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Exploring the Biocontrol Potential of Insecticidal Fluorescent Pseudomonads Applied Alone and in Combination with Entomopathogenic Nematodes and Fungi

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«When one tugs at a single thing in nature,
he finds it attached to the rest of the world.»

– John Muir

«The best thing you can possibly do with your life is
to tackle the motherfucking shit out of it.»

– Cheryl Strayed

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Summary

This thesis focuses on the biocontrol of insect pests using insecticidal *Pseudomonas* bacteria. Fluorescent pseudomonads are well-known for their abilities to promote plant growth, suppress pathogens and induce systemic resistance. In the last decade, research focused on the mechanisms underlying oral insecticidal activity of *P. chlororaphis* and *P. protegens* subgroups as well as their ecological interactions with insects. We aimed at exploiting the insecticidal activity of pseudomonads for controlling belowground insect pests alone and in combination with entomopathogenic nematodes and fungi. Furthermore, we explored the phyllosphere competence of fluorescent pseudomonads, a prerequisite to control foliar pests and pathogens.

In the first part of this thesis, we investigated the potential of *P. chlororaphis* and *P. protegens* strains to control the cabbage maggot *Delia radicum*, an important pest of Brassicacean crops for which no satisfactory control exists. We then combined the most potent strain, *P. chlororaphis* PCLRT03, with the entomopathogenic nematode *Steinernema feltiae* RS5 and the entomopathogenic fungus *Metarhizium brunneum* Bip5 in screening, greenhouse, semi-field experiments and a field trial. The consortium of *P. chlororaphis*, *S. feltiae* and *M. brunneum* could successfully reduce *D. radicum* damage in the field trial and the individual members had no impact on the survival of each other on roots and in the soil. The biocontrol agents applied alone were also effective, yet the *Pseudomonas* strain was more efficient than the nematode and the fungus. Under screening and semi-field conditions, combinations of pseudomonads with either nematodes or fungi resulted in synergistic interactions.

In a next step, the consortium was applied in laboratory assays against the leaf-feeding large cabbage white *Pieris brassicae* and the root-feeding banded cucumber beetle *Diabrotica balteata* and the interaction between the three biocontrol agents inside the larvae was investigated. The triple consortium was the most lethal and fastest killing treatment against both insects. A combination of plating and qPCR approaches allowed us to simultaneously monitor all biocontrol agents including the nematode-associated bacterium *Xenorhabdus bovienii* in the same insect. After simultaneous application, all three agents as well as the xenorhabds established inside the larvae in the early

stages of the infection. *P. chlororaphis* seems to profit from the other BCA and reached the highest colonisation densities in co-infections in both insects. *S. feltiae* and *M. brunneum*, however, seemed to be mutually exclusive in double applications of nematodes and fungi. Interestingly, all four insecticidal organisms could be detected in several individual larvae. These results suggest that *P. chlororaphis*, *S. feltiae* and *M. brunneum* can indeed co-infect the same insect.

In the last part of this thesis, fluorescent pseudomonads were isolated from radish leaves and screened for their abilities to kill insects, suppress pathogens and persist in the phyllosphere. Unfortunately, no *P. chlororaphis* and *P. protegens* strains were discovered, but strains from the subgroups *P. fluorescens*, *P. koreensis* and the group *P. putida*. Several strains showed insecticidal activity upon injection into *Galleria mellonella* larvae. Two leaf isolates of the *P. fluorescens* subgroup showed potent oral insecticidal activity against the diamondback moth *Plutella xylostella*, comparable to *P. chlororaphis* PCLRT03 and *P. protegens* CHA0. Furthermore, the new leaf isolates persisted better in the phyllosphere than the tested *P. chlororaphis* and *P. protegens* strains.

The results obtained in this thesis suggest that insecticidal pseudomonads from the *P. chlororaphis* subgroup can be used to control below-ground insect pests. Furthermore, our findings show that insecticidal pseudomonads are compatible and co-operate with entomopathogenic nematodes and fungi. The consortium of *P. chlororaphis*, *S. feltiae* and *M. brunneum* can potentially be used to control a variety of below-ground insect pests. Applying the consortium might improve efficacy or stability of biocontrol. However, further research on performance in the field and efficacy against different insect pests is needed. The approaches used in this thesis can be used to build and evaluate further consortia against other pests. Finally, the insights gained in this thesis are highly valuable for the development of biocontrol strategies based on insecticidal pseudomonads and consortia of biocontrol agents.

// Zusammenfassung

Diese Arbeit befasst sich mit der biologischen Bekämpfung (Biokontrolle) von Schadinsekten mit Hilfe von insektiziden *Pseudomonas*-Bakterien. Fluoreszierende Pseudomonaden sind bekannt für ihre Fähigkeit, das Pflanzenwachstum zu fördern, Pflanzenpathogene zu unterdrücken und systemische Resistenz zu induzieren. In den letzten zehn Jahren konzentrierte sich die Forschung auf die Mechanismen, die der oralen insektiziden Aktivität der Untergruppen *P. chlororaphis* und *P. protegens* zugrunde liegen, sowie auf ihre ökologischen Interaktionen mit Insekten. Unser Ziel war es, die insektizide Wirkung von Pseudomonaden zur Bekämpfung von Bodeninsekten allein oder in Kombination mit entomopathogenen Nematoden und Pilzen zu nutzen. Ausserdem untersuchten wir die Phyllosphären-Kompetenz von fluoreszierenden Pseudomonaden, eine Voraussetzung für die Bekämpfung von Blattschädlingen und -pathogenen.

Im ersten Teil dieser Arbeit untersuchten wir das Potenzial von *P. chlororaphis* und *P. protegens* Stämmen zur Bekämpfung der Kleinen Kohlfliege *Delia radicum*, einem wichtigen Schädling von Kreuzblütler-Kulturen, für den es keine zufriedenstellende Bekämpfung gibt. Anschliessend haben wir den wirksamsten Stamm, *P. chlororaphis* PCLRT03, mit dem entomopathogenen Nematoden *Steinernema feltiae* RS5 und dem entomopathogenen Pilz *Metarhizium brunneum* Bip5 kombiniert und in Screening-, Gewächshaus-, Semifeldexperimenten und einem Feldversuch angewendet. Das Konsortium aus *P. chlororaphis*, *S. feltiae* und *M. brunneum* konnte die Schäden durch *D. radicum* im Feldversuch erfolgreich reduzieren, und die einzelnen Mitglieder hatten keinen Einfluss auf das Überleben der jeweils anderen auf den Wurzeln und im Boden. Die Biokontroll-Organismen, die allein angewendet wurden, waren ebenfalls wirksam, wobei der *Pseudomonas*-Stamm effizienter war als der Nematode und der Pilz. Unter Screening- und Semifeldbedingungen führten Kombinationen von Pseudomonaden mit Nematoden oder Pilzen zu synergistischen Wechselwirkungen.

In einem nächsten Schritt wurde das Konsortium in Laborversuchen gegen den blattfressenden Grossen Kohlweissling *(Pieris brassicae*) und den wurzelfressenden Gebänderten Gurkenkäfer (*Diabrotica balteata*) eingesetzt und die Wechselwirkung zwischen

den drei Biokontroll-Organismen in den Larven untersucht. Das Dreierkonsortium war die tödlichste und am schnellsten wirksame Behandlung gegen beide Insekten. Eine Kombination aus Kultivierungs- und qPCR-Ansätzen ermöglichte es uns, alle Biokontroll-Organismen, einschliesslich des Nematoden-assoziierten Bakteriums *Xenorhabdus bovienii*, gleichzeitig in demselben Insekt zu überwachen. Nach der gleichzeitigen Anwendung etablierten sich alle drei Organismen sowie die Xenorhabden in den Larven in den frühen Stadien der Infektion. *P. chlororaphis* scheint von den anderen BCA zu profitieren und erreichte die höchsten Kolonisationsdichten bei Koinfektionen in beiden Insekten. *S. feltiae* und *M. brunneum* schienen sich jedoch bei Doppelanwendungen gegenseitig auszuschliessen. Interessanterweise konnten alle vier insektiziden Organismen in mehreren *P. brassicae* und *D. balteata* Larven nachgewiesen werden. Diese Ergebnisse deuten darauf hin, dass *P. chlororaphis*, *S. feltiae* und *M. brunneum* dasselbe Insekt koinfizieren können.

Im letzten Teil dieser Arbeit wurden fluoreszierende Pseudomonaden von Rettichblättern isoliert und auf ihre Fähigkeit untersucht, Insekten zu töten, Pflanzenpathogene zu unterdrücken und in der Phyllosphäre zu persistieren. Leider wurden keine *P. chlororaphis*- und *P. protegens*-Stämme entdeckt, sondern Stämme aus den Untergruppen *P. fluorescens*, *P. koreensis* und der Gruppe *P. putida*. Mehrere Stämme zeigten bei der Injektion in *Galleria mellonella*-Larven eine insektizide Wirkung. Zwei Blattisolate der Untergruppe *P. fluorescens* zeigten starke orale insektizide Aktivität gegen die Kohlschabe *Plutella xylostella*, die mit *P. chlororaphis* PCLRT03 und *P. protegens* CHA0 vergleichbar war. Ausserdem persistierten die neuen Blattisolate besser in der Phyllosphäre als die getesteten *P. chlororaphis*- und *P. protegens*-Stämme.

Die in dieser Arbeit erzielten Ergebnisse deuten darauf hin, dass insektizide Pseudomonaden der Untergruppe *P. chlororaphis* zur Bekämpfung von Insektenschädlingen im Boden eingesetzt werden können. Ausserdem zeigen unsere Ergebnisse, dass insektizide Pseudomonaden mit entomopathogenen Nematoden und Pilzen kompatibel sind und mit ihnen zusammenarbeiten. Das Konsortium aus *P. chlororaphis*, *S. feltiae* und *M. brunneum* kann potenziell zur Bekämpfung einer Vielzahl von unterirdischen Insektenschädlingen eingesetzt werden. Der Einsatz des Konsortiums könnte die Wirksamkeit oder Stabilität der biologischen Schädlingsbekämpfung verbessern. Es sind jedoch weitere Untersuchungen zur Wirksamkeit im Feld und gegen verschiedene Schädlinge erforderlich. Die in dieser Arbeit verwendeten Ansätze können für den Aufbau und die Bewertung weiterer Konsortien gegen andere Schädlinge genutzt werden. Schliesslich sind die in dieser Arbeit gewonnenen Erkenntnisse sehr wertvoll für die Entwicklung von Biokontrollstrategien auf der Grundlage von insektiziden Pseudomonaden und Konsortien von Biokontrollorganismen.

Resumaziun

Jeu dedicheschel la translaziun en romontsch a mia tatta
Agnes Spescha-Guetg (18.5.1924 – 13.9.2022).

Quella dissertaziun s'occupescha cun il cumbat biologic ed insectizid encunter parasits cun agid da bacterias *Pseudomonas*. Igl ei enconuschent che pseudomonads fluoreszents ein habels da promover il carschament da plontas ni era stinschentar excitaders che fan donn e san inducir ina resistenza systematica. Ils davos diesch onns ei la perscrutaziun scientifica seconcentrada sin mecanismen en moda da cumbatter l'activitad da vermanegls ord las subgruppas *P. chlororaphis* e *P. protegens*, sco era siu effect reciproc cun ils insects. Nossa finamira era d'anflar, tgei mortificau effect che pseudomonads han per cumbatter insects che vivan el terren, e quei els sulets ni en combinaziun cun nematods che mazzan insects e bulius e co ins savess nezegiar quei effect. Plinavon havien nus intercuretg la competenza phyllosphära da pseudomonads fluoreszents, la cundiziun per cumbatter parasits ed auters schierms nuschents sin la feglia.

