Figure S9), overall no differences in the GI_{50} on fenitrothion were observed between the previously MBOA or control selected strains ($F = 0.5$; $p = 0.475$, Figure S9). This finding is underlined by the non-significant correlation between the GI_{50} of MBOA and fenitrothion ($R = 0.039$, $p = 0.81$, Figure S13).

Resistance to sinigrin. Sinigrin did not affect bacterial growth as no significant differences in bacterial growth after 48 hours were observed between the two concentrations, 0 and 1000 µg/ml (two-tailed t-test, $p = 0.95$). Nevertheless, at a concentration of 1000 µg/ml sinigrin in the growing medium, bacterial growth reduction compared to growth in LB varied among strains ($F = 4.2$; $p < 0.001$, Table S1), and the previous selection on MBOA did have a significant impact ($F = 10.4$, $p = 0.002$, Table S1). For the MBOA-selected strains TT01, S5P8, S15 and IL9, bacterial growth was less reduced compared to the control selected strains ($p \leq 0.019$). In contrast, MBOA-selected IR2 seems to be more susceptible than control selected IR2 ($p = 0.029$).

Resistance to pyrithione. All the tested strains were highly susceptible to the compound 2-mercaptopyridin-N-oxid sodium salt (pyrithione) at a concentration of 5 µg/ml (two-tailed t-test, $p < 0.001$). Even at this low concentration, bacterial growth was reduced almost completely for all the tested strains (Table S2). Previous selection on MBOA did not have an impact ($F = 0.06$, $p = 0.94$, Table S2).

Resistance to rutin. Surprisingly, rutin trihydrate at a concentration of 1000 µg/ml increased bacterial growth by average of 35 % compared to bacteria growing only in LB. The increase varied among strains ($F = 3.5$; $p < 0.001$), but the previous selection on MBOA did not have an impact ($F = 1.1$, $p = 0.3$, Figure S10). A significant increase of MBOA selected strains was observed for the strains S15 ($p = 0.005$) and IL9 ($p = 0.016$) with a percentual growth increase of 313 and 68 % respectively compared to bacterial strains that passed the selection growing in LB only (Figure S10).

Table 2: Percentual changes of GI_{50} (Growth Inhibition 50) on a variety of chemical compounds between *Photorhabdus* bacterial strains selected on MBOA and non-selected strains. Positive values indicate per cent increase in GI_{50} values, and negative values indicate per cent reduction in GI_{50} values of MBOA selected bacteria (M-) compared to control selected bacteria (C-). Strains are ordered by their MBOA-resistance degree. On the bottom, mean per cent changes in GI_{50} values across all the strains are presented. Values in bold indicate that the increase or decrease in GI_{50} is statistically significant (by two-way ANOVA with Holm's multiple-comparisons test).

Strain	Chemical compound								
	MBOA	***BOA***	***Nicotine***	***Caffeine***	***6-Chloroacetyl-2-benzoxazolinone***	***Digitoxin***	***Fenitrothion***	***Ampicillin***	***Kanamycin***
S15	**102.5**	**71.3**	**95.4**	**25.1**	-22.5	-2.8	**45.0**	23.3	19.7
TT01	**95.5**	10.1	**67.0**	1.8	15.7	4.3	3.0	**-48.5**	**-44.5**
IR2	**81.9**	6.0	**27.7**	-12.6	**-39.3**	-15.2	**-33.7**	**-43.9**	**-53.8**
CN4	**81.7**	-12.7	8.9	0.1	28.8	-7.4	17.5	-8.1	-11.7
MEX20	**73.8**	**28.0**	**52.9**	-0.5	**137.4**	-5.0	4.0	**19.2**	-3.5
DE2	**73.6**	-0.8	**34.1**	**35.0**	22.0	10.8	-5.1	-3.5	-29.4
HU2	**69.6**	**34.6**	1.4	12.5	-7.6	-10.5	**-32.4**	-7.2	-22.3
MG6286	**63.8**	**74.7**	**56.0**	13.7	-16.8	-1.7	11.5	**-45.3**	**-58.3**
S5P8	**63.5**	**58.7**	**203.4**	**42.3**	17.2	8.0	-2.1	**-28.3**	-7.5
LJ	**54.4**	14.3	0.3	1.4	13.1	0.1	-9.1	15.0	0.0
S10	**41.7**	**33.0**	**49.7**	-6.5	**94.0**	-14.7	**-20.7**	**10.4**	44.0
B	**40.9**	**20.2**	4.0	-2.3	**87.6**	-1.9	-11.3	0.7	-5.0
DIA	**40.2**	**63.0**	**98.7**	-2.3	**92.8**	-10.3	16.4	**-46.8**	-2.8
IL9	**38.6**	**37.8**	**46.6**	**61.3**	-13.2	**-24.7**	-6.5	**-33.9**	**59.2**
EN01	**35.6**	**44.6**	**19.4**	10.1	58.8	-12.3	-1.3	**-26.5**	18.9
DE6	**35.4**	4.7	**35.6**	**122.3**	-31.6	3.1	7.7	18.9	17.1
S12	**33.9**	-2.5	11.6	30.9	44.5	3.6	-8.9	1.5	-15.4
IT6	**30.9**	3.8	**26.4**	4.8	56.7	6.6	-14.7	-19.2	-5.4
BIO	**30.0**	13.2	4.0	19.0	-24.5	13.6	16.2	**-45.2**	**-46.3**
S14	**25.7**	2.0	**-17.5**	-15.4	8.7	-6.1	12.6	**-25.0**	-11.4
Overall	**44.3**	**25.2**	**41.3**	**17.0**	**26.1**	-3.1	-0.6	**-14.6**	**-7.9**

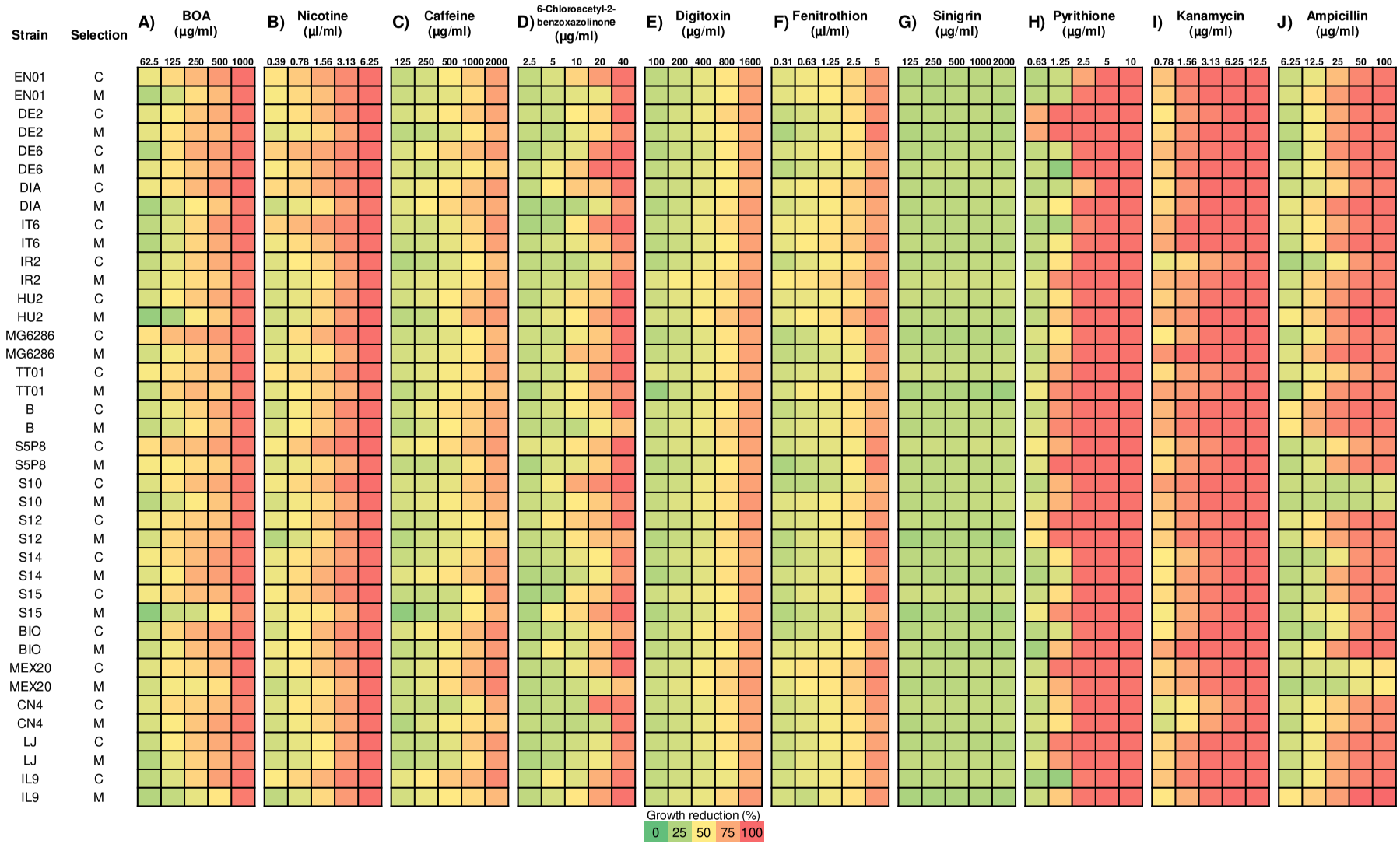

Figure 1: Heatmaps illustrating the strength of bacterial growth reduction and resistance patterns cause by A) 2-benzoxazolinone (BOA); B) nicotine; C) caffeine; D) 6-chloroacetyl-2-benzoxazolinone; E) digitoxin; F) fenitrothion; G) sinigrin; H) 2-mercaptopyridine N-oxide sodium salt (pyrithione); I) kanamycin and J) ampicillin for LB- (C) or MBOA-selected (M) strains. Values are represented on a red-yellow-green colour scale. Green colour indicates no change in bacterial growth (0%). Yellow colour indicates a reduction in bacterial growth by 50 %. Red colour indicates a complete inhibition of bacterial growth (i.e.: bacterial growth is reduced 100%). All measurements were taken two to three independent times with one replicate each time (n=2-3).

Resistance to ampicillin. To test for possible costs of increased MBOA resistance, we let the bacteria grow in the presence of the antibiotic ampicillin and we observed growth trade-offs: GI_{50} for ampicillin were lower, when the bacterial strains were previously selected on MBOA compared to the non-selected strains ($F = 56.9$; $p < 0.001$, Figure S11). The decrease of the GI_{50} on ampicillin was on average -14.6 % lower for the MBOA selected strains compared to the control selected strains (greatest decrease of the resistance by -48.5 % for the strain TT01, Table 2). The correlation between the GI_{50} of MBOA and ampicillin was not significant ($R = -0.24$, $p = 0.16$, Figure S13). Two *Photorhabdus* strains were found with a naturally occurring ampicillin resistance, S10 (*P. kleinii*) and MEX20 (*P. khaini* subsp*. guanajuatensis*). GI_{50} of these two strains were up to 20 times higher compared with the other tested strains and the significant effect of the selection regime was therefore analysed and presented separately ($F = 13.7$; $p = 0.006$, Figure S11 inlet).

Resistance to kanamycin. Similar, GI_{50} for kanamycin were lower, when the bacterial strains were previously selected on MBOA compared to the non-selected strains ($F = 13.5$; $p < 0.001$, Figure S12). The decrease of the GI_{50} on kanamycin was on average -7.9 % lower for the MBOA selected strains compared to the control selected strains (greatest decrease of the resistance by -58.3 % for the strain MG6286, Table 2). A lower GI_{50} for MBOA-selected strains was observed for the strains IR2, MG6286, TT01 and BIO ($p \leq 0.012$). On the other hand, the GI_{50} on kanamycin of the strains IL9 increased significantly when the strain was previously selected on MBOA ($p = 0.018$). The correlation between the GI_{50} of MBOA and kanamycin was not significant ($R = 0.027$, $p = 0.87$, Figure S13).