En l'emprema part da nossa lavur havein nus intercuretg il potenzial da *P. chlororaphis* e *P. protegens* per cumbatter la mustga-baguos pintga *Delia radicum*, in impurtont parasit en las culturas da cruciferas, encunter il qual ei dat aunc negins mieds che cuntentan. Silsuenter havein nus combinau il pli efficient tschep, *P. chlororaphis* PCLRT03, cun nematods che portan il bacil *Steinernema feltiae* RS5 ed il buliu cun *Metarhizium brunneum* Bip5 e havein observau cun tests systematics, cun experiments en siara ed auters expriments da pilot, tgei che capetta. Il consorzi ord *P. chlororaphis*, *S. feltiae* e *M. brunneum* muossa en experiments da pilot, ch'ins sa reduzir cun success ils donns dalla mustga-baguos. Ils singuls commembers dal conzorzi han negina influenza sil surviver d'in e l'auter sin las ragischs sco era el terren.

En in proxim pass vein nus sclariu giu co il consorzi secomprovescha encunter la tschittabaguos gronda *Pieris brassicae* ed encunter il bau da cucumeras *Diabrotica balteata* e co igl effect reciproc denter ils treis mieds biologics en las larvas. Il conzorzi da treis ei

la pli sperta e pli mortala sort encunter omisdus insects. La combinaziun da metodas da cultivar bacterias e bulius e tests da qPRC han dau a nus la pusseivladad da survigilar organissems biologics, inclusiv vermegls assoziai cun bacterias *Xenorhabdus bovienii*, da medem temps el medem insect. Nos resultads lain sminar che *P. chlororaphis*, *S. feltiae* e *M. brunneum* ensemen san infectar il medem insect.

En la davosa part da quella lavur vein nus intercuretg co pseudomonads fluoreszents isolai ord feglia da ravanet ein habels da mazzar insects, sco era stinschentar excitaders da plontas e d'insumma secasar sin las plontinas. Deplorablamein vein nus anflau negins tscheps da *P. chlororaphis* e *P. protegens*, mobein tscheps ord las subgruppas *P. fluorescens*, *P. koreensis* e dalla gruppa *P. putida*. Dus isolats ord feglia dalla subgruppa *P. fluorescens* han muossau ina ferma activitad orala encunter la tschittabaguos *Plutella xylostella*, ch'ins sa paregliar cun *P. chlororaphis* PCLRT03 e cun *P. protegens* CHA0. Plinavon muossan ils experiments cun ils novs isolats ord feglia megliers effects sin las plontinas, che quels ord ils tscheps *P. chlororaphis* e *P. protegens*.

Ils resultads che nus vein contenschiu en quella lavur indicheschan, che pseudomonads insecticids dalla subgruppa *P. chlororaphis* ein adattai per cumbatter parasits d'insects el terren. Plinavon muossan nos resultads ch'ils effects da pseudomonads insecticids cun nematods che mazzan insects e bulius ein compatibels e ch'ei lavuran ensemen. Il consorzi ord *P. chlororaphis*, *S. feltiae* e *M. brunneum* ei probabel habels da cumbatter ina gronda part dals parasits d'insects el terren. Il consorzi savess insumma ver in bien effect e sa stabilisar la controlla biologica. Denton ein aunc ulteriuras perscrutaziuns necessarias sur las pusseivladads per cumbatter cun success differents insects el terren. Las consideraziuns ch'ein vegnidas fatgas en quella lavur ein adattas per metter ensemen e valetar auters consorzis encunter differents parasits. Plinavon ein ils da present enconuschents resultads ord quella dissertaziun fetg impurtonts pil svilup da strategias e controllas sil fundament da pseudomonads insecticids e d'auters consorzis da vivents organissems.

A Robert Hitz engraziel jeu da cor per translatar questa resumaziun en romontsch.

Chapter 1

General Introduction

1.1. Biological Control – a key factor to overcome challenges in food production

Despite the efforts by international organisations, global hunger is again on the rise [1]. The main drivers of food insecurity and malnutrition are conflict, climate change, economic shocks and the increasing inequality [2]. The CoViD-19 pandemic has stripped many vulnerable people from their access to food and thereby further exposed various problems across the world food system [3]. Many food production systems are not sustainable and contribute to land degradation, biodiversity loss and greenhouse gas emissions [4]. In high income countries, highly industrialised large-scale agricultural food production relies on high yielding crop varieties, chemical inputs and heavy machinery. In low income countries, small-scale farmers have little access to inputs, therefore achieve low harvests and additionally, are extremely vulnerable to yield losses [2]. One direct threat to crops and thereby to food security are plant diseases, namely weeds, pathogens and pests [5, 6]. Savary et al. [7] estimate yield losses due to plant diseases at 17-30% for potato, soybean, wheat, maize and rice. Highest yield losses occur in low-income countries, which can be explained by the poor access to control strategies as farmers might not afford pesticides or certified seeds and lack knowledge about disease control methods [7]. In high-income countries, the prevalent planting of susceptible cultivars as monocultures and the high fertiliser input provide ideal conditions for pathogen spread [8]. In conventional agriculture, mainly pesticides are used to protect crops [9]. However, pesticides pose a great threat to human and animal health and the environment [10]. Furthermore, the emergence of resistant pathogens and pests renders pesticides ineffective. Alternative pest management methods include cultural methods, crop rotation, resistant cultivars and biological control [11].

Biological control can suppress pathogens and pests below economic damage thresh-

olds and can, in combination with other methods, reduce or even replace pesticide use [12]. Biological control aims at reducing the pest population and its damage by using living organisms [13]. Classical biocontrol refers to the introduction of a natural enemy of an introduced invasive pest, whereas conservation biocontrol specifies the modification of the agroecosystem to enhance natural enemy populations. In augmentative biocontrol, large numbers of biocontrol agents (BCA) are released to achieve a temporary pest control and can be divided in inundative (no BCA reproduction after application) and inoculative (a few generations of BCA reproduction) biocontrol [14]. There are different classes of BCA: Predators (e.g. arthropods, birds and snails) of different pests, parasitoids and entomopathogens (e.g. bacteria, viruses, fungi and nematodes) of insect pests, herbivores and plant pathogens against weeds, antagonists (e.g. bacteria and fungi) of plant pathogens as well as diseases of vertebrate pests [14]. One main advantage of biological control is its safety for farmers, consumers and the environment [15]. There are numerous successful examples of biological control, yet commercial augmentative biocontrol still faces many challenges especially in regard to product registration [16]. In comparison, classical biocontrol is used on 10% of cultivated land, whereas commercial augmentative biological control is applied only on 0.4% of suitable crop land [16]. Despite the hurdles of augmentative biocontrol, the market is growing rapidly with an estimated annual growth rate of nearly 15% [15, 17].

Soil-borne pathogens and below-ground pests are especially difficult to control. Due to their lifestyle in the soil, it is challenging to diagnose soil-borne diseases. Heavy damage to the root system can lead over time to visible wilting symptoms, yet minor damage is difficult to spot and might only be detected at harvest [18]. Furthermore, below-ground pests and pathogens can hardly be targeted by pesticide spraying. Several ubiquitous plant pathogens (e.g. *Pythium* spp. and *Rhizoctonia* spp. damping-off, *Fusarium oxysporum* wilt) and pests (e.g. wireworms, chafers, rootworms) cause considerable damage because little to no pesticides are available for disease control. The effective seed-coating with neonicotinoids to control below-ground pests was banned due to their harmful effects on pollinators [19]. Other effective systemic or semi-systemic pesticides were also banned recently due to environmental concerns or their registrations are under discussion by the authorities [20, 21]. Soil-inhabiting BCA are a promising method to control soil-borne pathogens and pests. The soil is inhabited by a vast amount of bacteria, fungi, oomycetes, nematodes, arthropods, earthworms and other organisms. Plants shape the area around their roots, the rhizosphere, by root exudates. The microbiome around the roots is richer than in the bulk soil and includes plant beneficial microbes [22]. Plants can assemble commensal and mutualistic microbes upon biotic and abiotic stress [23]. Recently, microbiome engineering or inoculation with synthetic communities has been suggested to increase plant health [24, 25]. However, until now,

biocontrol strategies usually consist of the application of one specific BCA strain or one biocontrol product. *Trichoderma* spp., *Bacillus* spp. and *Pseudomonas* spp. are among the most promising BCA against fungal diseases, whereas entomopathogenic fungi (EPF) from the genera *Metarhizium* and *Beauveria* as well as entomopathogenic nematodes (EPN), i.e. *Steinernema* spp. and *Heterorhabditis* spp., are widely used to control below-ground insect pests [26, 27]. Since the discovery of insecticidal *Pseudomonas* species 15 years ago, their potential for dual pest and disease control is explored [28].

1.2. Fluorescent pseudomonads for plant health

Pseudomonas are aerobic, gram-negative γ–proteobacteria that inhabit a wide range of ecological niches and include beside plant beneficial species (mainly *P. fluorescens* and *P. putida* groups) also plant pathogens (*P. syringae*) and opportunistic human pathogens (*P. aeruginosa*) [29]. Fluorescent pseudomonads are competitive root-colonisers and are frequently discovered in disease-suppressive soils [30]. Agricultural soils are rich in pseudomonads and *Pseudomonas* spp. producing specific antimicrobial metabolites are abundant [31]. Many strains with biocontrol abilities belong to the *P. chlororaphis*, *P. protegens* and *P. corrugata* subgroups, while many strains with plant-growth promoting properties belong to the *P. fluorescens*, *P. mandelii*, *P. jessenii* and *P. koreensis* subgroups within the *P. fluorescens* group [32]. Fluorescent pseudomonads owe their fluorescence to a pigment called pyoverdin (Pvd), an extremely potent siderophore (iron-carrier) that increases their competitiveness in the rhizosphere against fungi and bacteria with less potent siderophores [33]. To promote plant growth, fluorescent pseudomonads can produce phytohormones and solubilise plant nutrients, especially phosphorus [34]. For disease suppression, fluorescent pseudomonads can produce a wide variety of antimicrobial substances: 2,4-diacetylphloroglucinol (DAPG), phenazines, pyoluteorin (PLT), pyrrolnitrin (PRN), hydrogen cyanide (HCN) and cyclic lipopeptides [35]. Furthermore, several fluorescent pseudomonads are able to elicit induced systemic resistance (ISR) in plants.

Nearly 15 years ago, a gene cluster encoding an insecticidal toxin was discovered in *P. protegens* and *P. chlororaphis* genomes and termed *P. fluorescens* insecticidal toxin (Fit) [45]. Shortly afterwards, the first studies reported oral insecticidal activity by strains of the *P. protegens* and *P. chlororaphis* subgroups, which will be referred to as entomopathogenic pseudomonads (EPP) [46, 47]. The Fit toxin is expressed by EPP only in insects and there, mainly in the haemolymph [47, 48, 49]. Fit knockout-mutants still expressed limited insecticidal activity and *P. fluorescens* subgroup strains that do not carry the Fit cluster were also able to kill insects [40, 43, 46]. Accordingly, other secondary metabolites and toxins were described to contribute to the insecticidal activity,