Variant calling analyses reveals potential genetic regulators of the observed resistance patterns

We sequenced complete genomes of the different *Photorhabdus* strains to evaluate potential genetic mechanisms conferring resistance to the different toxins. We focused on mutations only present in the MBOA-selected strains that were absent from LB-selected strains. By screening for mutations that were predicted to change gene or encoded protein function, we identified a variety of mutations, which were either altering DNA transcription, membrane architecture or membrane channels (Table 3). There was only little overlap in the identified genes between strains, suggesting that resistance to the different environmental toxins can be acquired by multiple mechanisms in *Photorhabdus*.

The most abundant change over all MBOA-selected strains was a mutation in the multidrug efflux pump subunit *acrB*, present in the strains B, S5P8, S12, S15 and MEX20 (Table 3). This drug efflux protein complex with broad substrate specificity uses a proton motive force to export a wide range of substrates. MBOA selected strains had a single point mutation in different subunits of this protein (B: c.1390G>C p.Gly464Arg; S5P8: c.1421T>C p.Ile474Thr; S12: c.1208G>A p.Gly403Asp; S15: c.2999G>A p.Gly1000Asp; MEX20: c.2924G>A p.Arg975His). The mutation in the *acrB* gene of strain B may contribute to increased BOA and 6-chloroacetyl-2-benzoxazolinone resistance. For all the other tested chemical compounds, no increase in resistance was observed. Interestingly, the two strains S10 and MEX20, which exhibit significantly and solely higher antibiotic resistance in MBOA-selected strains, exhibit also mutations in this particular gene family (S10: *acrZ*, c.7G>C p.Glu3Gln; MEX20: *acrB*) (Table 3). In addition, the mutation in the *acrB* gene in MBOA-selected S10 did also contribute to increased resistance to BOA, nicotine, 6-chloroacetyl-2-benzoxazolinone and ampicillin, but decreased resistance to fenitrothion.

By comparing resistance patterns between the two structurally similar compounds 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxalinone (BOA), four genes were found that contribute resistance only to MBOA but not BOA, suggesting for a very specialised mode of action: A single point mutation in the HTH-type transcriptional repressor *fabR* gene in IR2 (c.116G>A p.Arg39His), a single point mutation leading to gain of a stop codon in the coding sequence for the *wzy* protein in TT01 (c.965G>A p.Trp322*), a single point mutation the *waaA* gene of MBOA selected TT01 (c.668C>T p.Thr223Ile), coding for the 3-deoxy-D-manno-octulosonic acid transferase which is responsible for lipopolysaccharide biosynthesis and as already previously mentioned a single point mutation in the *acrB* gene in S12 (c.1208G>A p.Gly403Asp) (Table 3). When the resistance pattern of 2-benzoxalinone (BOA) and 6-chloroacetyl-2-benzoxazolinone are compared, we can infer, which mutations may be responsible for a specific resistance mechanism, as the two chemicals only differ in an added chloroacteyl group to the benzene ring. For example, EN01, HU2, MG6286, S5P8, S15 and IL9 show increased resistance for BOA but not for 6-chloroacetyl-2-benzoxazolinone in previously on MBOA selected strains. Possible mutations in the strain EN01 that can explain this might be point mutations in the *nhaB,* the *ompR* and the *mppO* genes (Table 3). A single point mutation in the Na(+)/H(+) antiporter *nhaB* gene (c.1061C>T p.Pro354Leu) was detected. This antiporter extrudes sodium in exchange for external protons. A single point mutation was observed in the *mppO* gene (c.842G>T

p.Arg281Leu), known to code for an oxidoreductase. In MBOA-selected EN01, a single point mutation in the *ompR* gene was detected (c.614T>G p.Val205Gly). This gene has been described as a transcriptional regulator of porins. In IL9 a single point mutation in the beta subunit of the DNA-directed RNA polymerase *rpoB* gene (c.1655C>T p.Pro552Leu) was detected. Lastly, for the strains S5P8 and S15, a single point mutation in different positions of the multidrug efflux pump subunit *acrB* gene (S5P8: c.1421T>C p.Ile474Thr; M-S15: c.2999G>A p.Gly1000Asp) was detected that may lead to increased resistance for BOA but not for 6-chloroacetyl-2-benzoxazolinone (Table 3).

Table 3: Mutations in MBOA-selected (M-) strains that may contribute to cross-resistance and collateral sensitivity against a variety of environmental chemicals in *Photorhabdus* bacteria.

Strain	Gene	Mutation type	Gene product name	Putative function
M-EN01	*nhaB*	SNP (c.1061C>T p.Pro354Leu)	Na(+)/H(+) antiporter	Extrudes sodium in exchange for external protons
	ompR	SNP (c.614T>G p.Val205Gly)	Transcriptional regulatory protein	Regulation of porins
	mppO	SNP (c.842G>T p.Arg281Leu)	Enduracididine beta-hydroxylase	Oxidoreductase
M-IT6	*envZ*	SNP (c.1016G>A p.Arg339Gln)	Osmolarity sensor protein	Osmoregulation
M-IR2	*fabR*	SNP (c.116G>A p.Arg39His)	HTH-type transcriptional regulator	Negative regulator of un-saturated fatty acid biosynthesis
M-IL9	*rpoB*	SNP (c.1655C>T p.Pro552Leu)	DNA-directed RNA polymerase subunit beta	Transcription of DNA to RNA
M-TT01	*waaA*	SNP (c.668C>T p.Thr223Ile)	3-deoxy-D-manno-octulosonic acid transferase	Lipopolysaccharide biosynthesis
	wzy	Stop gain (c.965G>A p.Trp322*)	O-antigen polymerase	Polymerization of O-antigens
M-B	*acrB*	SNP (c.1390G>C p.Gly464Arg)	Multidrug efflux pump subunit	Part of drug efflux protein complex
M-S5P8	*acrB*	SNP (c.1421T>C p.Ile474Thr)	Multidrug efflux pump subunit	Part of drug efflux protein complex
M-S10	*acrZ*	SNP (c.7G>C p.Glu3Gln)	Multidrug efflux pump accessory protein	Part of drug efflux protein complex
M-S12	*acrB*	SNP (c.1208G>A p.Gly403Asp)	Multidrug efflux pump subunit	Part of drug efflux protein complex
M-S15	*acrB*	SNP (c.2999G>A p.Gly1000Asp)	Multidrug efflux pump subunit	Part of drug efflux protein complex
	ychP	SNP (c.379A>G p.Thr127Ala)	Inverse autotransporter beta domain-containing protein	Autotransporter
	panF	Frameshift (c.1167dupC p.Glu390fs)	Sodium/pantothenate symporter	Pantothenate transmembrane transport
	envZ	Stop gain (c.373G>T p.Glu125*)	Osmolarity sensor protein	Osmoregulation
	rpoD	SNP (c.421G>A p.Glu141Lys)	RNA polymerase sigma factor	Promotes the attachment of RNA polymerase
	C6H65_03095	SNP (c.52C>T p.Arg18Cys)	XRE family transcriptional regulator	DNA-binding transcription factor
M-MEX20	*acrB*	SNP (c.2924G>A p.Arg975His)	Multidrug efflux pump subunit	Part of drug efflux protein complex

MBOA-selected IT6 bacteria show only increased resistance against MBOA and nicotine. For all other tested chemicals, no or only negative effects were observed. A possible mutation that could explain increased resistance for MBOA and nicotine may be a single point mutation in the *envZ* gene (c.1016G>A p.Arg339Gln), coding for a osmolarity sensor protein (Table 3).

By controlling unsaturated fatty acids production, the HTH-type transcriptional repressor *fabR* directly influences the physical properties of the membrane bilayer. Selected IR2 bacteria with

a single point mutation in this gene (c.116G>A p.Arg39His) have an advantage growing in the presence of MBOA or nicotine, but their GI_{50} is significantly reduced in the presence of the two antibiotics ampicillin and kanamycin. In addition, MBOA-selected IR2 seems to be more susceptible to sinigrin than control selected IR2, suggesting a possible trade-off that could be induced by this particular mutation of the *fabR* gene (Table 3).

The *Photorhabdus* strain with the most mutations is MBOA-selected S15 (Table 3). Single point mutations were found in the multidrug efflux pump subunit *acrB* (c.2999G>A p.Gly1000Asp), in the inverse autotransporter beta domain-containing protein *ychP* (c.379A>G p.Thr127Ala), the RNA polymerase sigma factor *rpoD* (c.421G>A p.Glu141Lys) and in the XRE family transcriptional regulator *C6H65_03095* (c.52C>T p.Arg18Cys). In addition, an insertion resulting in a frameshift was detected in the sodium/pantothenate symporter gene *panF* (c.1167dupC p.Glu390fs). Insertion mutations were observed to shift stop codons, which dramatically changes protein secondary and tertiary structure, similar to stop gains by single point mutations. Lastly, a stop gain for the osmolarity sensor protein *envZ* (c.373G>T p.Glu125*) was detected, resulting in a shorter protein product compared to the wild type. S15 previously selected on MBOA is the only strain that exhibits increased resistance for the majority of the tested chemicals (BOA, nicotine, fenitrothion and caffeine) except for 6-chloroacetyl-2-benzoxazolinone where a dose-dependent growth reduction was observed. S15 could even increase its growth in the presence of rutin by 313 % compared to growing in LB only. MBOA-selected S15 grew better in the presence of sinigrin than the control selected strain and no significant growth trade-offs in the presence of the two antibiotics ampicillin and kanamycin were observed.

MBOA-selected IL9 shows increased resistance against BOA, nicotine, caffeine, sinigrin, rutin and even for kanamycin, suggesting that the single point mutation in a subunit of the DNA-directed RNA polymerase (*rpoB*, c.1655C>T p.Pro552Leu) contributes to increased resistance against a variety of chemical compounds.

Discussion

Evolution of resistance in bacteria to one antibiotic or drug is frequently associated with increased resistance to another antibiotic, termed cross-resistance (Sanders et al. 1984, Chuanchuen et al. 2001, Obolski et al. 2015). Conversely, increased resistance to one antibiotic can often result in decreased resistance to another, a phenomenon referred to as collateral sensitivity antibiotic (Obolski et al. 2015, Pál et al. 2015). By combining experimental evolution, whole-genome sequencing of laboratory-evolved bacteria and biochemical assays, our work charted a map of cross-resistance and collateral-sensitivity interactions in MBOA-selected *Photorhabdus* bacteria. We found that MBOA-selected *Photorhabdus* bacteria were associated with a variety of genomic mutations, which translated to increased MBOA-resistance. As expected and confirmed by whole-genome sequencing, the major genetic targets were the translational machinery, membrane transport, phospholipid synthesis, and cell envelope homeostasis. MBOA-resistance can be acquired by genetic alterations at different loci across the different strains. In addition, these mutations resulted in either cross-resistance for BOA, 6-chloroacetyl-2-benzoxazolinone, nicotine, caffeine and sinigrin, and in collateral sensitivity against the two antibiotics ampicillin and kanamycin.

There are four major resistance mechanisms in bacteria: Modification of cellular targets so that antibiotic binding is diminished, reduced cellular uptake, physical removal of an antibiotic from the cell through modification of efflux pumps and enzymatic inactivation of the antibiotic or drug (Mayers et al. 2009). We found that a mutation in the multidrug efflux pump subunit *acrB*, present in five different selected strains, was the most abundant genetic change after selection on MBOA. This drug efflux protein complex with broad substrate specificity uses a proton-motive-force to catalyse the active efflux of many antimicrobial agents (Nikaido 1998, Pos 2009). We observed an increase in resistance for the majority of tested chemical compounds, suggesting for an increased efflux of the tested chemical compounds from the peri- or cytoplasm into the extracellular space. Nikaido *et al.* showed that among β-lactam antibiotics only those with lipophilic side chains were efficiently pumped out by AcrB (Nikaido et al. 1998). As we observed increased resistance for MBOA, BOA, 6-chloroacetyl-2-benzoxazolinone and nicotine when mutations in *acrB* were present, we suggest that the resulting changes may affect substrate specificity and therefore increase the efflux of those mainly hydrophilic molecules.

We showed that the chemical compounds MBOA, BOA, 6-chloroacetyl-2-benzoxazolinone, nicotine, caffeine, digitoxin and fenitrothion have antimicrobial properties and reduce bacterial growth in a linear and dose-dependent manner. With regard to benzoxazinoids and similar structures, 6-chloroacetyl-2-benzoxazolinone showed highest bactericidal activity against *Photorhabdus* strains, followed by MBOA and BOA with mean GI_{50} of 25, 240 and 450 µg/ml, respectively. Similar results were observed when testing antibacterial activity of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its derivatives against *Ralstonia solanacearum*: 2-mercaptobenzothiazole (MBT) showed the strongest antibacterial activity, followed by 6-chloro-2-benzoxazolinone (CDHB) and DIMBOA, with minimum inhibitory concentrations (MICs) of 50, 100 and 200 mg/L, respectively, better than BOA with 300 mg/L (Guo et al. 2016). We showed that chloroacetic substituent highly enhanced the bactericidal activity against *Photorhabdus* species. Nevertheless, selection for MBOA significantly increased resistance for most of the tested strains against BOA and 6-chloroacetyl-2-benzoxazolinone, suggesting for a similar mode of action in the resistance against benzoxazinoids and structurally similar synthetic compounds. Furthermore, we observed reduced bacterial growth in the presence of nicotine, caffeine, digitoxin and pyrithione, which are known to act as antimicrobial agents (Guthery et al. 2005, Benli et al. 2009, Nonthakaew et al. 2015, Salman et al. 2016). Selection for MBOA increased resistance for most of the tested strains to nicotine and caffeine. Evolution of resistance is often constrained by physiological or ecological trade-offs. For example, resistance to xenobiotics has been associated with different types of costs in bacteria, including reduced growth, impaired competitive performance and metabolic imbalance (Lenski 1998, Andersson et al. 2007). We found that MBOA resistance in *Photorhabdus* bacteria can be associated with substantial costs. In particular, we observed growth trade-offs in the presence of two antibiotics: MBOA-selected strains showed reduced growth in the presence of ampicillin and kanamycin compared to the same strains that do not show increased MBOA resistance. This collateral sensitivity is predominantly observed between antibiotics that have different killing mechanisms and mode of actions. For the two widely used antibiotics ampicillin and kanamycin, the modes of action are well studied: Ampicillin acts as a competitive inhibitor of the enzyme transpeptidase, which is needed by bacteria to build cell walls. It inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to cell lysis (Waxman and Strominger 1983). Kanamycin, an aminoglycoside antibiotic, interferes with bacterial protein synthesis by specifically targeting the bacterial ribosome, which disrupts

the proper assembly of the initiation complex during the translation process (Konno et al. 1973). In contrast, the exact mechanisms of the antimicrobial activity for other environmental chemicals such as benzoxazinoids, other secondary plant metabolites such as nicotine, caffeine and sinigrin, are very complex and still not fully understood but may involve disruption of cellular membranes, interference with metabolic processes, induction of oxidative stress and inhibition of other essential cellular processes. This fact should be encouraging for future studies to investigate in exact mechanisms of antimicrobial activity of a variety of environmental chemicals.

Last, we need to emphasize the limitations of our work. Although we may infer which mutations are responsible for an increase or decrease in resistance towards toxins, we are lacking the detailed mechanistic knowledge behind the effects of the observed mutations. Firstly, we are not aware of the actual levels of gene expression as genomic changes alone do not account for this. The regulation of gene expression involves complex mechanisms beyond the DNA sequence, including transcriptional and post-transcriptional processes. Secondly, we neglect complex genetic interactions which are often involved between multiple genes and their products. Studying individual genes in isolation may not capture the synergistic effects or compensatory mechanisms that can occur within a biological system. Lastly, we do not know the extent to how the detected mutations actually affect the gene products. This could be increase of activity, conformational changes in the binding site or inhibition or increase of activity by certain substrates. By additionally creating mutant strains and executing expression analysis and functional assays, we could gain more detailed insight into the characterization of the observed mutations. Nevertheless, we provided with our work the direct effects of genomic mutations caused by MBOA selection and their effect on the resistance towards other toxins.

In summary, this work demonstrates the effects of directed selection of *Photorhabdus* strains on the benzoxazinoid MBOA towards a variety of chemical compounds. We tested if certain strains of *Photorhabdus* spp. bacteria that were previously adapted to increased 6-methoxy-2-benzoxazolinone (MBOA) concentrations exhibit cross resistance to chemically structurally similar benzoxazinoids, but also to insecticides, pesticides, secondary plant metabolites and antibiotics. Our results suggest that strains that were previously selected on MBOA are more resistant to the structurally similar benzoxazinoid 2-benzoxalinone (BOA) and the synthetic

compound 6-chloroacetyl-2-benzoxazolinone as well as to caffeine, nicotine and sinigrin. On the other hand, the pre-selected strains showed a negative trade-off in growth towards the ability of tolerating the antibiotics ampicillin and kanamycin. Our results suggest that specific mechanisms that are involved in MBOA resistance that can also be expanded to other chemicals but with no risk for possible cross-resistances towards antibiotics. We acquired interesting new insights into the mechanisms of cross-resistance and collateral sensitivity after selection of *Photorhabdus* bacteria on MBOA and highlighted the importance of resistance assessment after a directed evolution approach.

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Competing interests

The authors declare no competing interests.

References of Chapter III

Abril M-A, Michan C, Ramos JL (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. Journal of Bacteriology 171: 6782–6790.

Andersson DI, Patin SM, Nilsson AI, Kugelberg E (2007) The biological cost of antibiotic resistance. Enzyme-Mediated Resistance to Antibiotics: Mechanisms, Dissemination, and Prospects for Inhibition: 339–348. https://doi.org/10.1128/9781555815615.ch21

Barbosa C, Trebosc V, Kemmer C, Rosenstiel P, Beardmore R, Schulenburg H, Jansen G (2017) Alternative evolutionary paths to bacterial antibiotic resistance cause distinct collateral effects. Molecular Biology and Evolution 34: 2229–2244. https://doi.org/10.1093/molbev/msx158

Benli M, Yi N, Bingöl Ü (2009) Antimicrobial activity of endemic *Digitalis lamarckii Ivan* from Turkey. Indian Journal of Experimental Biology 47: 218–221.

Bennett AF, Lenski RE (1993) Evolutionary adaption to temperature II. Thermal niches of experimental lines of *Escherichia coli*. Evolution 47: 1–12. https://doi.org/10.1111/j.1558-5646.1993.tb01194.x

Best DJ, Roberts DE (1975) Algorithm AS 89: the upper tail probabilities of Spearman's Rho. Applied Statistics 24: 377. https://doi.org/10.2307/2347111

Bridges BA (2001) Hypermutation in bacteria and other cellular systems. Wood RD, Gearhart PJ, Neuberger MS (Eds). Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 356: 29–39. https://doi.org/10.1098/rstb.2000.0745

Bryan LE (1988) General mechanisms of resistance to antibiotics. Journal of Antimicrobial Chemotherapy 22: 1–15. https://doi.org/10.1093/jac/22.Supplement_A.1

Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP (2001) Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. Antimicrobial Agents and Chemotherapy 45: 428–432. https://doi.org/10.1128/AAC.45.2.428-432.2001

Davies J (1994) Inactivation of antibiotics and the dissemination of resistance genes. Science 264: 375–382. https://doi.org/10.1126/science.8153624

Dever LA, Dermody TS (1991) Mechanisms of bacterial resistance to antibiotics. Archives of Internal Medicine 151: 886–895. https://doi.org/10.1001/archinte.1991.00400050040010

Dhar R, Sägesser R, Weikert C, Yuan J, Wagner A (2011) Adaptation of *Saccharomyces cerevisiae* to saline stress through laboratory evolution: Adaptation of *S. cerevisiae* to saline stress. Journal of Evolutionary Biology 24: 1135–1153. https://doi.org/10.1111/j.1420-9101.2011.02249.x

Duetz WA, Wind B, van Andel JG, Barnes MR, Williams PA, Rutgers M (1998) Biodegradation kinetics of toluene, m-xylene, p-xylene and their intermediates through the upper TOL pathway in *Pseudomonas putida* (pWWO). Microbiology 144: 1669–1675. https://doi.org/10.1099/00221287-144-6-1669

Guo B, Zhang Y, Li S, Lai T, Yang L, Chen J, Ding W (2016) Extract from maize (*Zea mays* L.): antibacterial activity of DIMBOA and its derivatives against *Ralstonia solanacearum*. Molecules 21: 1397. https://doi.org/10.3390/molecules21101397

Guthery E, Seal LA, Anderson EL (2005) Zinc pyrithione in alcohol-based products for skin antisepsis: persistence of antimicrobial effects. American Journal of Infection Control 33: 15–22. https://doi.org/10.1016/j.ajic.2004.07.012

Hollander M, Wolfe DA, Chicken E (2013) Nonparametric statistical methods. John Wiley & Sons.

Ishikawa T, Li Z-S, Lu Y-P, Rea PA (1997) The GS-X pump in plant, yeast, and animal cells: structure, function, and gene expression. Bioscience Reports 17: 189–207. https://doi.org/10.1023/A:1027385513483

Kapil A (2005) The challenge of antibiotic resistance: need to contemplate. Indian Journal of Medical Research 121: 83–91.

Kolss M, Vijendravarma RK, Schwaller G, Kawecki TJ (2009) Life-history consequences of adaptation to larval nutritional stress in *Drosophila*. Evolution 63: 2389–2401. https://doi.org/10.1111/j.1558-5646.2009.00718.x

Konno K, Oizumi K, Kumano N, Oka S (1973) Mode of action of kanamycin on *Mycobacterium bovis* BCG. American Review of Respiratory Disease 108: 101–107.

Koprivnjak T, Peschel A (2011) Bacterial resistance mechanisms against host defense peptides. Cellular and Molecular Life Sciences 68: 2243–2254. https://doi.org/10.1007/s00018-011-0716-4

Kumar A, Schweizer H (2005) Bacterial resistance to antibiotics: active efflux and reduced uptake. Advanced Drug Delivery Reviews 57: 1486–1513. https://doi.org/10.1016/j.addr.2005.04.004

Lázár V, Pal Singh G, Spohn R, Nagy I, Horváth B, Hrtyan M, Busa-Fekete R, Bogos B, Méhi O, Csörgő B, Pósfai G, Fekete G, Szappanos B, Kégl B, Papp B, Pál C (2013) Bacterial evolution of antibiotic hypersensitivity. Molecular Systems Biology 9: 700. https://doi.org/10.1038/msb.2013.57

Lenski RE (1998) Bacterial evolution and the cost of antibiotic resistance. Int Microbiol 1: 265–270.

Machado RAR, Thönen L, Arce CCM, Theepan V, Prada F, Wüthrich D, Robert CAM, Vogiatzaki E, Shi Y-M, Schaeren OP, Notter M, Bruggmann R, Hapfelmeier S, Bode HB, Erb M (2020) Engineering bacterial symbionts of nematodes improves their biocontrol potential to counter the western corn rootworm. Nature Biotechnology 38: 600–608. https://doi.org/10.1038/s41587-020-0419-1

Mayers DL, Lerner SA, Ouellette M, Sobel JD (2009) Antimicrobial drug resistance. Springer. https://doi.org/10.1007/978-3-319-47266-9.