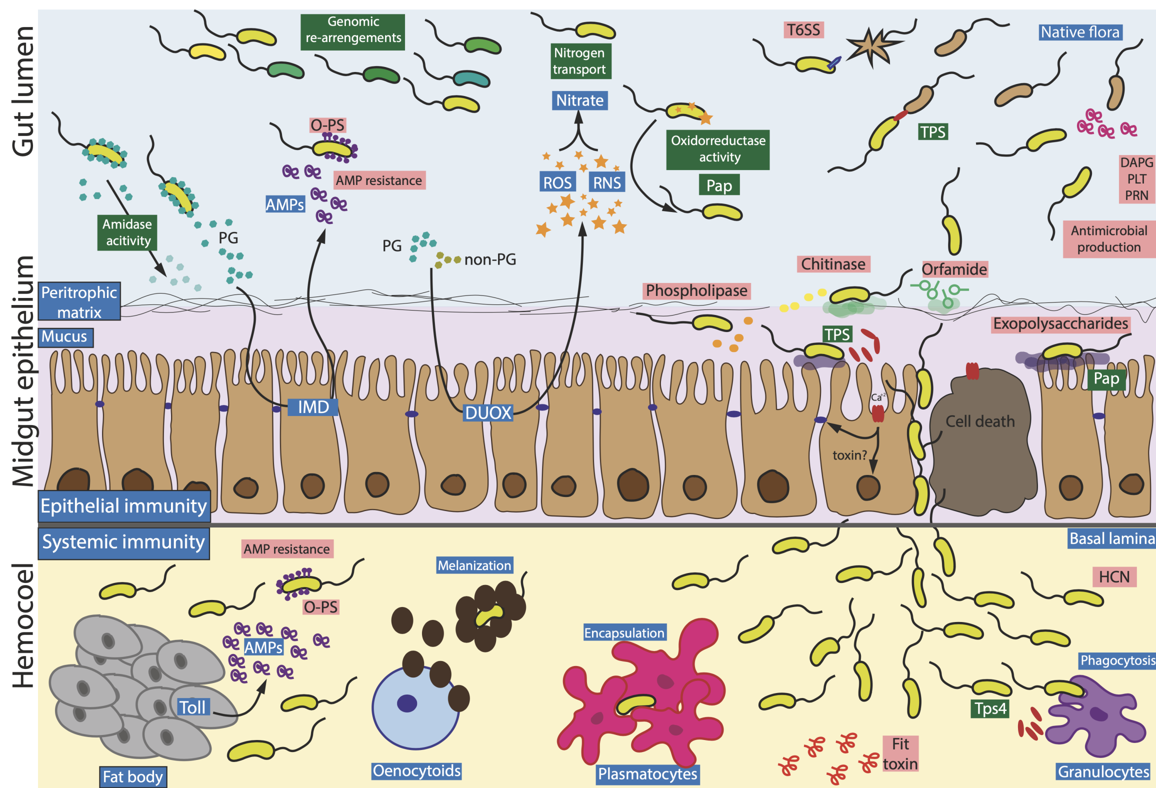

Fig. 1. **Proposed pathogenesis model of *P. protegens* CHA0 infecting a Lepidopteran insect pest after oral uptake drawn by Pilar Vesga [36]**

Vesga et al. [36] proposed the following pathogenesis model with insect immune responses marked in blue and CHA0 factors discovered in their study in dark green and factors known prior to their study in pink. Upon pathogen invasion, the insect will detect bacterial components and trigger the immune response, namely activate the production of reactive oxygen and nitrogen species (ROS and RNS) and antimicrobial peptides (AMPs) [37, 38]. CHA0 tries to avoid immune recognition through amidase activity, protects itself from ROS and RNS using oxidoreductases and the Pap protein and resists AMPs due to its O-polysaccharide surface conformation [39]. CHA0 competes with the resident gut flora by producing antimicrobials [40] and using the type VI secretion system (T6SS) [41]. CHA0 then adheres to the peritrophic matrix, a physical and biochemical barrier [42], using orfamide A [40] and disrupts it using chitinases [43]. To breach the gut epithelial cells, CHA0 relies on different two-partner secretion proteins (TPS) that trigger host cell death. In the hemocoel, CHA0 is attacked by granulocytes, plasmatocytes, oenocytoids and AMPs produced by the fat body [44] and responds by producing the Fit toxin, hydrogen cyanide and TpsA proteins that, together with bacterial multiplication, kill the insect [36].

namely rhizoxin [50], two-partner secretion proteins (TPS) [36], chitinases, phospholipases and orfamide [43]. The Type VI secretion system (T6SS) and antimicrobial exoproducts are important to suppress the resident gut microbiome that is composed of bacteria, fungi, viruses, archaea and protozoa [40, 41, 51, 50, 52]. For the *P. protegens* type strain CHA0 a pathogenesis model was proposed by Vesga et al. [36] as shown in detail in Fig. 1. Shortly, CHA0 is taken up by an insect feeding on a colonised plant root. In the insect gut, it has to defend itself against the first insect immune response and the

resident gut flora. Subsequently, CHA0 breaches through the gut epithelium and multiplies in the haemolymph after defeating the insect's immune response, finally leading to septicaemia and insect death [36]. Furthermore, EPP were shown to persist through different insect stadia (from larva to pupa to adult) and be transmitted by adults to new host plants [53, 54]. Additionally, EPP have been isolated from healthy arthropods in agricultural fields [55]. These studies suggest that EPP are naturally associated with insects and might even have a commensal relationship with insects.

Despite the vast amount of studies on the biocontrol activity of fluorescent pseudomonads - mainly against plant pathogens - there are relatively few biocontrol products available [28]. In Europe, only the *Pseudomonas* sp. DSMZ 13134 and MA342 are registered and sold as products called Cedress, Proradix, Cedomon and Cerall. The renewal of the approval of MA342 is under criticism due to the production of a rhizoxin analog and additionally, its taxonomic identification as *P. chlororaphis* is doubtful. In the US, two *P. chlororaphis* strains are commercially available against various oomycete and fungal pathogens. Remarkably, *P. chlororaphis* AFS009 (product Howler) was selected by AgBiome among 60'000 isolated microorganisms and might become available in Europe in the next few years [56]. Several other fluorescent pseudomonads are marketed or registered in the US [28]. Interestingly, in the effort to identify a bacterial or natural product to control the invasive quagga and zebra mussels, the fluorescent pseudomonad strain CL145A was discovered to be highly lethal against both mussels. The product Zequanox containing killed CL145A cells has a low risk of non-target impact and is registered for controlling quagga and zebra mussels in limited open-water environments and in industrial water systems facilities [57]. In South Korea, Russia and India, several *Pseudomonas* spp. products are commercially available [28]. In South Korea, products containing *P. chlororaphis* O6 are marketed for controlling aphids and nematodes and as microbial fertiliser, which is the first EPP product for insect control [58]. However, the overall availability of *Pseudomonas* biocontrol products is very limited against pathogens and nearly absent against insect pests.

Possible bottlenecks for successful product commercialisation are unreliable performance of BCA in the field, BCA formulation, regulatory issues and reluctant farmers [59]. Product formulation and shelf life of non-sporulating bacteria are challenging but have improved in the last few years [60]. To increase uptake of BCA products by farmers, efficient products and a dialogue with farmers about the expected effect of BCA applications are important. Höfte [28] identified the inconsistent field performance and the 'slow, difficult and costly registration process' as the main factors limiting the development of commercial *Pseudomonas* products. The EU registration process is not adapted for microbial BCA and has no clear guidelines for appropriate toxicity tests. For example, it does not differentiate between BCA secondary metabolites and degradation

products of chemical pesticides, even though there is a huge difference between BCA producing low amounts of secondary metabolites that are commonly found in the rhizosphere and a broad-range application of a chemical that is degraded into products that do not occur in nature. The regulation needs to be updated to include a clear terminology and appropriate data requirements [61]. To improve the consistency and reliability of field application, several approaches should be explored, including the use of BCA consortia [62].

1.3. Biocontrol agent consortia for improved crop protection

Overall, biocontrol is a suitable and effective method for controlling below- and aboveground pests and pathogens in agricultural fields. The bacterium *Bacillus thuringiensis* (Bt) is the most widely applied BCA and a successful example of augmentative biocontrol [63]. Bt is a gram-positive soil bacterium that forms spores and, during sporulation, forms δ-endotoxins better known as Cry proteins which have a narrow host range. The application mainly against leaf-feeding Lepidopteran insect pests, either as bacterial inoculant or by expressing its Cry toxins in plants, led to resistant pest populations [64]. Granuloviruses are very specific and effective BCA against codling moth larvae, yet emergence of resistances after intense application forces companies to find new resistance-breaking virus isolates [65]. However, resistances are overall rarely reported against BCA and the reported cases were linked to a high exposure of pests to one single BCA with one single mode of action. Fortunately, most BCA rely on multiple modes of action, for example the above described fluorescent pseudomonads as well as the entomopathogenic fungi and nematodes described below. The inconsistent field performance described for fluorescent pseudomonads is frequently observed for many BCA, including EPN and EPF [66]. For effective control, BCA must establish in sufficient densities at the target site, which fails at times due to adverse environmental conditions, the present microbiome or predators of the BCA [67]. Several measures can be taken to improve BCA establishment in the field, ranging from improved formulation to repeated applications. Furthermore, farmers should not solely apply BCA for disease control, but combine biocontrol with other methods to suppress disease incidence, e.g. crop rotation, resistant cultivars, mechanical control or field hygiene [15]. A different yet promising approach is to apply consortia of two or more BCA [62, 67]. The theory behind is simple: in case one BCA fails, the other can still control the pest. BCA consortia have, however, more advantages. BCA consortia can have synergistic effects, especially when BCA inhabit different niches and/or have different modes of action. Applying BCA with different modes of action also limits the likelihood of resistance development. On the other hand, BCA that attack the same pathogen or pest could also inhibit each other e.g. due to competition for resources or direct antibiosis.

The vast majority of biocontrol studies test the single application of different BCA species or strains and only little research was conducted on BCA consortia, i.e. the combined application of two or more BCA. Consortia of bacterial and fungal BCA (often including *Pseudomonas* spp., *Bacillus* spp. and *Trichoderma* spp.) for controlling soil-borne pathogens were reviewed in two different studies. Xu et al. [68] calculated whether combined BCA applications resulted in synergistic, additive or antagonistic effects according to the Bliss formula of independence. From their selected combinations, only in 2% of cases synergistic effects were detected. Comparing the combination to the best performing single BCA application, efficacy was improved in 15% and reduced in 9% of cases [68]. Niu et al. [69] found mainly improved efficacy upon application of microbial BCA consortia and linked these to increased rhizosphere colonisation and increased suppression of pathogens. By combining fluorescent pseudomonads and EPF *B. bassiana*, simultaneous below-ground pest and disease control was achieved in rice and groundnut [70, 71, 72]. Several studies explored combined applications of EPF and EPN against a variety of insect pests in laboratory, greenhouse or field trials and mainly discovered additive and/or synergistic effects [73, 74, 75, 76, 77, 78, 79, 80, 81]. For example, Bueno-Pallero et al. [77] found that co-infections of *B. bassiana* and *S. feltiae* in the model insect *Galleria mellonella* resulted mainly in additive effects. Shapiro-Ilan et al. [82], on the other hand, observed that combined application of the EPN *H. indica* and *S. carpocapsae* with EPF *M. anisopliae*, *B. bassiana*, *Cordyceps fumosoroseus* or EPB *Serratia marcescens* against the pecan weevil resulted mainly in antagonistic effects, except for the combination of *H. indica* with *M. anisopliae*, which was additive. Ansari et al. [73] achieved synergistic effects when combining *S. kraussei* and *M. anisopliae* to control black vine weevils. These examples illustrate that EPN and EPF can increase control efficacy yet it depends on the applied species and strains, the targeted insect and the application (dose and method). Similarly, the combined application of EPN and Bt against different pests resulted in additive and synergistic interactions [81, 83, 84, 85, 86, 87]. In two field studies, combinations of EPN, EPP and arbuscular mycorrhizal fungi (AMF) could protect plants from insect damage [88, 89]. The literature indicates that BCA consortia have the potential to achieve a more stable or even more efficient pest control depending on the chosen consortia members.

The review by Xu et al. [68], focusing on biocontrol of soil-borne pathogens using rhizosphere competent bacteria and fungi, reports antagonistic interactions amongst BCA in the majority of cases. These BCA inhabit, on a large scale, the same habitat - the rhizosphere - and might compete with each other for resources, resulting in antagonistic effects [68]. Niu et al. [69], on the other hand, describe an increase in rhizosphere colonisation by consortia, which was linked to enhanced biofilm formation (i.e. multi-species biofilms), syntrophy (i.e. cross-feeding) and facilitated migration. Furthermore,

the increased pathogen suppression by consortia was explained by higher competition for resources between a pathogen and a consortium compared to a single BCA and also by a greater number of produced antimicrobial compounds [69]. These contrasting reviews both highlight the importance of selecting BCA species and strains that are compatible with each other. The listed studies focusing on below-ground pest control using EPF, EPN and Bt rarely reported antagonistic effects, but mainly additive and synergistic effects. The increased lethality against insects when combining EPF and EPN is speculated to arise from the cocktail of insecticidal and antimicrobial substances that both BCA produce, and possibly by the EPF damaging the tissue to facilitate EPN infection [77, 90]. The different lifestyles in the soil might further make EPF and EPN compatible for combined application. However, to construct a consortium of soil-inhabiting BCA against below-ground pests, the ecology and mode of action of each BCA must be explored.