McMurry LM, Oethinger M, Levy SB (1998) Overexpression of *marA, soxS* , or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. FEMS Microbiology Letters 166: 305–309. https://doi.org/10.1111/j.1574-6968.1998.tb13905.x

Moken MC, McMurry LM, Levy SB (1997) Selection of multiple-antibiotic-resistant (mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the mar and *acrAB* loci. Antimicrobial Agents and Chemotherapy 41: 2770–2772. https://doi.org/10.1128/AAC.41.12.2770

Nichol D, Rutter J, Bryant C, Hujer AM, Lek S, Adams MD, Jeavons P, Anderson ARA, Bonomo RA, Scott JG (2019) Antibiotic collateral sensitivity is contingent on the repeatability of evolution. Nature Communications 10: 334. https://doi.org/10.1038/s41467-018-08098-6

Nikaido H (1998) Multiple antibiotic resistance and efflux. Current opinion in microbiology 1: 516–523. https://doi.org/10.1016/S1369-5274(98)80083-0

Nikaido H (2001) Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Seminars in Cell & Developmental Biology 12: 215–223. https://doi.org/10.1006/scdb.2000.0247

Nikaido H, Vaara M (1985) Molecular basis of bacterial outer membrane permeability. Microbiological Reviews 49: 1–32. https://doi.org/10.1128/mmbr.49.1.1-32.1985

Nikaido H, Basina M, Nguyen V, Rosenberg EY (1998) Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those β-lactam antibiotics containing lipophilic side chains. Journal of Bacteriology 180: 4686–4692. https://doi.org/10.1128/jb.180.17.4686-4692.1998

Nonthakaew A, Matan N, Aewsiri T, Matan N (2015) Caffeine in foods and its antimicrobial activity. International Food Research Journal 22. http://www.ifrj.upm.edu.my/22%20(01)%202015/(2).pdf.

Obolski U, Stein GY, Hadany L (2015) Antibiotic restriction might facilitate the emergence of multi-drug resistance. PLoS computational biology 11: e1004340. https://doi.org/10.1371/journal.pcbi.1004340

Pagès J-M, James CE, Winterhalter M (2008) The porin and the permeating antibiotic: a selective diffusion barrier in gram-negative bacteria. Nature Reviews Microbiology 6: 893–903. https://doi.org/10.1038/nrmicro1994

Pál C, Papp B, Lázár V (2015) Collateral sensitivity of antibiotic-resistant microbes. Trends in Microbiology 23: 401–407. https://doi.org/10.1016/j.tim.2015.02.009

Paulsen IT, Brown MH, Skurray RA (1996) Proton-dependent multidrug efflux systems. Microbiological Reviews 60: 575–608. https://doi.org/10.1128/mr.60.4.575-608.1996

Peschel A, Sahl H-G (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nature Reviews Microbiology 4: 529–536. https://doi.org/10.1038/nrmicro1441

Poole K (2000) Efflux-mediated resistance to fluoroquinolones in gram-positive bacteria and the mycobacteria. Antimicrobial Agents and Chemotherapy 44: 2595–2599. https://doi.org/10.1128/AAC.44.10.2595-2599.2000

Poole K (2001) Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. Journal of Molecular Microbiology and Biotechnology 3: 255–264.

Poole K (2002) Mechanisms of bacterial biocide and antibiotic resistance: antibiotic and biocide resistance. Journal of Applied Microbiology 92: 55S-64S. https://doi.org/10.1046/j.1365-2672.92.5s1.8.x

Pos KM (2009) Drug transport mechanism of the AcrB efflux pump. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1794: 782–793. https://doi.org/10.1016/j.bbapap.2008.12.015

Ramos JL, Duque E, Gallegos M-T, Godoy P, Ramos-González MI, Rojas A, Terán W, Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. Annual Review of Microbiology 56: 743–768. https://doi.org/10.1146/annurev.micro.56.012302.161038

Robert CAM, Zhang X, Machado RAR, Schirmer S, Lori M, Mateo P, Erb M, Gershenzon J (2017) Sequestration and activation of plant toxins protect the western corn rootworm from enemies at multiple trophic levels. eLife 6: e29307. https://doi.org/10.7554/eLife.29307

Roemhild R, Linkevicius M, Andersson DI (2020) Molecular mechanisms of collateral sensitivity to the antibiotic nitrofurantoin. Bollenbach T (Ed.). PLOS Biology 18: e3000612. https://doi.org/10.1371/journal.pbio.3000612

Rosenkilde CEH, Munck C, Porse A, Linkevicius M, Andersson DI, Sommer MOA (2019) Collateral sensitivity constrains resistance evolution of the CTX-M-15 β-lactamase. Nature Communications 10: 618. https://doi.org/10.1038/s41467-019-08529-y

RStudio Team (2020) RStudio: Integrated development environment for R. RStudio, PBC., Boston, MA. http://www.rstudio.com/.

Ruiz L, Margolles A, Sánchez B (2013) Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. Frontiers in Microbiology 4. https://doi.org/10.3389/fmicb.2013.00396

Russell AD, Tattawasart U, Maillard J-Y, Furr JR (1998) Possible link between bacterial resistance and use of antibiotics and biocides. Antimicrobial Agents and Chemotherapy 42: 2151–2151. https://doi.org/10.1128/AAC.42.8.2151

Ryan BM, Dougherty TJ, Beaulieu D, Chuang J, Dougherty BA, Barrett JF (2001) Efflux in bacteria: what do we really know about it? Expert Opinion on Investigational Drugs 10: 1409–1422. https://doi.org/10.1517/13543784.10.8.1409

Salman S, Idrees F, Pervaiz S, Shah FH, Badshah S, Usman M, Halimi SMA, Idrees J (2016) Evaluation of antimicrobial activities of harmine, harmaline, nicotine and their complexes. Pakistan Journal of Pharmaceutical Sciences 29: 1317–1320.

Sanders CC, Sanders WE, Goering RV, Werner V (1984) Selection of multiple antibiotic resistance by quinolones, 1- lactams, and aminoglycosides with special reference to cross-resistance between unrelated drug classes. Antmicrobial Agents and Chemotherapy 26: 797–801. https://doi.org/10.1128/aac.26.6.797

Santos M, Borash DJ, Joshi A, Bounlutay N, Mueller LD (1997) Density-dependent natural selection in *Drosophila*: evolution of growth rate and body size. Evolution 51: 420–432. https://doi.org/10.1111/j.1558-5646.1997.tb02429.x

Smith J (2019) SigmaPlot (Version 15). Systat Software Inc., San Jose CA.

Suzuki H, Sugiyama Y (1998) Excretion of GSSG and glutathione conjugates mediated by MRP1 and CM0AT/MRP2. In: Seminars in liver disease. Thieme Medical Publishers, Inc., 359–376. https://doi.org/10.1055/s-2007-1007170

Tattawasart U, Maillard J-Y, Furr JR, Russell AD (1999) Development of resistance to chlorhexidine diacetate and cetylpyridinium chloride in *Pseudomonas stutzeri* and changes in antibiotic susceptibility. Journal of Hospital Infection 42: 219–229. https://doi.org/10.1053/jhin.1999.0591

terHorst CP (2011) Experimental evolution of protozoan traits in response to interspecific competition: evolution in response to competition. Journal of Evolutionary Biology 24: 36–46. https://doi.org/10.1111/j.1420-9101.2010.02140.x

Trieber CA, Taylor DE (2002) Mutations in the 16S rRNA genes of *Helicobacter pylori* mediate resistance to tetracycline. Journal of Bacteriology 184: 2131–2140. https://doi.org/10.1128/JB.184.8.2131-2140.2002

Ullah I, Khan AL, Ali L, Khan AR, Waqas M, Hussain J, Lee I-J, Shin J-H (2015) Benzaldehyde as an insecticidal, antimicrobial, and antioxidant compound produced by *Photorhabdus temperata* M1021. Journal of Microbiology 53: 127–133. https://doi.org/10.1007/s12275-015-4632-4

Van Bambeke F, Balzi E, Tulkens PM (2000) Antibiotic efflux pumps. Biochemical Pharmacology 60: 457–470. https://doi.org/10.1016/S0006-2952(00)00291-4

Waxman DJ, Strominger JL (1983) Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. Annual Review of Biochemistry 52: 825–869. https://doi.org/10.1146/annurev.bi.52.070183.004141

Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols 3: 163–175. https://doi.org/10.1038/nprot.2007.521

Supplementary Information Chapter III

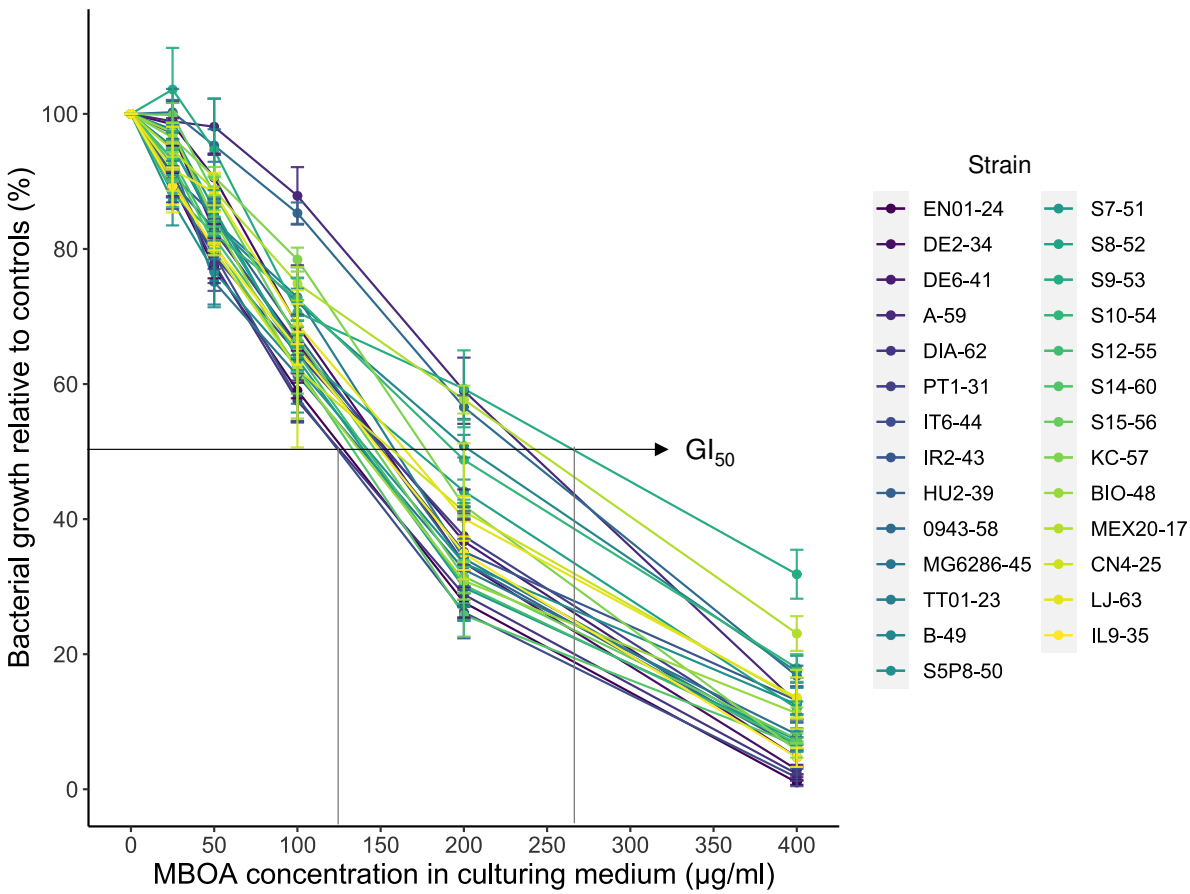

Figure S1

MBOA reduces the growth of all the tested strains in a dose-dependent and linear manner. Mean (± s.e.m.) bacterial growth of twenty-seven *Photorhabdus* strains in Luria-Bertani liquid medium containing different concentrations of MBOA relative to controls. Data of dose-dependent reduction was used to calculate growth inhibition 50 values (half-maximal growth, GI_{50}). Bacterial growth was measured four to five independent times with one replicate each time (n=4-5).