The focus of this thesis is the biocontrol of below-ground insect pests using insecticidal pseudomonads. Because of the unstable performance of pseudomonads in the field and the indications that consortia can achieve more stable or more efficient biocontrol, EPP were combined with two different entomopathogens, namely EPN and EPF. At the start of this thesis, EPP and EPN had only been combined in two field studies [88, 89] and no literature was available on EPP and EPF combinations, only on fluorescent pseudomonads and EPF [70, 71, 72]. These studies gave a first insight that EPP might be compatible with EPN and EPF. Recently, EPP were isolated from EPN and EPN-infested cadavers, suggesting a frequent association in nature [91, 92, 93]. Because a major part of this thesis focuses on applying EPP with EPN and EPF to control insect pests, EPN and EPF will be shortly introduced in the following sections.

1.3.1. Entomopathogenic nematodes

Steinernematidae and Heterorhabditidae nematodes live in the soil, hunt for insects for nutrition and kill them rapidly, which makes them suitable BCA against below-ground insect pests [94]. Though other nematode species also exhibit insecticidal activity, the term entomopathogenic nematodes is mainly used to refer to *Steinernema* and *Heterorhabditis* species. Some EPN species are found only in specific habitats, but others like *S. feltiae*, *S. carpocapsae*, *H. bacteriophora* and *H. indica* have a cosmopolitan distribution [95]. In the last decades, companies developed and improved product formulations and industrial rearing processes for EPN [96, 97, 98, 99]. The insecticidal effect of EPN is due to a symbiosis between EPN and entomopathogenic bacteria (EPB): *Steinernema* spp. are associated with *Xenorhabdus* spp. and *Heterorhabditis* spp. with *Photorhabdus* spp., both gram-negative γ-proteobacteria. The symbiosis between EPN and nematode-associated bacteria (NB) was long believed to be monoxenic, i.e. that

one EPN species is associated with one NB species, yet recent studies suggest that the association is less fixed [100]. Maher et al. [101] found that one EPN species was associated with two NB species and Ogier et al. [91] observed that EPN infective juveniles (IJ) carry beside their NB core symbiont around a dozen frequently associated Proteobacteria including pseudomonads.

In order to infect an insect host, the free-living stage, the IJ, carry the NB and search for a host. Different EPN species deploy different hunting schemes: ambushers (e.g. *S. carpocapsae*) wait in the soil for an approaching host, while cruisers (e.g. *H. bacteriophora*) actively search for a new host, yet the schemes vary considerably between species and some species show intermediate foraging schemes (e.g. *S. feltiae*) [102]. EPN then enter their host through natural openings, i.e. mouth, anus and spiracles, though some Heterorhabditidae can also penetrate the insect cuticle [103]. The IJ move to the hemolymph and release the NB, where the NB then produce several insect toxins. 'makes caterpillars floppy' (Mcf) toxins are only active upon injection and are a dominant factor for insecticidal activity [104]. Toxin complexes (Tc) are large three-component toxins with oral activity and were also detected in genomes of other EPB [105]. These are employed both by *Photorhabdus* and *Xenorhabdus* species, while *Photorhabdus* additionally produce '*Photorhabdus* insect-related' (PirAB) and '*Photorhabdus* virulence cassettes' (Pvc) toxins [105]. The Mcf toxins show high sequence similarity with the *Pseudomonas* Fit toxin, whereas PirAB has similarity to Bt Cry toxins [47, 105]. NB compete with and suppress the resident insect microflora by producing a variety of antimicrobial substances, the T6SS and tailocins [106, 107, 108, 109, 110]. The EPN themselves also produce insecticidal toxins [100, 111]. Insects usually succumb within 24-48 h to EPN infection and are degraded by the NB. The EPN feed on the NB and the degraded insect tissue and develop through the 4th and 5th juvenile stage to adults. Most *Steinernema* species have males and females that sexually reproduce (i.e. gonochorism), while *Heterorhabditis* species have a first hermaphroditic generation followed by a gonochoristic second generation [95]. After several generations of reproduction, resources become scarce in the cadaver and the second juvenile stage develops into infective juveniles (also called dauer juveniles) instead of the third juvenile stage. IJ are characterised by a double cuticle layer for improved desiccation tolerance and the closing of all body openings, i.e. mouth and anus, and hence do not feed anymore [112]. *Heterorhabditis* species only form IJ upon a process called 'endotokia matricida' where eggs hatch inside the female or hermaphrodite nematode and the juveniles feed on the maternal tissue [113]. Upon IJ formation, IJ take up roughly 100 NB cells: *Steinernema* form a specialised structure called receptacle for *Xenorhabdus*, while *Photorhabdus* locate in the intestinal lumen of *Heterorhabditis* [95]. The NB rely on the IJ to be carried to a new insect host, whereas the EPN benefit from the NB killing the host and depend

on the NB for successful reproduction [100].

EPN products containing, among others, *H. bacteriophora*, *S. carpocapsae* and *S. feltiae* strains are marketed to control several insect pests from the orders Coleoptera, Diptera, Lepidoptera, Orthoptera and Thysanoptera in crops, vegetables, greenhouses, orchards, pastures and forestry [114]. Successful application depends mainly on selecting a suitable EPN species or strain for the target host and according to the environmental conditions [115]. IJ are very sensitive to UV light and need moist soil to be able to move. To improve field applications, UV protectants are added to product formulations and applications early in the morning or in the evening reduce direct exposure to sunlight [115]. EPN virulence can be increased by creating stable populations through hybridisation (EPN breeding) and by adaptation to abiotic stress factors, e.g. by selecting for cold stress or desiccation tolerant populations [96, 97, 98, 99]. Mass production *in vitro* in solid or liquid fermentation or *in vivo* as well as formulation and application as powders, gels or using cadavers are continuously improved. Koppenhöfer et al. [115] identify efficacy, costs and ease of use (short shelf-life) as the most limiting factors for EPN use that need further improvement.

1.3.2. Entomopathogenic fungi

Over 750 species of entomopathogenic fungi were discovered, but around 80% of commercial products contain strains from the genus *Metarhizium* and *Beauveria* [116] belonging to the order Hypocreales (Ascomycota). The order Entomophthorales (Entomophthoromycota) also contains several highly virulent species that cause epizootic disease outbreaks in natural insect populations. *Entomophthora muscae* was observed to infect *Delia* spp. pest insects, yet it is an obligate pathogen and cannot be easily cultured in the laboratory and is so far not used in biocontrol [117]. The Hypocreales order contains three famous genera of EPF, namely *Metarhizium* (family Clavicipitaceae), *Beauveria* and *Cordyceps* (family Cordycipitaceae). Most EPF used in biocontrol have a wide host range, e.g. *M. anisopliae*, *B. bassiana* or *C. fumosorosea* (formerly *Isaria fumosorosea*) [118], while *B. brongniartii*, for example, is mainly effective against *Melolontha* grubs [119, 120, 121]. Both *M. anisopliae* and *B. bassiana* are globally distributed, yet *M. anisopliae* is abundant in agricultural soils, while *B. bassiana* is more commonly found in natural habitats [122, 123, 124]. In recent years, *Metarhizium* and *Beauveria* were both discovered to be closely related to distinct grass endophytes and were shown to grow endophytically themselves, i.e. inside asymptomatic plant tissues [125]. Negative effects were reported when insects were feeding on plant tissue endophytically colonised by EPF, but the mechanisms are not yet understood [126]. EPF were further reported to colonise the rhizosphere, promote plant growth, induce systemic resistance and suppress fungal plant diseases [127].

Butt et al. [116] and Moonjely et al. [128] thoroughly reviewed and described how the EPF *Metarhizium* and *Beauveria* infect insect hosts. The infection process starts with the adhesion of conidia to the insect cuticle. Conidia possess a layer of hydrophobin proteins that ease adhesion to the hydrophobic cuticle and cell wall proteins also contribute to adhesion. The spores then germinate on the cuticle and grow as hyphae on the insect. To invade the host, EPF form an appressorium and use an array of proteases and chitinases to penetrate the cuticle by mechanical pressure and enzymatic degradation. Once the hyphae reach the hemocoel, the fungus switches to blastospore growth, which are yeast-like cells that are formed in nutrient rich environments. EPF kill the insect within one week of proliferation in the hemocoel by absorbing the nutrients in the haemolymph, invading tissues and organs and producing insecticidal toxins. After taking up all the nutrients, EPF grow as hyphae throughout the cadaver and form conidia on the cadaver surface, which are green for *Metarhizium* and white for *Beauveria*. During the infection process, EPF evade or limit the host immune response by repressing proteases that trigger melanisation, hiding its surface carbohydrates from detection, expressing immune evasion proteins and suppressing host immune system signalling pathways. To inhibit microbes and kill the insect, EPF produce a wide range of antimicrobial and insecticidal metabolites, which include cyclosporine, swainsonine, and destruxin for *Metarhizium* and bassianin, bassiacridin, bassianolid, tenellin, and oosporein for *Beauveria*.

EPF products are applied against locusts and grasshoppers, soil-dwelling Coleoptera, Lepidoptera and Diptera, as well as Hemipteroidea (piercing and sucking pests) [129]. A main advantage of using EPF over bacteria and viruses to control insect pests are that they do not need to be taken up orally. On the other hand, the slow killing speed limits their use for pest control [123]. Similar to EPP and EPN, EPF are sensitive to abiotic conditions, mainly to UV light, temperature and moisture. To decrease the effects of soliar radiation, UV protectants are added to formulations with limited success. EPF are usually either applied as spray treatment on the soil or plants using liquid formulations, or incorporated into the soil as granules. In recent years, ‘attract-and-infect’ devices were developed to attract pests to EPF-inoculated granules or beads. In products, EPF are mainly present as conidia, yet microsclerotia and blastospores are also being tested for product formulation [129]. EPF grow and sporulate on many simple substrates such as barley kernels or rice grains, but it is challenging to produce high quality products with a high number of viable conidiospores [130, 131].

1.4. Aims of this thesis and thesis outline

Fluorescent pseudomonads are aggressive root colonisers with plant-beneficial and disease suppressive traits. *Pseudomonas chlororaphis* and *P. protegens* additionally possess potent oral insecticidal activity. While their potential as biocontrol agents against plant pathogens was explored extensively, their use as BCA against insect pests was little studied so far. One major aim of this thesis was to explore the efficacy of insecticidal pseudomonads to control an important below-ground root pest. To improve reliability of biocontrol, entomopathogenic pseudomonads were combined with entomopathogenic nematodes and entomopathogenic fungi. To gain more insight into how the BCA interact, the co-existence of EPP, EPN and EPF was investigated during joint infection of an insect. Fluorescent pseudomonads are mainly applied against soil-borne diseases, yet EPP are also efficient in killing above-ground pests. In the last part of the thesis, the focus lay on the quest to discover pseudomonads with insecticidal properties and a good survival potential on leaves. The following four major aims were addressed in the different chapters:

1. *Biocontrol of a below-ground pest using insecticidal pseudomonads*
   P. chlororaphis and *P. protegens* were shown to infect, colonise and kill several insect pests from different orders, yet prior to this thesis, these bacteria were applied only in one study to control a root-feeding insect pest in the field. In **chapter 2**, different strains of these two *Pseudomonas* species were evaluated for their efficacy in killing the cabbage maggot *Delia radicum* and the most promising EPP *P. chlororaphis* strain was further tested for its ability to control this pest in pot, semi-field and field trials. Comparing its efficacy with other biocontrol agents which are already registered for below-ground pest control revealed that *P. chlororaphis* PCLRT03 has an even greater potential for cabbage maggot control. **Chapter 2** describes *P. chlororaphis* as new and promising biocontrol agent for below-ground pests.

2. *Development of a reliable control method for root pests based on combinations of insecticidal pseudomonads with other entomopathogenic biocontrol agents*
   Since the unstable performance of single BCA in the field is a major limitation of biocontrol, a BCA consortium was sought by combining the pseudomonads with other soil-inhabiting entomopathogens used for biocontrol. EPN and EPF are both widely applied against below-ground insect pests and were shown to have additive and synergistic effects when applied in combination. In **chapter 2**, EPP were combined with EPN *S. feltiae* and EPF *M. brunneum*. All biocontrol agents alone and in combination provided significant control and were able to reduce *D. radicum* survival in pot experiments and damage in the field. In the field trial, EPP

performed significantly better than EPN and EPF alone, and similar to the triple combination. Synergistic effects were observed for double combinations with EPP in the screening (EPP-EPN and EPP-EPF) and the semi-field trial (EPP-EPN). The results of **chapter 2** indicate that EPP are compatible with EPF and EPN, that combinations of EPP with EPF and EPN can lead to synergism and that a consortium of EPP, EPN and EPF can be used to efficiently control below-ground pests in the field.

3. *To gain insight into the interaction between EPP, EPN and EPF in co-infections*
   The limited research on biocontrol consortia rarely examined the interaction between the BCA in detail. The consortium selected in chapter 2 was used to study the insecticidal effect and the colonisation of insects by BCA during co-infection of the leaf-feeding large cabbage white *Pieris brassicae* and the root-feeding banded cucumber beetle *Diabrotica balteata* in **chapter 3**. The triple combination reached the highest mortality and killing speed against both insects, yet EPN-EPP were more efficient against *P. brassicae* and EPN-EPF against *D. balteata*. All BCA were found in the larvae at early stages of the infection and over time, less co-colonisation events were detected. This indicated that all BCA contributed to insect killing but competed for resources in the decaying cadaver. Altogether, the increased efficacy of the triple combination against a Lepidopteran and a Coleopteran pest insect observed in **chapter 3** indicates that the selected consortium is suitable for biocontrol against various insect pests.

4. *Discovery of fluorescent pseudomonads with insecticidal activity that are adapted to survival in the phyllosphere*
   EPP root strains were shown to be highly efficient in killing Lepidopteran pest insects feeding on plant leaves [47, 53] but persisted poorly on leaves, which raised the question whether we can discover strains on leaves with good phyllosphere persistence and activity against foliar pests and diseases. In **chapter 4**, fluorescent pseudomonads were isolated from radish leaves and investigated for their phylogeny, insecticidal activity, disease suppression *in vitro* and *in planta*, and persistence in the phyllosphere. Several isolates of the *P. fluorescens* subgroup exhibited insecticidal activity upon injection and ingestion and provided protection against an oomycete plant pathogen. Remarkably, two isolates reached similar mortality and killing speed against *P. xylostella* as tested *P. chlororaphis* and *P. protegens* strains. The new leaf isolates persisted better on wheat leaves than the tested reference strains. The findings from **chapter 4** indicate that, in addition to the control of root pests and diseases, certain fluorescent pseudomonads might also be suitable to control foliar pests and diseases.

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Chapter 2

The More, the Merrier: Combining entomopathogenic *Pseudomonas* bacteria, nematodes and fungi for biocontrol of a below-ground insect pest

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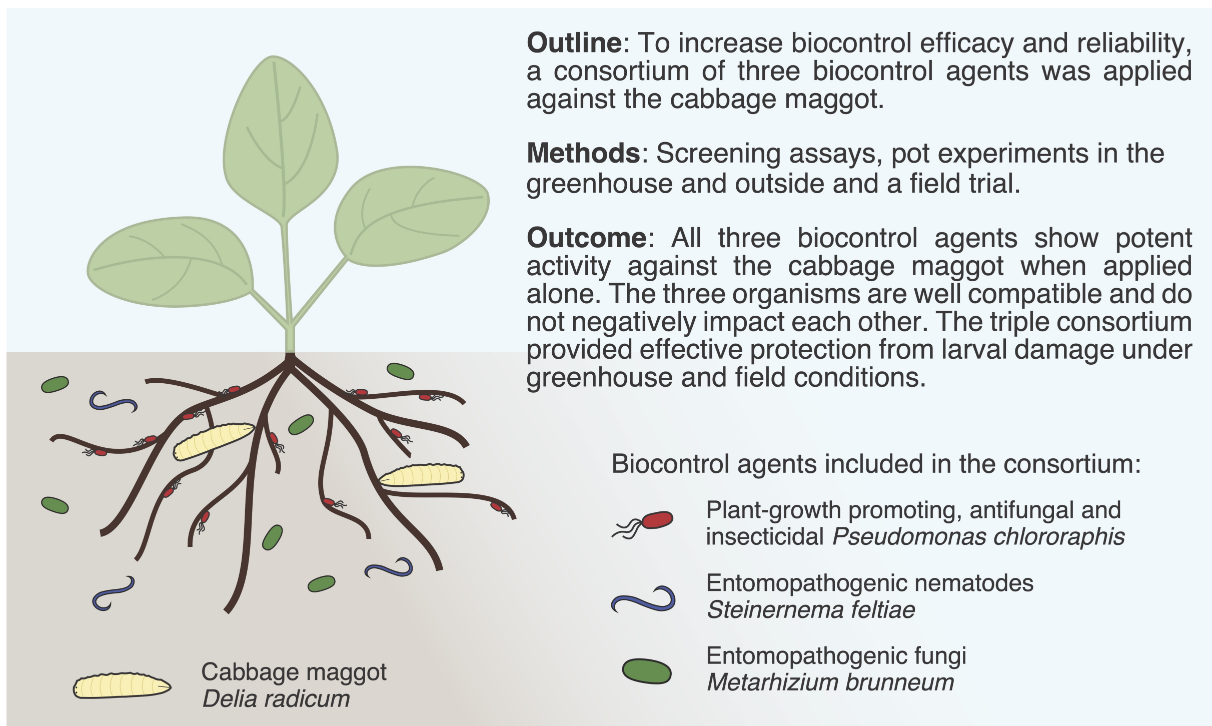
Abstract

Below-ground pests are challenging to control because they are well concealed in their subterranean environment and difficult to target with control measures. Moreover, broad spectrum soil insecticides are or will soon be banned due to their negative effects on non-target organisms. For these reasons, alternatives to synthetic insecticides are urgently needed. The application of soil-inhabiting biocontrol agents (BCAs) is a promising approach to control root-feeding insects. However, the efficacy of biocontrol products based on a single BCA is often unsatisfactory because their performance can vary strongly depending on prevailing environmental conditions. One approach to overcome the limited stability of single BCA is to use multiple BCAs with different modes of action. To evaluate the efficacy of this approach we tested how three BCAs (bacteria, nematodes, and fungi) alone and in combination controlled the cabbage maggot *Delia radicum* (Diptera: Anthomyiidae), a significant pest of Brassicacean crops, using a combination of lab, greenhouse, and field-based trials.

First, we tested if fluorescent *Pseudomonas* bacteria with antifungal and insecticidal activity can control below-ground *D. radicum*. These bacteria have the advantage that they can be used in a dual strategy against insect pests and fungal plant diseases. Second, we investigated the compatibility of insecticidal pseudomonads (*Pseudomonas chlororaphis*) with entomopathogenic nematodes (*Steinernema feltiae*) and entomopathogenic fungi (*Metarhizium brunneum*), two well-established biocontrol organisms used against below-ground insect pests. Third, we tested the effect of combinations of these BCAs on *D. radicum* development and maggot-induced damage on radish bulbs in a series of pot experiments with artificial cabbage maggot infection performed in the greenhouse and outdoors and in a field trial with natural infestation. Our results show that i) insecticidal pseudomonads are highly efficient in *D. radicum* control, ii) the three BCAs are compatible and neither inhibit each other's infectiousness nor survival in the soil or on the roots and iii) the triple combination reduced both pest survival in greenhouse experiments and maggot-induced damage on radish bulbs in the field, by 50%. The strategy we present here is a promising step forward to a reliable and efficient environmentally friendly biological control method for the cabbage maggot, which can also be adapted to other problematic below-ground pests.

Graphical Abstract



Introduction

Since the rise of agricultural food production, crop yields have been under constant threat from pathogens and pests, both above and below-ground [1, 2]. Monocropping is particularly beneficial for the spread of these pathogens and pests as it provides a high density of suitable host plants within a close range [3]. Conventional large-scale agriculture relies heavily on pesticides to protect yields [4] but pesticide use is linked to a multitude of problems. The widespread and excessive application of pesticides containing the same group of active ingredients leads inevitably to the development of resistances, rendering the product less- or ineffective [5]. Pesticide use also affects the environment, for example, by harming non-target organisms, many of which are essential to our food production [6]. The most well-known example is the massive lethal and sub-lethal effects of neonicotinoids on bees as well as many other species [7]. However, there are many pesticides that have detrimental non-target effects and this has led to bans of multiple pesticides, including insecticides targeting below-ground pests, [8, 9] and an increasing demand for alternative control measures.

Biological control agents (BCAs) are a promising alternative control measure and BCA products are a rapidly expanding market [10, 11, 12]. The application of BCAs can reduce pest or pathogen damage below an economic threshold [13, 14, 15]. The most widely used biological control agent (BCA) is the entomopathogenic bacterium (EPB) *Bacillus thuringiensis* (Bt) [16]. It produces effective, small-host-range Cry toxins that are also commonly expressed in transgenic crop plants [17]. However, the widespread use of Cry toxins either as BCA or in transgenic plants has led to the emergence of insects that are resistant to these toxins [18].

One approach to overcome the limitations of a single BCA application is to combine BCAs with other management methods, or to combine different biocontrol agents [19]. Only a few studies have explored this option so far with mixed results. These involved some lab, greenhouse and field studies using combinations of nematodes with either fungi or bacteria against different insect pests [20, 21, 22, 23, 24, 25, 26]. Combinations of BCAs sometimes had improved or neutral effects, others reported antagonistic effects, and results often varied strongly depending on the year (e.g. for field trials) or with the application technique. To increase biocontrol efficacy and consistency we need to develop effective combinations of BCAs i.e. biocontrol consortia, which have been rigorously tested using *in vitro*, greenhouse and field trials for not only their biocontrol efficacy but their compatibility. Thus, the goal of this study was to test three BCAs with different modes of actions for their compatibility and for their combined effect on an important root pest. We chose to test and combine entomopathogenic pseudomonads (EPP), entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF)

for controlling the cabbage maggot *Delia radicum*.

Pseudomonas protegens and *Pseudomonas chlororaphis* are root-colonizing bacteria which are especially interesting for agriculture because they possess plant-growth promoting and disease suppressive capacities [27, 28, 29, 30] and additionally have potent oral insecticidal activity [31, 32, 33]. The relationship of entomopathogenic pseudomonads (EPP) with insects and the determinants of their insecticidal activity have been extensively studied over the last 15 years, especially for the model strains *P. protegens* CHA0 [34] and Pf-5 [35]. EPP rely on multiple factors to infect and kill insects: toxins like Fit (*P. fluorescens* insecticidal toxin) [36, 37, 31], rhizoxin [35] and TPSA's (two-partner secretion proteins) [38], enzymes such as chitinases and phospholipases [32], the Type 6 secretion system (T6SS) [39], as well as antimicrobial exoproducts [40, 41, 35]. The versatile life-style and multifactorial mode of action of EPP makes them ideal biocontrol agents with little risk of resistance development and dual or even triple use: they can be applied against fungal pathogens and insect pests while at the same time promoting plant growth and vigour in general.