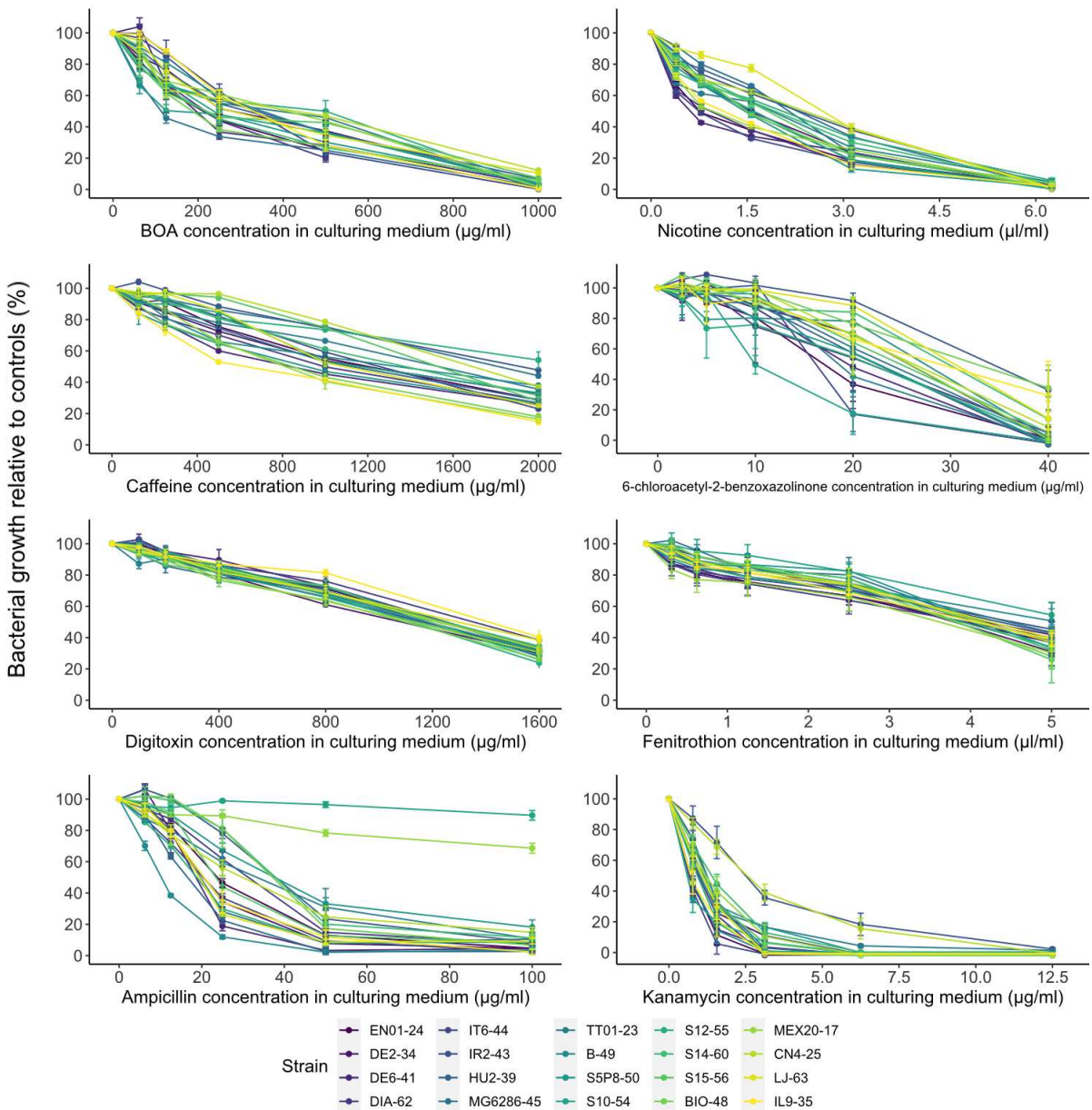

Figure S2

A subset of the tested chemical compounds reduces the growth of the tested strains in a dose-dependent and linear manner. Mean (± s.e.m.) bacterial growth of twenty *Photorhabdus* strains in Luria-Bertani liquid medium containing different concentrations of 2-benzoxazolinone (BOA), nicotine, caffeine, 6-chloroacetyl-2-benzoxazolinone, digitoxin, fenitrothion, ampicillin and kanamycin. Bacterial growth was measured two to three independent times with one replicate each time (n=2-3).

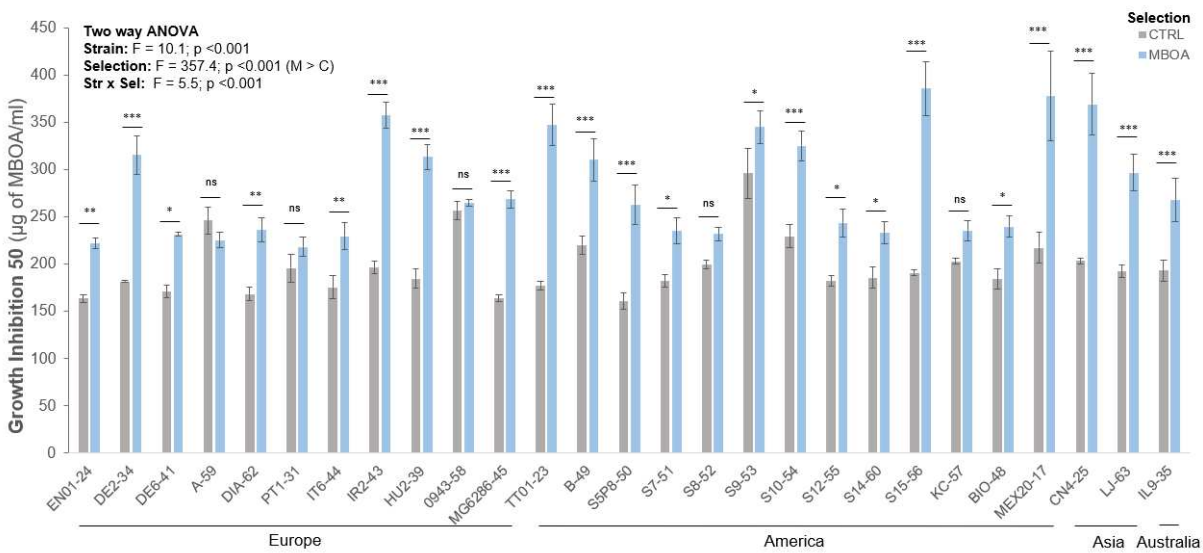

Figure S3

Selection on MBOA increases MBOA resistance of different *Photorhabdus* strains. Mean (± s.e.m.) MBOA concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) in the strains selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars) after 24h of incubation time. Bacterial species are grouped by their continental origin. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). Experiment was conducted four to six independent times with one replicate each time (n=4-6).

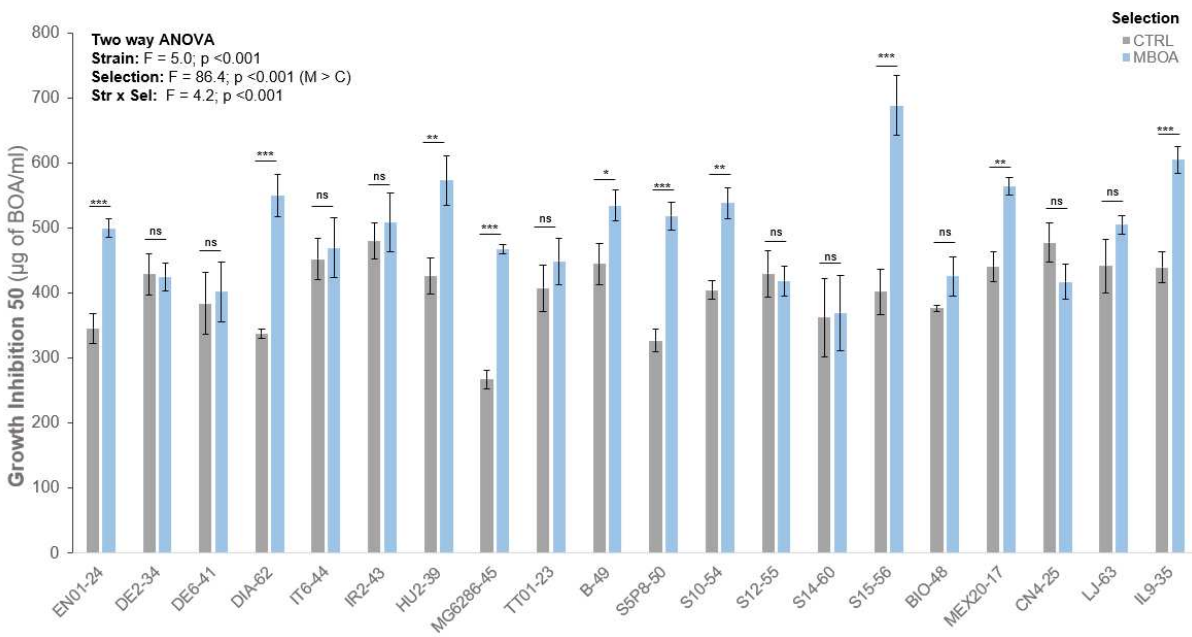

Figure S4

Previous selection on MBOA increases 2-benzoxazolinone (BOA) resistance of different *Photorhabdus* strains. Mean (± s.e.m.) BOA concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) in the strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars) after 24h of incubation time. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Experiment was conducted three independent times with one replicate each time (n=3).

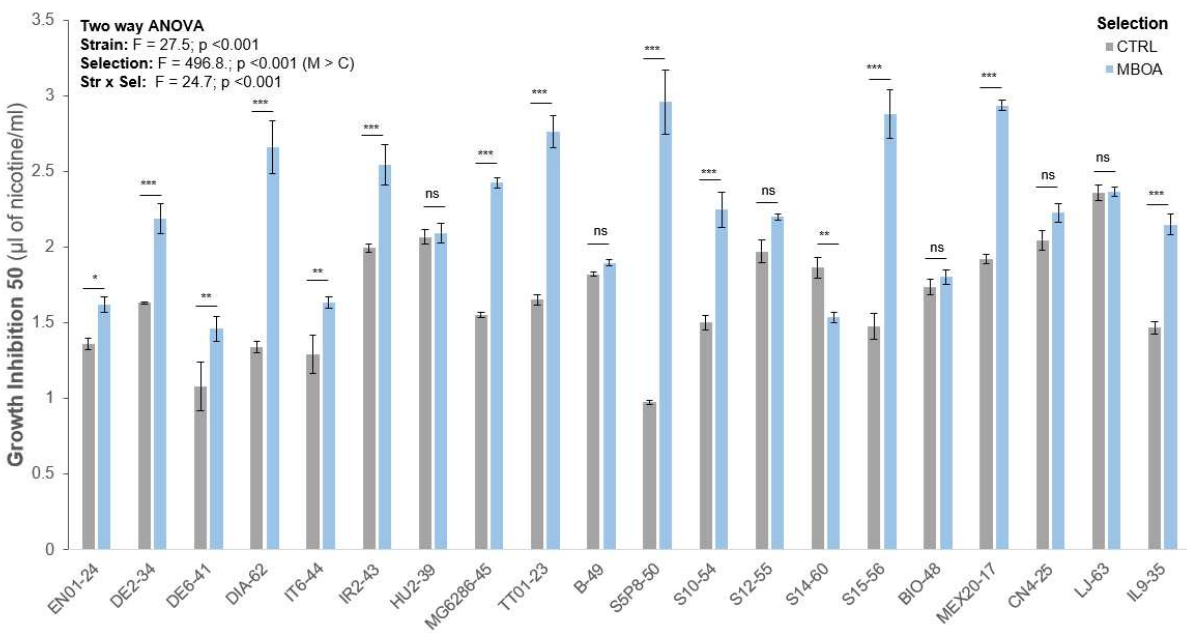

Figure S5

Previous selection on MBOA increases nicotine resistance of different *Photorhabdus* strains. Mean (± s.e.m.) nicotine concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) in the strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars) after 24h of incubation time. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). Experiment was conducted three independent times with one replicate each time (n=3).