Despite their many plant-beneficial activities, there are only a few *Pseudomonas*-based products on the market, and these are registered as antifungal agents (e.g. Cerall / Cedomon and Proradix in the EU and in Switzerland) or are ingredients in biofertilizers [13, 15]. So far, the biocontrol potential of pseudomonads against insect pests has not been commercially explored. Yet, DuPont Pioneer has inserted an insecticidal protein derived from a *P. chlororaphis* strain into a corn variety, rendering plants resistant to corn rootworms (*Diabrotica* spp.) [42, 43, 44, 45, 46].

Entomopathogenic fungi and nematodes are widely used to control insect pests and hundreds of EPF- and EPN-based biocontrol products are commercially available. The most commonly applied EPF species are *Metarhizium anisopliae* (Ma) and *Beauveria bassiana* (Bb) [12]. Both Hypocrealean EPF rely on multiple exoproducts to overcome the insect's defence mechanisms and kill it, such as proteases, chitinases, lipases, immunomodulation and transcription factors, as well as beauvericin, bassianin and oosporein for Bb, and cyclosporine and destruxin for Ma [47, 48, 49, 50]. EPN are associated with specific bacteria (nematode-associated bacteria, NB) that play the most important part in killing the insect, e.g. *Steinernema feltiae* with *Xenorhabdus bovienii* and *Heterorhabditis bacteriophora* with *Photorhabdus luminescens* [51]. EPN enter the insect mainly through natural openings and carry their NB into the haemolymph [52, 53]. In the haemolymph, NB express different insecticidal toxins, e.g. Tc's (toxin complexes) and Mcf (makes caterpillar floppy), and suppress both the hosts immune system as well as other microbes, i.e. using antimicrobials and T6SS, while EPN express venom proteins or ESPs (excreted/secreted products) [54, 55, 56, 57, 58]. As in EPP, the ento-

mopathogenic activity of EPF and EPN relies on multiple mechanisms, which renders the evolution of resistance in insects very unlikely.

The pest used in this study is the cabbage root fly or cabbage maggot *Delia radicum* L. (Diptera: Anthomyiidae) which poses a big challenge for producers of Brassicacean crops. The larvae feed on the below-ground parts of several crops such as canola, cabbage, radish, broccoli and cauliflower. Yield losses occur when larvae feed on the produce, e.g. radish or turnip, or reduce plant growth and seed numbers, or cause seedling death due to heavy root damage, e.g. in broccoli or canola. *D. radicum* infestations can be devastating for vegetable and oilseed producers in temperate regions, with estimated annual economic losses of $100 million in Western Europe and Northern America [59]. Only very few insecticides are available for controlling the cabbage maggot and their efficacy is often limited. For example, cyantraniliprole (registered for *D. radicum* control in Canada) is highly toxic to bees [60] and is less efficient than the formerly widely used chlorpyrifos [61] that is now banned in the EU, Canada and the USA [62, 63, 64]. In Switzerland, only Spinosad is registered for control of the cabbage maggot [65] but this has limited efficacy and may exhibit toxic effects on non-target insects [61, 66]. As a cultural measure, besides crop rotation and weed management, the use of nets is recommended to keep the flies from laying eggs in the field [67]. Though this measure may be very effective, it also complicates field management [68]. There have been several attempts to control the cabbage maggot with entomopathogenic fungi and nematodes. Although laboratory and greenhouse studies identified promising candidates, the efficacy was generally low in field trials [69, 70, 71, 72]. A durable and effective method to control the cabbage maggot based on BCAs would be environmentally and economically valuable.

In order to establish an effective biological control method using multiple BCAs we conducted a series of field and lab experiments to 1) evaluate the potential of insecticidal pseudomonads of the species *P. chlororaphis* and *P. protegens* as novel biocontrol agents for controlling *D. radicum*, 2) investigate the compatibility of selected entomopathogenic *Pseudomonas* strains with entomopathogenic nematodes and entomopathogenic fungi for improved control of below-ground pests, and 3) explore BCA consortia for their potential to control *D. radicum* in comparison to each single BCA under different experimental conditions from the lab to the field. As a first step, EPP and EPN strains effectively killing *D. radicum* and promising EPP-EPN and EPP-EPF combinations were selected under lab screening conditions. The most promising strains were used to form a tripartite consortium, which was tested against the cabbage maggot on radish in greenhouse pot experiments, outdoor pot experiments and a field trial. The impact of individual BCAs on the survival of the other consortium members was monitored at all stages. Using this systematic approach, we have developed an effective

consortium based on BCAs with different modes of action for controlling the cabbage maggot.

Material and Methods

Rearing of organisms

– Cabbage maggot *Delia radicum*

Pupae were obtained from Swiss field sites and from research groups at the Julius Kühn Institute in Braunschweig (Germany) and the University of Rennes (France). Pupae were stored in sand (0.3-0.9 mm) at 3 °C in the dark. To induce fly emergence, the pupae were placed in an insect cage within a climate chamber with the following rearing conditions: 16 h daytime at 20 °C and 15 kLux and 8 h nighttime at 18 °C, and 80% relative humidity. Emerging flies were provided with water by adding wet sand (0.3-0.9 mm) and fed on dry (10 g glucose, 10 g milk powder, 1 g soy flour, 1 g dry yeast) and wet food (5 g honey, 5 g soy flour, 1 g dry yeast, ∼6.5 ml ddH_2O) (all ingredients except for glucose were purchased at Coop Supermarket, Switzerland). To induce egg laying, kohlrabi pieces were placed on the wet sand. Eggs were harvested by pouring the sand in an 800 ml beaker, adding water and filtering the water through a ø 185 mm filter paper folded in a funnel. Approx. 80 eggs were transferred on a ø 90 mm filter paper with a brush and placed in an 800 ml beaker on a 2 cm sand layer. Half a kohlrabi was added on top of the eggs and covered with sand. After four weeks, maggots had completed larval development and the pupae were harvested by rinsing the remainder of the kohlrabi and the sand over a 2 mm and then over a 1.5 mm sieve.

– *Pseudomonas chlororaphis* and *P. protegens*

Bacteria strains (Table 1) were stored at -80 °C in 44% glycerol. Colonies were grown on King's B medium with antibiotics (KB^{+++} with cycloheximide 100 mg/l, chloramphenicol 13 mg/l and ampicillin 40 mg/l resp. KB^{++G} with gentamycin 10 mg/l instead of ampicillin for *gfp*-tagged strains) [73, 74]. For experiments, overnight Lysogeny broth (LB; 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 0.25 g $MgSO_4$ x $7H_2O$, 8 g NaCl dissolved in 1 L ddH_2O) liquid cultures were prepared [75]. These were either used directly or to inoculate KB plates (without antibiotics), where bacteria grow to high numbers within 24 hours. The bacteria were washed in ddH_2O, the optical density at 600 nm (OD_{600}) was measured (Ultrospec 3300 pro, Amersham Biosciences, UK) and suspensions adjusted to the desired concentration with an OD_{600} = 0.125 corresponding to approx. 10^8 cfu/ml.

– *Steinernema* spp. and *Heterorhabditis bacteriophora*

The commercially available formulated product of nematode populations (Table 1) were kindly provided by e-nema (Schwentinental, Germany) and Andermatt Biocontrol (Gross-

dietwil, Switzerland). The powder was dissolved in tap water and suspensions used to infect *Galleria mellonella* (Lepidoptera: Pyralidae) larvae (from Hebeisen Fisher or Andy's Fisher store, Zurich, Switzerland). The other EPN populations were isolated from field studies during 2013-2015 in Switzerland [23, 76, 77]; Table 1). All nematode populations (also referred to as strains) used in this study were regularly multiplied in *G. mellonella* larvae using the White Trap method [78, 79]. Emerging infective juveniles (IJ) were stored at 500 IJ/ml in tap water in filter cap cell culture flasks (75 cm^2, CELL-STAR®, Greiner Bio-One, Austria) at 15 °C for up to four months. Fresh IJ (no older than three weeks) were used for experiments. The concentration was determined by counting IJs in 20 or 50 µl suspension under a stereomicroscope and suspensions were adjusted with tap water to the desired concentration (1000 IJ/ml for most experiments).

– *Metarhizium brunneum* and *Beauveria bassiana*

The fungi *M. brunneum* BIPESCO5/F52 (Bip5) and *B. bassiana* ART2587 (Table 1) were stored as conidia on plates with SM medium [80] for up to one year at 3 °C. The infectivity of fungal isolates was maintained by frequent passaging through host insects and subsequent single spore isolation as described by Reinbacher et al. [81]. For experiments, SM plates were inoculated by transferring fungal spores from a stored culture using an inoculation loop [80] and incubated for two weeks at 24 °C in the dark. Conidiospores were scraped off the plates using sterile inoculation loops or Drigalski spatula and suspended in 0.01% Tween80. For the semi-field trial, sterilized barley kernels were inoculated with Bip5 and incubated for several weeks at 22 °C in the dark as described by Reinbacher et al. [82]. The spores were dried and harvested from the kernels using a myco-harvester MH5 (VBS Agriculutre Ltd., Beaconsfield, UK). Concentration was measured by counting conidiospores suspended in 0.01% Tween80 under the microscope using KOVA or Thoma chambers and adjusted to the desired concentration with ddH_2O.

Table 1: **Biocontrol agents used in this study.**

Species	Strain/ population	Origin	Described biocontrol activity	Reference
P. chlororaphis	PCLRT03 (P)	Potato root	Px	Vesga et al. [74]
P. chlororaphis	PCLRT03-gfp	Derivative of PCLRT03, PCLRT03::miniTn7-gfp2; Gm^R	-	This study; Provided by Jordan Vacheron, Université Lausanne
P. chlororaphis	PCL1391	Tomato root, ESP	Fol, Px	Chin-A-Wong et al. [27], Flury et al. [32]

Table 1: **Biocontrol agents used in this study.**

P. chlororaphis	PCLAR03	Potato invertebrate	Px	Vesga et al. [74]
P. protegens	CHA0	Tobacco root	Fol, Ggt, Pu, Px, Tb	Flury et al. [32]
P. protegens	PF	Wheat leaf, USA	Gm, Px, Zt	Flury et al. [32]
P. protegens	PPRAR04	Agriotes sp.	Px	Vesga et al. [74]
P. protegens	PPRAR03	Bank invertebrate	Px	Vesga et al. [74]
Pseudomonas sp.	PILAR01	Bank invertebrate	Px	This study; Isolated by Pilar Vesga (see Vesga et al. [74])
S. feltiae	RS-5 (RS5) (N)	Soil, wheat field	Ta	Jaffuel et al. [77, 79]
S. feltiae	MG-594*	Soil, grassland	ND	Jaffuel et al. [77]; this study
S. feltiae	MG-608*	Soil, forest (caduca)	ND	Jaffuel et al. [77]; this study
S. feltiae	nemaplus	e-nema AG	Cp, Sci	e-nema AG
S. affine	OG-656*	Soil, wheat field	ND	Imperiali et al. [83]; this study
S. carpocapsae	MG-596a	Soil, grassland	Ta	Jaffuel et al. [76], Campos-Herrera et al. [84]
S. carpocapsae	nemastar	e-nema AG	Ag, Cp, Gg, Ti	e-nema AG
S. poinarii	MG-617*	Soil, forest (caduca)	ND	Jaffuel et al. [76]; this study
S. poinarii	MG-646*	Soil, forest (conifer)	ND	Jaffuel et al. [76]; this study
H. bacteriophora	MG-618b	Soil, grassland	Ta	Jaffuel et al. [76], Campos-Herrera et al. [84]
H. bacteriophora	nematop	e-nema AG	O, Ph	e-nema AG
M. brunneum	BIPESCO5/F52 (Bip5) (F)	*Cydia pomonella*, Austria	A, Mm, O, Ph, Sci	EFSA [85]
B. bassiana	ART2587	*Meligethes* sp.	Ma	Meyling et al. [86]; Pilz [87]

Table 1: **Biocontrol agents used in this study.**

Origin: if not otherwise indicated, strains were isolated in Switzerland. Abbreviations: A *Amphimallon majalis & solstitialis*; Ag *Agrotis* spp; Cp *Cydia pomonella*; Fol *Fusarium oxysporum* f. sp. *lycopersici*; Ggt *Gaeumannomyces graminis* var. *tritici*; Gg *Gryllotalpa gryllotalpa*; Ma *Meligethes aeneus*; Mm *Melolontha melolontha*; O *Otiorhynchus* spp.; Ph *Phyllopertha horticola*; Pu *Pythium ultimum*; Px *Plutella xylostella*; Sci *Sciaridae* flies; Ta *Tuta absoluta*; Tb *Thielaviopsis basicola*; Ti *Tipula* spp.; ND not determined. The list of known biocontrol activity does not claim to be complete.
*These populations were isolated within the frame of the respective studies, but first individually described in this study

Screening assay

The screening assay was performed as described by Flury et al. [88] for pseudomonads and adapted for nematodes and fungi. In all assays, 8-10 *D. radicum* eggs were added to plastic pots (45 x 276 x 80 mm; Bachmann Plantec AG, Switzerland) each containing autoclaved quartz sand (0.3-0.9 mm diameter) and two small round red radish bulbs (Coop, Switzerland). For the bacterial treatment, bulbs were submerged in bacterial suspension (OD_{600} = 0.47) for 10 min (Figs. 1A, C, D, 3, Tables S1, S2, S3, S6), or for the bacteria-control in ddH_2O. For the nematode treatments (Figs. 1B, C, D, 3, Tables S1, S2, S3), 4000 IJ in 4 ml tap water were pipetted on top of the sand right before egg addition (exp. 1-6, combination exp. 1) or 1 week later (exp. 7-10, combination exp. 2). For the nematode-control, 4 ml tap water was added on the sand. For fungal treatments (Fig. 3, Table S6), 10^6 conidia/g were mixed into the top sand layer (30 g) after radish burial before egg addition. For the fungal-control, 6.25 ml ddH_2O was mixed into the top 30 g sand. Eight to twelve pots were prepared per assay and treatment. The pots were incubated in a climate chamber (20 °C, 15 kLux, 16 h; 18 °C, 0 kLux, 8 h; 80% rH) for 3.5 weeks. Then, the sand was sieved through a 2 mm sieve to obtain the pupae and bulbs were cut open to retrieve remaining larvae or pupae. All pupae from one pot were stored in a petri dish (ø 30 mm) and incubated for at least one month to allow flies to emerge.

Greenhouse experiments

We used red bulb forming radish *Raphanus sativus* var. *sativus* cultivar 'Riesenbutter' (Samen Mauser or Coop, Switzerland) in the greenhouse with the following settings: 21 °C (16 h, day) and 18 °C (night) at 70% humidity. Radish seeds were sown in 11 x 11 x 12 cm pots (Lamprecht-Verpackungen, Göttingen, Germany) into a mixture (1:1) of Jiffy peat substrate (Jiffy Products International, Moerdijk, the Netherlands) and Allmig substrate (Trog- und Topferde, Allmig, Baar, Switzerland) and watered by adding water to the trays containing the pots. There were three plants grown in each pot and four pots were grouped into a tray. For each treatment we had four trays (16 pots, 48 plants).

In general, plants were grown for four weeks and then inoculated with *D. radicum* eggs and then grown for another four weeks until final evaluation. For egg addition, 12-15 freshly harvested eggs were placed onto a small piece of paper and all eggs were washed onto the soil using 1 ml ddH_2O. For final evaluation, all pupae and larvae were collected by sieving soil through a 2 mm sieve. All pupae were stored in a ø 30-mm petri dish for at least one month to allow flies to emerge. For fungal treatments, Bip5 conidia were mixed into the soil immediately before sowing at 10^6 conidia/g soil. For bacterial treatments, 20 ml PCLRT03-gfp at 10^8 cfu/ml were spread around the plants to reach a density of 10^7 cfu/g soil. This procedure was done twice, first one week after sowing and the second time one week before egg addition. For nematode treatments, 5 ml of 1000 IJ/ml RS5 were pipetted on the soil to reach approx. 50 IJ/cm^2. EPN were added 2 days before egg addition for the triple combination experiments and 3 days after egg addition for the double combination experiments, respectively.

Semi-field trials

Two semi-field trials were performed in 2020 and 2021: Trial 1 (April – May) with EPN and EPP single treatments and a EPN/EPP combination and Trial 2 (August – September) with EPN, EPP, EPF single, double and triple combinations. We used the same radish cultivar 'Riesenbutter' as in the greenhouse experiments. Treatments with and without bacteria were kept in separate blocks in order to prevent bacteria migrating to pots of the bacteria-free treatments (see Fig. S1). Treatments were distributed randomly over the length of the seedbed within blocks. Data loggers were buried in six *Delia*-free pots to record soil temperature and humidity. Radish seeds were pre-germinated in 273-hole Quick-Pots (gvz-rossat, Switzerland) for one week in Jiffy substrate in the glasshouse. Pots (20 x 20 x 23 cm, Growland, Germany) were filled with a mixture of field soil and Allmig substrate (1:2 for trial 1 resp. 1:1 trial 2). For EPF treatments, spores of *M. brunneum* Bip5 were mixed into the top third ($\approx$ 1.8 kg) at 10^6 conidia/g soil. Four seedlings were transferred into one pot and pots were placed outside on a 3 x 20 m seedbed at Agroscope in Zurich, Switzerland (47.250413 N, 8.305810 E). Plants were watered and covered with a shading net (Accura, Germany) according to weather conditions. The day after planting the seedlings into the pots, EPP (*P. chlororaphis* PCLRT03) were inoculated by distributing to each pot 50 ml of a suspension containing 4 x 10^8 cfu/ml around the radishes and on the soil, resulting in approx. 10^7 cfu/g soil. This procedure was repeated after one week in trial two and after two weeks in trial one. The infestation with *D. radicum* eggs took place one week after the second EPP inoculation. For trial 1 48 eggs and for trial 2 30 eggs were added to each pot except for the *Delia*-free treatment. For EPN (*S. feltiae* RS5) inoculation, 40 ml of a suspension containing 500 IJ/ml were added to each pot three days before egg addition to reach a density of 50 IJ/cm^2. Four weeks after egg infestation, the pots were transferred into

the glasshouse to reduce moisture before harvest. Three days later, the radishes were harvested, washed, the roots weighed, and the bulbs rated for *D. radicum* damage. Then, the top two thirds of the soil was mixed and soil samples (approx. 250 g) were taken to monitor BCA populations. Soil samples were stored at 10 °C and root samples at 3 °C until processing the next day (EPP and EPF monitoring) or two to three days later (EPN monitoring) as described below. In the days after egg addition in trial 2, soil temperatures reached 30 °C which strongly affected the survival of the insects. As a result, pest pressure was too low to have any effect on plants, therefore only data on BCA populations are presented (Fig. 6A, Table S12).

Field trial

The field trial was conducted in Windisch (47.476110 N, 8.227799 E; Aargau, Switzerland) in a field sown with radish *Raphanus sativus* L. cultivar 'Andes F1' (Enza Zaden, Germany) that forms around 35-40 cm long cylindric white bulbs. The seeds were coated with the fungicide Saphire (active ingredient Fludioxonil; Fenaco, Switzerland). The field was divided into 5 x 5 10 m long by and 3.2 m wide plots, representing eight planting rows. The treatments were distributed according to a Latin square design (Fig. S2). Only the inner area, four rows (1.6 m) of 5 m length, of each plot was treated with biocontrol agents and sampled. The rest served as buffer zone to avoid cross-contamination. Five different treatments were each applied to five plots: 1) control with no application, 2) EPP *P. chlororaphis* PCLRT03, 3) EPN *S. feltiae* RS5, 4) EPF *M. brunneum* Bip5, and 5) EPP x EPF x EPN with an application of all three agents (PFN). All BCAs were applied using a watering can distributing 2.5 l inoculum suspension for each 5-m planting row. The suspensions were prepared in the lab and diluted by 1:10 at the field site using tap water resulting in final concentrations of 10^7 conidia/ml for EPF, 150 IJ/ml for EPN and 10^8 cfu/ml for EPP. EPF were applied one day after sowing at 2.5 x 10^{10} spores/5-m row (corresponding to approx. 1.3 x 10^{14} spores/ha). One week later, first EPN and then EPP were applied at 3.75 x 10^5 IJ/5-m row (1.9 x 10^9 IJ/ha) and 2.5 x 10^{11} cfu/5-m row (1.3 x 10^{15} cfu/ha), respectively. EPP were applied a second time four weeks after sowing. Two and eight weeks after sowing, soil and root samples were taken to monitor BCA colonization. For this, five samples (three for the control) were taken from each plot. Root samples consisted of one root system and soil samples of three scoops of soil down to 15 cm depth. Samples were taken uniformly over the whole plot. Soil samples were stored at 10 °C and root samples at 3 °C until processing the next day (EPP and EPF monitoring) or two to three days later (EPN monitoring) as described below. The final sampling was done nine weeks after sowing when all radish plants were harvested to evaluate damage. The green was cut off and the white bulbs were washed and rated for damage on a scale from 0-3 with 0 = no *D. radicum* specific mining; 1 = light damage, 1 mining; 2 = heavy damage, 2-5 minings; 3 = very severe

damage, bulb partly or completely destroyed by > 5 minings. We also noted how much each bulb was rotten due to water logging in the field. Heavily rotten bulbs (> 70%) were later excluded from analysis. All treatments were harvested within two days.

Monitoring of biocontrol agents

For EPP and EPF soil colonization, 10 g soil was suspended in 50 ml sterile 0.9% NaCl solution in a 100 ml Erlenmeyer flask and shaken using a rotary shaker at 200 rpm for 30 min at 3 °C. For root colonization, the roots were weighed and incubated in 15 or 50 ml falcon tubes with 10 or 40 ml 0.9% NaCl solution, and shaken as described for the soil samples. After shaking, samples were serially diluted in 0.9% NaCl solution and plated on selective agar. For EPF, 100 µl of undiluted and 1:100 diluted samples were plated on SM plates and plates were incubated at 24 °C. Colony forming units (cfu) were counted after two weeks. *Metarhizium* colonies were identified morphologically, thereby also counting naturally occurring *Metarhizium* species. For EPP, 100 µl of the 10^0 to 10^{-3} dilutions were plated on KB^{+++} agar and plates were incubated at 24 °C for two days. *P. chlororaphis* PCLRT03 colonies can be distinguished from other pseudomonads since colonies turn green due to phenazine production.
EPN colonization was assessed as described in Campos-Herrera et al. [79]. Briefly, 200 g soil samples were suspended in tap water and sieved through a 125 and then through a 25 µm sieve. The EPN collected were sucrose-extracted [89, 79] and the samples reduced to 100 µl by centrifugation to allow for DNA extraction. Each sample was disrupted by a pellet pestle motor (KIMBLE®, DWK Life Sciences, Germany) and DNA extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, The Netherlands) according to the manufacturer's instructions. DNA concentration was measured using NanoDrop2000 (Thermo Fisher Scientific, MA, USA) and diluted to 1 ng/µl for qPCR. DNA from 300 RS5 IJ (pure culture) extracted using the same kit was used for the standard curve and miliQ water was used as a negative control. We used *S. feltiae* specific primers and probe as designed by Campos-Herrera et al. [90] and synthesised by Microsynth AG (Balgach, Switzerland). The reaction was performed using the TaqMan® polymerase (Thermo Fisher Scientific, MA, USA) in a 7500 Fast Lightcycler (Thermo Fisher Scientific, MA, USA) at the Genetic Diversity Center (GDC, Zurich, Switzerland) and analysed with the 7500 Software (v 2.0.6). Thermal cycling was performed as described in Campos-Herrera et al. [91] with 60 °C annealing temperature during 40 cycles. The total reaction volume was 10 µl and two technical replicates were run for each sample.
50 g soil samples were dried for 24 hours at 105 °C to calculate soil dry weight.