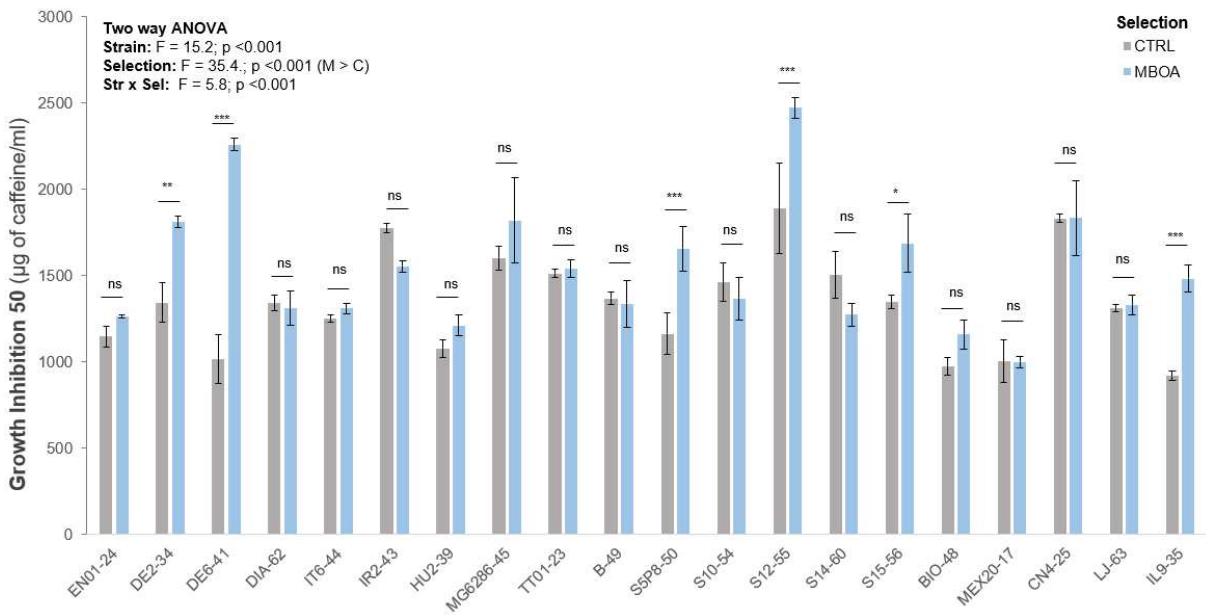

Figure S6

Previous selection on MBOA increases caffeine resistance of different *Photorhabdus* strains. Mean (± s.e.m.) caffeine concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) after 48h in the strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars) after 48h of incubation time. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Experiment was conducted three independent times with one replicate each time (n=3).

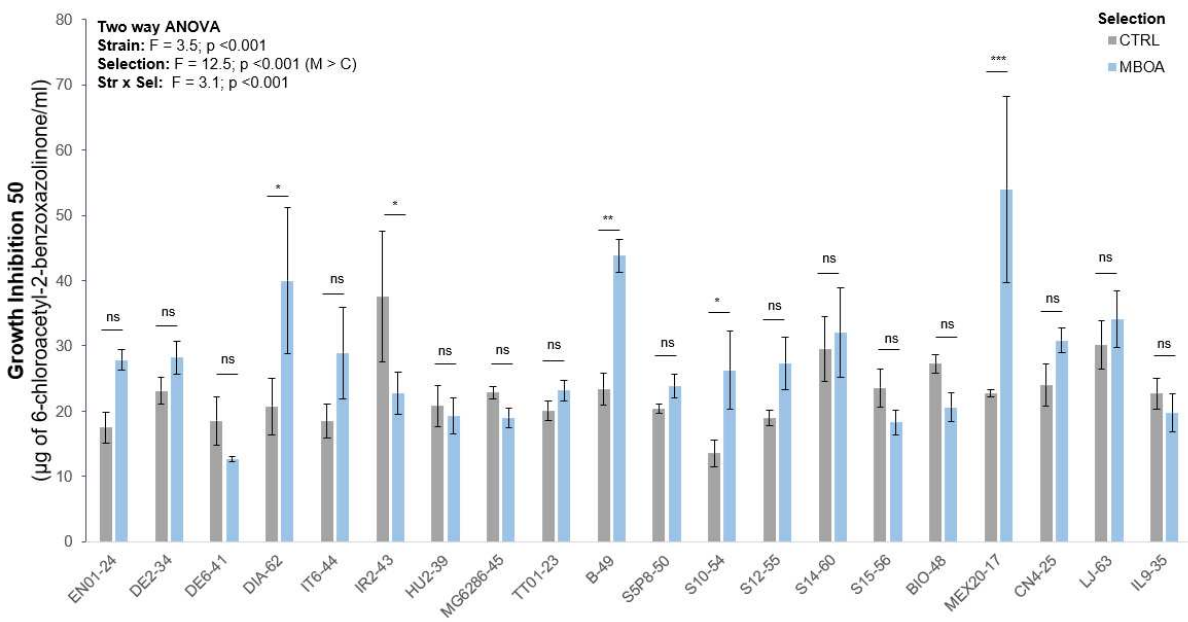

Figure S7

Previous selection on MBOA increases 6-chloroacetyl-2-benzoxazolinone resistance of some *Photorhabdus* strains. Mean (± s.e.m.) 6-chloroacetyl-2-benzoxazolinone concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) in the strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars) after 24h of incubation time. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). Experiment was conducted three independent times with one replicate each time (n=3).

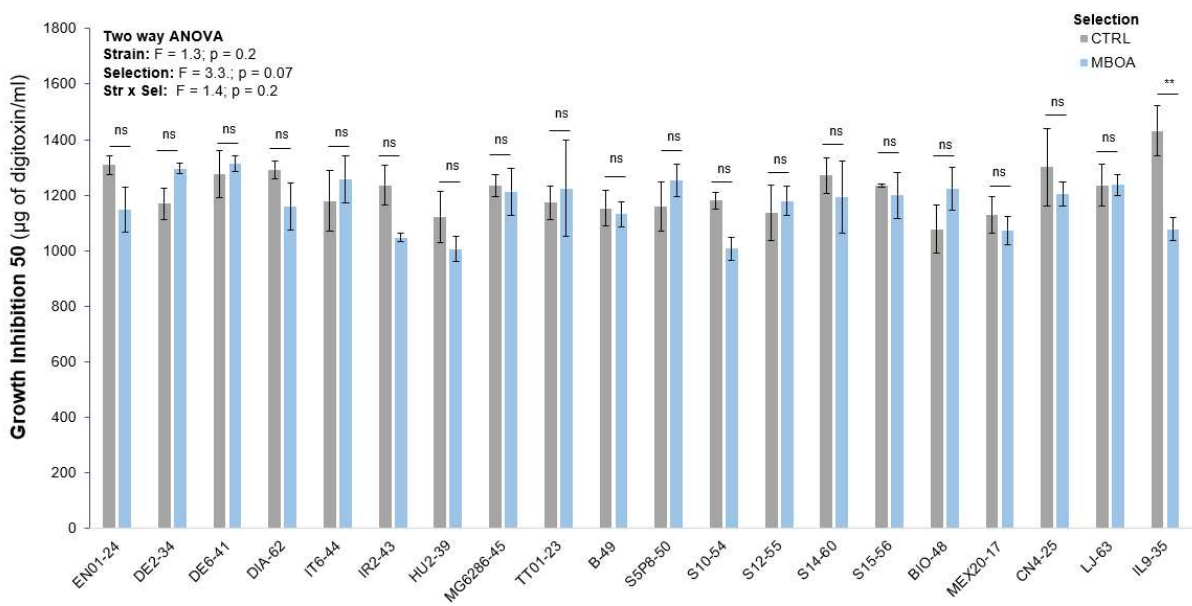

Figure S8

Previous selection of *Photorhabdus* strains on MBOA has overall no effect on the resistance towards digitoxin. Mean (± s.e.m.) digitoxin concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) in the strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars) after 24h of incubation time. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Experiment was conducted three independent times with one replicate each time (n=3).

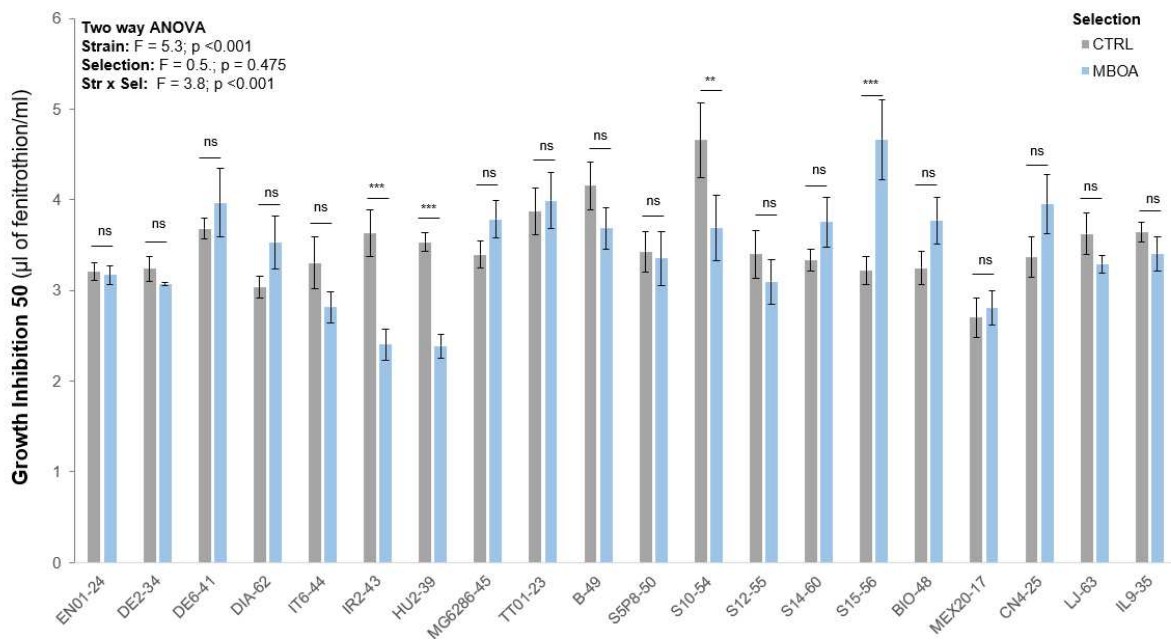

Figure S9

Previous selection of *Photorhabdus* strains on MBOA has overall no effect on the resistance towards fenitrothion. Mean (± s.e.m.) fenitrothion concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) in the strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars) after 24h of incubation time. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Experiment was conducted three independent times with one replicate each time (n=3).

Strain	LB-selected	MBOA-selected	Statistics
EN01-24	-0.7 ± 2.9	-2.0 ± 1.7	ns
DE2-34	-0.3 ± 0.8	-2.5 ± 1.5	ns
DE6-41	1.5 ± 0.1	1.8 ± 2.0	ns
DIA-62	2.0 ± 0.7	0.6 ± 2.1	ns
IT6-44	-2.4 ± 1.4	-0.8 ± 0.1	ns
IR2-43	1.7 ± 0.4	6.1 ± 0.1	*
HU2-39	1.6 ± 0.5	0.7 ± 0.4	ns
MG2686-4	-2.8 ± 0.3	0.1 ± 0.5	ns
TT01-23	2.3 ± 1.0	-5.6 ± 1.0	***
B-49	0.9 ± 1.0	1.8 ± 0.4	ns
S5P8-50	4.8 ± 2.4	0.1 ± 1.7	*
S10-54	1.0 ± 1.0	2.4 ± 0.5	ns
S12-55	-2.5 ± 1.8	-3.5 ± 2.3	ns
S14-60	0.4 ± 0.4	-3.1 ± 1.2	ns
S15-56	2.6 ± 1.7	-5.1 ± 3.0	***
BIO-48	-2.4 ± 2.4	-4.9 ± 0.8	ns
MEX20-17	1.9 ± 0.7	1.0 ± 0.3	ns
CN4-25	1.1 ± 1.0	-0.1 ± 0.2	ns
LJ-63	-2.4 ± 0.3	0.5 ± 1.3	ns
IL9-35	1.1 ± 0.5	-6.8 ± 2.9	***

Table S1

Sinigrin has only little effect on bacterial growth. Mean (± s.e.m.) % growth reduction in 1000µg/ml sinigrin compared to LB media for *Photorhabdus* strains previously selected in MBOA-free LB control medium (LB-selected), and the same strains selected in MBOA-containing LB medium (MBOA-selected) after 48h of incubation time. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Experiment was conducted three independent times with one replicate each time (n=3).

Strain	LB-selected	MBOA-selected	Statistics
EN01-24	101.0 ± 0.6	100.8 ± 0.5	ns
DE2-34	99.9 ± 0.1	100.5 ± 0.0	ns
DE6-41	100.9 ± 0.5	101.7 ± 0.4	ns
DIA-62	101.1 ± 0.8	100.5 ± 0.2	ns
IT6-44	100.8 ± 0.5	100.4 ± 0.4	ns
IR2-43	100.0 ± 0.4	101.6 ± 0.8	ns
HU2-39	100.3 ± 0.4	100.7 ± 0.3	ns
MG2686-4	100.0 ± 0.3	100.8 ± 0.2	ns
TT01-23	101.3 ± 0.6	100.0 ± 0.0	ns
B-49	100.4 ± 0.2	100.4 ± 0.0	ns
S5P8-50	101.5 ± 1.0	101.2 ± 0.5	ns
S10-54	100.5 ± 0.5	100.3 ± 0.4	ns
S12-55	100.6 ± 0.1	102.1 ± 2.6	ns
S14-60	100.8 ± 0.3	101.1 ± 0.5	ns
S15-56	101.1 ± 0.6	100.7 ± 0.1	ns
BIO-48	101.1 ± 0.7	100.8 ± 0.5	ns
MEX20-17	100.7 ± 0.7	98.9 ± 0.4	ns
CN4-25	101.1 ± 0.7	100.3 ± 0.7	ns
LJ-63	100.4 ± 0.0	100.4 ± 0.1	ns
IL9-35	100.8 ± 0.2	100.9 ± 0.4	ns

Table S2

Pyrithione completely inhibits bacterial growth. Mean (± s.e.m.) % growth reduction in 5 µg/ml pyrithione compared to LB media for *Photorhabdus* strains previously selected in MBOA-free LB control medium (LB-selected), and the same strains selected in MBOA-containing LB medium (MBOA-selected) after 48h of incubation time. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Experiment was conducted two independent times with one replicate each time (n=2).

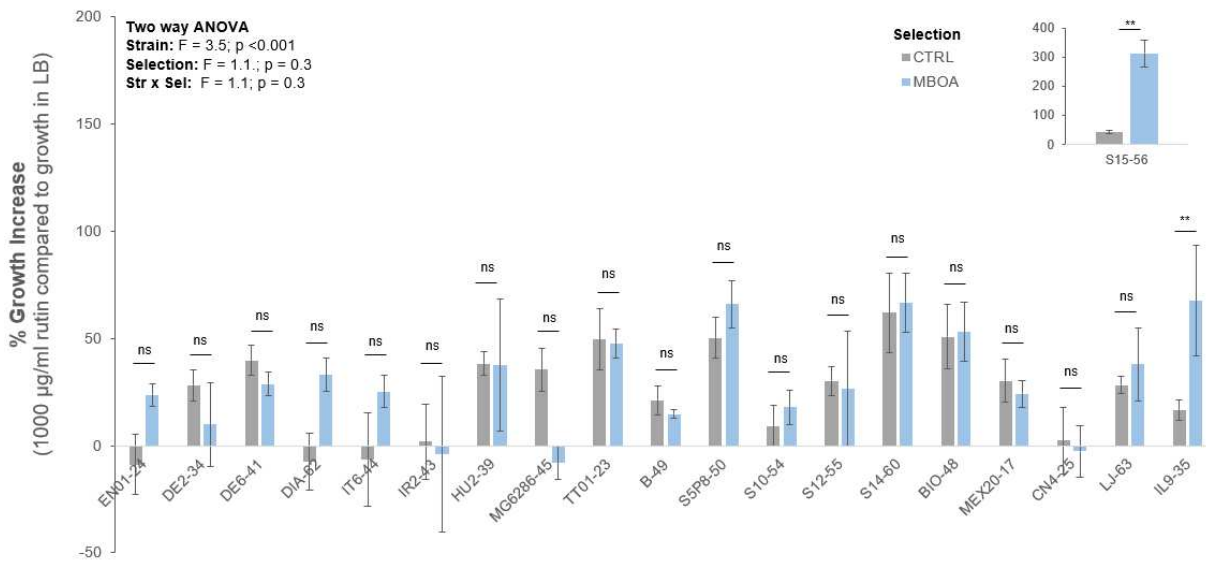

Figure S10

Rutin trihydrate increases bacterial growth. Mean (± s.e.m.) % growth increase in 1000 µg/ml rutin trihydrate compared to LB media for *Photorhabdus* strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars) after 24h of incubation time. Inlet shows strain with highest growth increase. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). Experiment was conducted three independent times with one replicate each time (n=3).

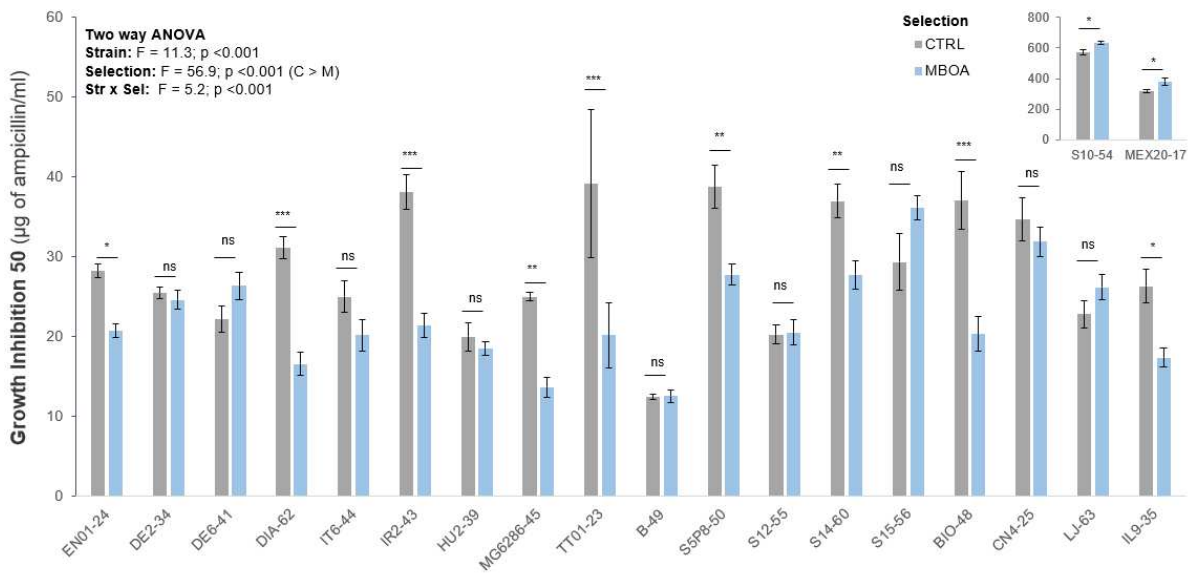

Figure S11

Previous selection on MBOA decreases ampicillin resistance of some *Photorhabdus* strains. Mean (± s.e.m.) ampicillin concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) in the strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars). Inlet shows two strains with naturally occurring ampicillin resistance, that were analysed separately. Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). Experiment was conducted three independent times with one replicate each time (n=3).

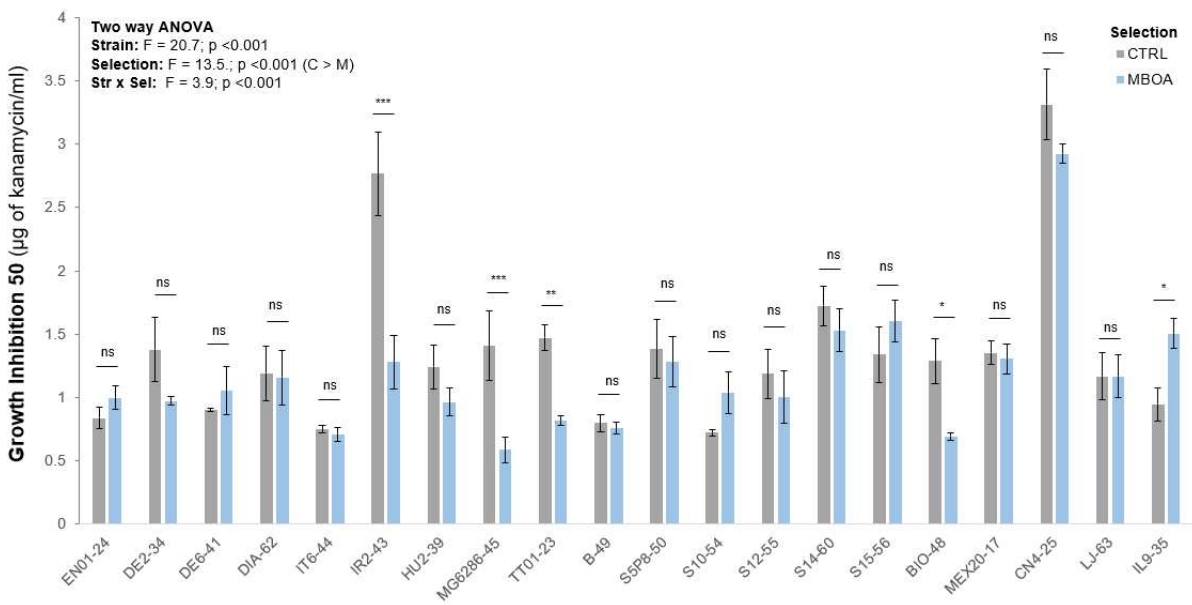

Figure S12

Previous selection on MBOA decreases kanamycin resistance of some *Photorhabdus* strains. Mean (± s.e.m.) kanamycin concentrations that inhibit bacterial growth by 50 % (Growth Inhibition 50, GI_{50}) in the strains previously selected in MBOA-free LB control medium (CTRL, grey bars), and the same strains selected in MBOA-containing LB medium (MBOA, blue bars). Asterisks indicate significant differences of the selection regime within the strains by two-way ANOVA with Holm's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). Experiment was conducted three independent times with one replicate each time (n=3).

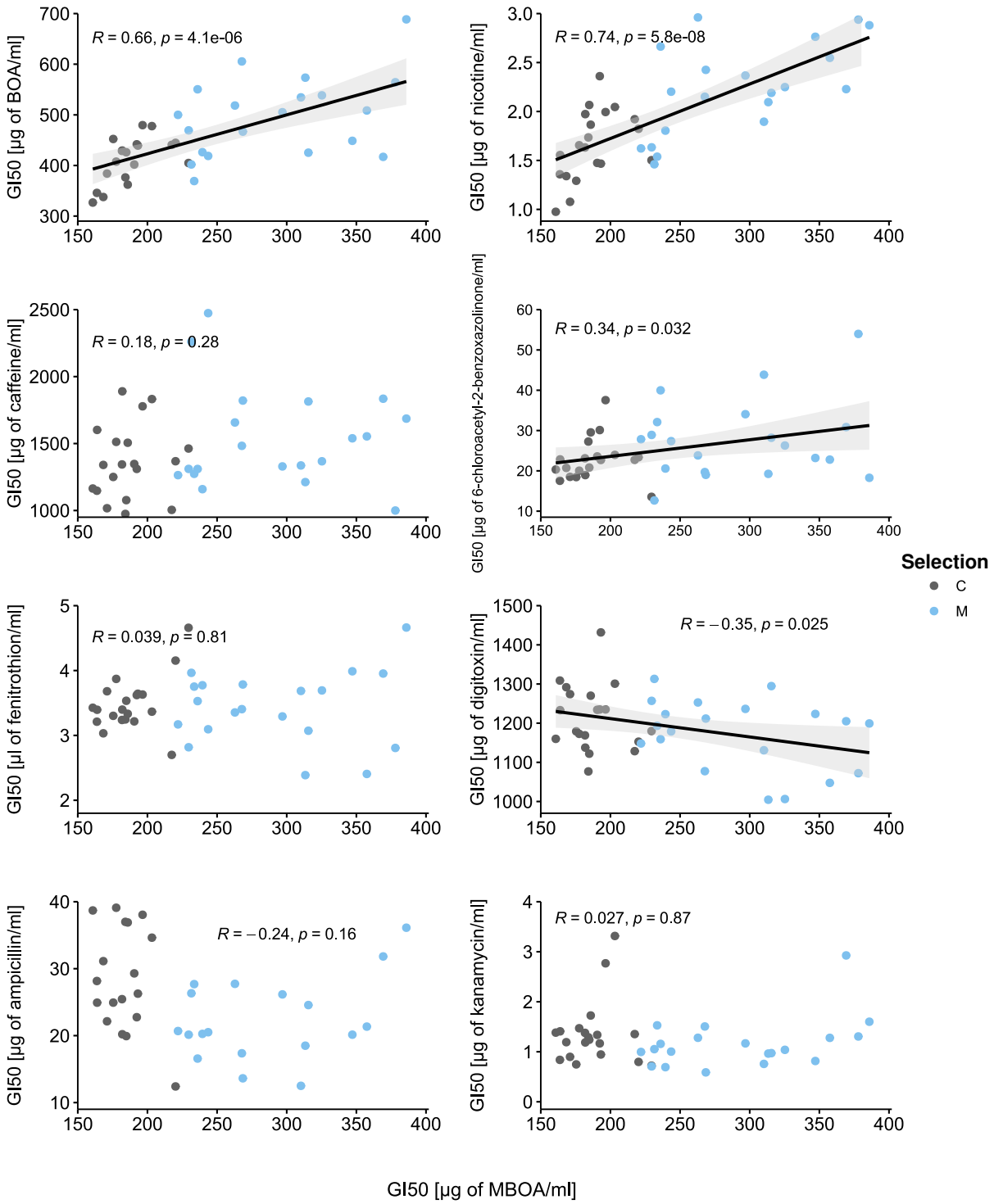

Figure S13

Correlations between GI_{50} of MBOA and the eight tested chemicals with dose-dependent and linear bacterial growth reduction. Strains that were previously selected in MBOA-free LB control medium are represented in grey points (C), and the same strains selected in MBOA-containing LB medium are represented in blue (M). Results of Pearson's correlation test are presented in the graphs (R-value and p-value).

Conclusion

Insect herbivores cause major yield losses in agriculture. The western corn rootworm (WCR) is among the most damaging maize pests and is likely to expand its range in the future (Aragón et al. 2010). The use of biological control agents is a promising strategy to combat root-feeding pests such as WCR without the need for synthetic pesticides. This includes entomopathogenic nematodes (EPNs) and their symbiotic bacteria. They are among the most common biological control agents against WCR in Europe (Toepfer et al. 2005). However, WCR larvae exhibit resistance to EPNs due to the sequestration of secondary plant metabolites from maize plants, such as benzoxazinoids (BX), rendering them to some extent resistant against nematode attacks (Robert et al. 2017). Research suggests that EPNs may evolve tolerance or resistance to plant defence metabolites over time (Zhang et al. 2019, Bruno et al. 2020). Engineering bacterial symbionts for desired traits, such as benzoxazinoid resistance, shows potential for enhancing the effectiveness of biological control agents (Machado et al. 2020). Understanding the complex interactions between *Photorhabdus*, *Heterorhabditis* nematodes, and their hosts is crucial for developing sustainable pest management strategies in agriculture and improving the efficacy of nematode biocontrol is needed to improve the economic viability of this approach.

With my PhD thesis, I confirmed that engineered bacterial symbionts do not negatively impact the infectivity of EPNs against WCR larvae, a major maize pest with the ability to sequester benzoxazinoids. Experimental evolution on 6-methoxy-2-benzoxazolinone (MBOA) increased benzoxazinoid resistance in *Photorhabdus* bacterial symbionts. Whole genome sequencing analysis revealed that MBOA resistance was associated with genetic alterations at different loci across the different strains. The detected genomic changes fell into three broad categories: DNA transcription, membrane architecture and membrane channels.

The benzoxazinoid-resistant *Photorhabdus* strains were reintroduced into two strains of *Heterorhabditis bacteriophora* EPNs, resulting in the identification of four nematode–symbiont pairs that effectively targeted and killed benzoxazinoid-sequestering WCR larvae under laboratory conditions. Encouragingly, these engineered pairs demonstrated pest control efficacy in field trials comparable to commercially available nematode strains. The study suggests that modifying bacterial symbionts is a promising strategy for efficient and rapid improvement in the biocontrol of agricultural pests. My research additionally delves into the

broader implications of altering bacterial symbionts, recognizing the intricate interplay between microbiota and pest resistance mechanisms.

My thesis explores the consequences of selecting *Photorhabdus* bacteria for increased tolerance to MBOA. The results illuminate the complex landscape of cross-resistance and collateral sensitivity, demonstrating that MBOA-selected *Photorhabdus* strains exhibit altered tolerance not only to structurally similar compounds like 2-benzoxalinone (BOA) and 6-chloroacetyl-2-benzoxazolinone but also to other environmental toxins such as caffeine, nicotine, and sinigrin. In contrast, the MBOA-selected strains showed increased susceptibility to antibiotics like ampicillin and kanamycin. With my work, I highlighted the importance of assessing cross-resistance and collateral sensitivity in directed evolution efforts.

The major aim of my PhD thesis was to improve the capacity to fight a major agricultural pest, the western corn rootworm, through biological control by engineering novel resistance-traits into bacterial symbionts of entomopathogenic nematodes. This project was expected to provide valuable insights into the dynamics governing interactions among plants, herbivores, nematodes and microbes. The anticipated major outcomes of this project included detailed insights into the potential of forward evolution to enhance both plant toxin resistance and the infectivity of diverse *Photorhabdus* bacterial symbionts. The research aimed to conduct a comprehensive evaluation of how plant toxin resistance impacts performance, cross-resistance and collateral sensitivity to other environmental chemicals, as well as the potential for successful symbiosis. Furthermore, the project achieved a milestone by genomic insights into the genetic basis of plant toxin resistance in *Photorhabdus* and the associated resistance patterns against other toxins.

My conducted research in the laboratory undertook a thorough validation and benchmarking process to assess the potential of plant-toxin resistant *Photorhabdus* symbionts in improving the biological control of WCR under field conditions. As a culmination of these findings, this project developed a roadmap facilitating the practical implementation of engineered symbionts in sustainable agriculture. In a further step, this may involve the engaging of relevant stakeholders to ensure the effective and ethical utilization of the research outcomes for the benefit of agricultural practices.

My PhD thesis incorporated various innovative elements. Firstly, it explored largely uncharted territory by attempting to enhance the services of bacterial symbionts through targeted bioengineering. Despite the prevalence of symbionts across different life forms and their vital functions, there have been limited attempts at targeted bioengineering to improve their services. This project stands among one of the first to showcase the potential of engineering symbionts and improving host performance. Secondly, while substantial efforts are dedicated to breeding biological control agents like parasitoids and entomopathogenic nematodes, microbial symbionts have been largely overlooked as targets for improvement. Existing biocontrol enhancement programs have not specifically addressed these symbionts, despite their common presence in biocontrol agents that carry bacteria and viruses to combat insect hosts. Consequently, this project sought to broaden the scope of biocontrol enhancement initiatives by emphasizing the untapped potential for improving microbial symbionts. Lastly, the project introduced innovative strategies to tackle the challenge posed by plant toxins, which can significantly hinder the effectiveness of natural enemies against herbivorous insects. By focusing on plant toxin resistance as a crucial trait, this research demonstrated how engineered resistance in herbivore natural enemies can empower them to combat herbivores that have ingested plant toxins. This approach did not only advanced current understanding but also underscored the potential of integrating plant toxin resistance into herbivore natural enemies to enhance their capacity for effective top-down control.

References of Conclusion

Aragón P, Baselga A, Lobo JM (2010) Global estimation of invasion risk zones for the western corn rootworm *Diabrotica virgifera virgifera*: Integrating distribution models and physiological thresholds to assess climatic favourability. Journal of Applied Ecology 47: 1026–1035. https://doi.org/10.1111/j.1365-2664.2010.01847.x

Bruno P, Machado RAR, Glauser G, Köhler A, Campos-Herrera R, Bernal J, Toepfer S, Erb M, Robert CAM, Arce CCM, Turlings TCJ (2020) Entomopathogenic nematodes from Mexico that can overcome the resistance mechanisms of the western corn rootworm. Scientific Reports 10: 8257. https://doi.org/10.1038/s41598-020-64945-x

Machado RAR, Thönen L, Arce CCM, Theepan V, Prada F, Wüthrich D, Robert CAM, Vogiatzaki E, Shi Y-M, Schaeren OP, Notter M, Bruggmann R, Hapfelmeier S, Bode HB, Erb M (2020) Engineering bacterial symbionts of nematodes improves their biocontrol potential to counter the western corn rootworm. Nature Biotechnology 38: 600–608. https://doi.org/10.1038/s41587-020-0419-1

Robert CAM, Zhang X, Machado RAR, Schirmer S, Lori M, Mateo P, Erb M, Gershenzon J (2017) Sequestration and activation of plant toxins protect the western corn rootworm from enemies at multiple trophic levels. eLife 6: e29307. https://doi.org/10.7554/eLife.29307

Toepfer S, Gueldenzoph C, Ehlers R, Kuhlmann U (2005) Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. Bulletin of Entomological Research 95: 473–482. https://doi.org/10.1079/BER2005379

Zhang X, van Doan C, Arce CCM, Hu L, Gruenig S, Parisod C, Hibbard BE, Hervé MR, Nielson C, Robert CAM, Machado RAR, Erb M (2019) Plant defense resistance in natural enemies of a specialist insect herbivore. Proceedings of the National Academy of Sciences 116: 23174–23181. https://doi.org/10.1073/pnas.1912599116

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Declaration of Consent

on the basis of Article 18 of the PromR Phil.-nat. 2019

Name/First Name:	**Anja Boss**
Registration Number:	**15-119-977**
Study program:	**PhD in Molecular Life Sciences**
	Dissertation
Title of the thesis:	**Engineering bacterial symbionts to improve the biological control potential of entomopathogenic nematodes against an important agricultural pest**
Supervisor:	**Prof. Dr. Matthias Erb**

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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