Statistical analysis

The analysis was conducted in Rstudio (version 1.4.1717) using R (ver. 4.1.2). The results from five to ten screening and greenhouse experiments were combined and analysed using a linear mixed-effect model comparing the fly emergence rates across treatments and controlling for experiment and experiment x treatment effects (package lme4 ver. 1.1-27.1). For analysis of single screening assays, Kruskal-Wallis and post-hoc Dunn test were performed using the package FSA (ver. 0.9.1). Radish damage ratings were analysed with an ordinal regression model using the function polr (package Mass ver. 7.3-55) and emmeans (package emmeans ver. 1.7.2) was used for post-hoc pairwise testing. Colonization data was log-transformed and tested for normal distribution using a Shapiro-Wilk Normality Test. Since data from the field trial did not follow a normal distribution, Kruskal-Wallis and post-hoc Dunn test were used for all colonization data sets (both field and semi-field trials). Differences were considered to be significant at $P < 0.05$ and were indicated by asterisks (*) when compared to the control. When multiple treatments were compared, treatments labelled with the same letter do not significantly differ. Boxplots and barplots were created using ggpubr (ver. 0.4.0) and ggplot2 (ver. 3.3.5) packages. Boxplots are standardized with the middle line representing the median, the upper and lower box edge the interquartile range between the 25th and the 75th percentile, the upper and lower line end the maximum resp. minimum values within the 1.5 interquartile range, and dots represent outliers. Biocontrol effect was calculated in excel as difference in mean value compared to the control. These values were used for assessing synergism according to the Bliss independence formula: $E_{12} = E_1 + E_2 - (E_1 \times E_2)$, with $E_{12} > E_{combo}$ = antagonistic, $E_{12} = E_{combo}$ = basic additivity, $E_{12} < E_{combo}$ = synergism [92, 93].

Results and Discussion

Evaluation of the biocontrol potential of entomopathogenic pseudomonads and nematodes against the cabbage maggot

To select the most efficient strains for controlling the cabbage maggot *D. radicum*, a screening was performed in a radish sand system. In the screening assays, all three tested *P. chlororaphis* strains significantly reduced the mean fly emergence rate per egg compared to the control by more than 50% and strain PCLRT03 even by 83.5% (Fig. 1A, Table S1). Three out of four tested *P. protegens* strains, namely CHA0, PPRAR03 and PPRAR04, reduced fly emergence by around one third, although this reduction was not significant. These findings support the results obtained by Flury et al. [88], where only a *P. chlororaphis* strain significantly reduced pupation rate of *D. radicum*, but not the two tested *P. protegens* strains. Interestingly, the opposite seems to be

the case for Lepidoptera: *Plutella xylostella* (Lepidoptera: Plutellidae) larvae are more susceptible to *P. protegens* strains than to *P. chlororaphis* [74, 88]. *P. protegens* and *P. chlororaphis* harbour the Fit (*P. fluorescens* insecticidal toxin) cluster in different regions of their genome and they have different exoproduct profiles [32, 74, 94]. This might contribute to the observed difference in specificity between these two closely-related insecticidal *Pseudomonas* species. Vesga et al. [74] compared *Pseudomonas* strains isolated from different environments and found that *P. protegens* were genetically more uniform and more consistent in killing larvae compared to *P. chlororaphis*, independent of their origin of isolation. When considering all strains that were used in more than one experiment, the same tendency can also be observed in our screening assays (Table S1). These findings indicate the importance of screening not only at the species but also at the strain level, especially for *P. chlororaphis*.

In the nematode screening assays, all *S. feltiae* and one *H. bacteriophora* population significantly reduced fly emergence rate compared to the control (Fig. 1B). The three most efficient populations, two belonging to *S. feltiae* and one to *H. bacteriophora*, reduced mean fly emergence by 40-45% compared to the control (Table S1). Thus, *S. feltiae* is the most suitable EPN species for *D. radicum* control as suggested by Beck et al. [95]. Chen et al. [96] discovered that *S. feltiae* performs better than other EPN species against the cabbage maggot under different temperatures, especially under cold conditions, which is in line with other temperature-sensitivity studies [97]. This adds to the suitability of *S. feltiae* for *D. radicum* control since most *Brassicaceae* vegetables can be sown early in the season. Our results suggest that selecting the population is as important as choosing the right species because efficacy varied greatly between species and within species, especially for *S. feltiae* and *H. bacteriophora* (Table S1). Campos-Herrera et al. [84] and Filgueiras & Willett [98] also observed a variability in virulence between populations of the same species. Bruno et al. [99] found a similar pattern and could link it partially to the sensitivity of different EPN populations to the insect's defence mechanism. Interestingly, in our assay, the commercially available populations of *S. feltiae* and *H. bacteriophora* had a lower potential to control *D. radicum* than the Swiss isolates of these species (Fig. 1B, Table S1). On the other hand, Kapranas et al. [100] found that e-nema's *S. feltiae* and *S. carpocapsae* population are effective in preventing *D. radicum* larvae from infecting radish bulbs under laboratory conditions. In our screening assay, these two populations reduced mean fly emergence only by 16-18% compared to the control and only causing a significant effect for *S. feltiae* nemaplus. However, differences between our results and those of the previous study could be due to differences in experimental design and measured values. Despite these differences, Kapranas et al. [100] also conclude that *S. feltiae* is the most efficient species for *D. radicum* control according to their results.

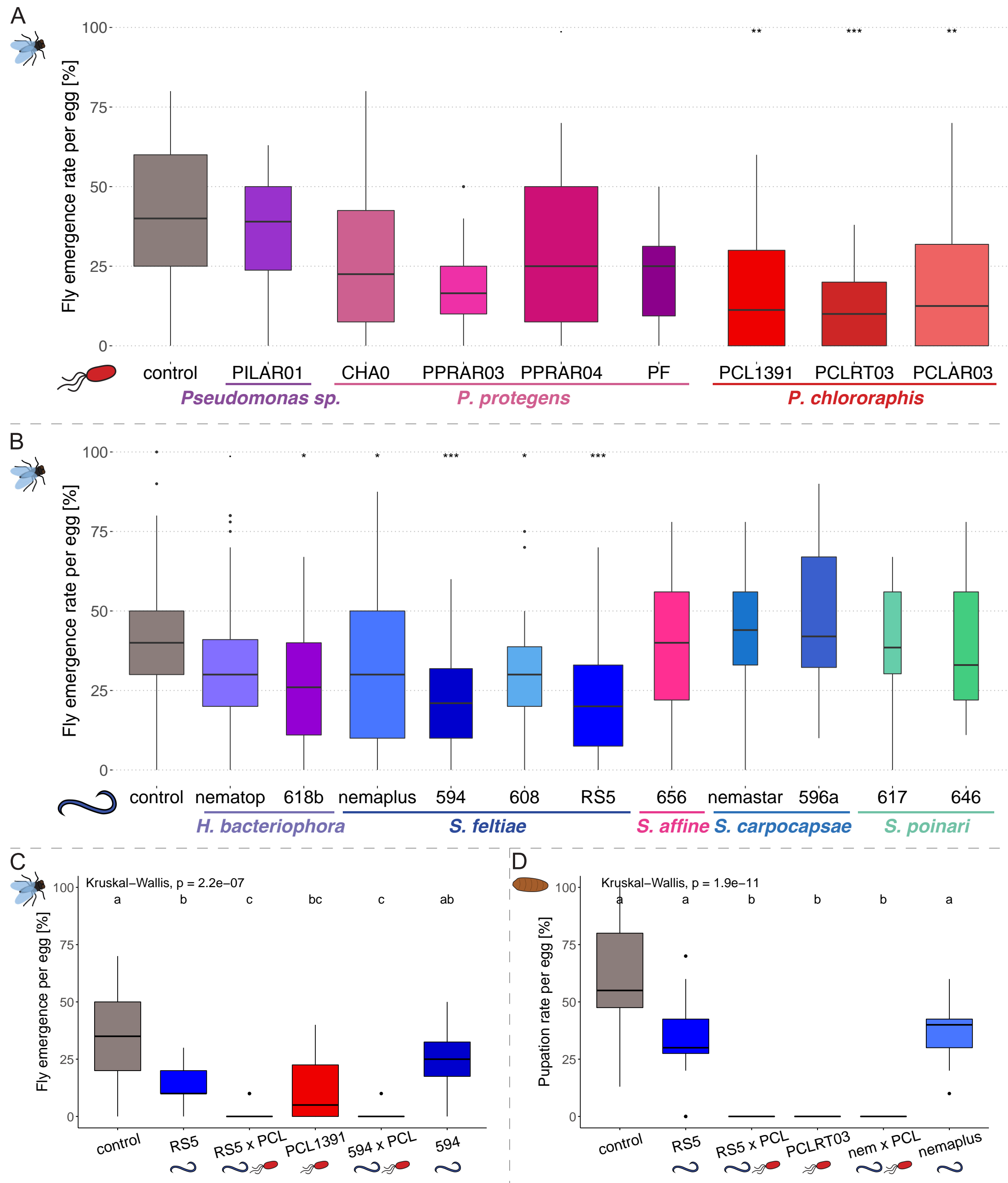


Fig. 1. **Effect of fluorescent pseudomonads and entomopathogenic nematodes on *D. radicum* development under screening conditions.**

Figures show *D. radicum* fly emergence and pupation rates per egg, respectively, obtained in a radish-sand screening assay. A) Pooled results of five independent replicates using eight different *Pseudomonas* spp. strains. B) Pooled results of ten independent replicates using eleven different entomopathogenic nematode populations of *Heterorhabditis bacteriophora* and *Steinernema* species. C & D) Two independent screening assays applying combinations of *P. chlororaphis* and *S. feltiae*.
Standard boxplots represent the pupation resp. fly emergence rate. In panels A & B) boxplot width indicates the number of assays in which a strain was tested in. Results of individual assays are shown in Table S2. Data was analysed with linear mixed effect model (R::lme4), asterisks indicate significant differences compared to the control with *P*-values . = <0.1, * = <0.05, ** <0.01, *** <0.001. In panels C & D) Kruskal-Wallis and post-hoc Dunn-test with bh-correction were conducted in Rstudio; different letters indicate significant differences between treatments ($P < 0.05$).

In summary, our screening resulted in the discovery of several *P. chlororaphis* stains and *S. feltiae* populations with promising potential to kill larvae of *D. radicum* and which we used further in our experiments.

Combination of entomopathogenic pseudomonads and nematodes is synergistic

After identifying suitable *Pseudomonas* and nematode strains, we tested these together under the same screening conditions in two experiments. In the first experiment, *P. chlororaphis* PCL1391 and *S. feltiae* RS5 and 594 applied alone reduced the average fly emergence rate by 65%, 65% and 30%, respectively, compared to the control (Fig. 1C, Table S3). The combination of PCL1391 with either *S. feltiae* population led to a reduction of over 95%. In the second experiment, *P. chlororaphis* PCLRT03 alone and in combination with either *S. feltiae* population reduced the pupation rate to 0%, whereas both *S. feltiae* populations alone insignificantly reduced the average pupation rate by around 40% compared to the control (Fig. 1D, Table S3). When considering the effects of the BCAs in combination, the experiments main results varied. The combination could not improve efficacy in the second experiment as PCLRT03 alone reached 100% control. However, the increased control efficacy in the first trial represents a synergistic interaction according to the Bliss formula ([92, 93]; Table S4). Although a synergistic effect of the BCA combination could only be observed in the first experiment, across both experiments there was no evidence of inhibitory effects, i.e. the combined application did not reduce the efficacy.

The combined application of pseudomonads and nematodes was then upscaled in a semi-field trial, where radishes were grown in pots under natural weather conditions and inoculated with *D. radicum* eggs. In the treatment without artificial inoculation, a few *D. radicum* larvae were discovered, implying a very small natural infestation. As a consequence, only 14 % of the plants were affected by the insect (Fig. 2). The artificial inoculation with *D. radicum* eggs, however, created a very high pest pressure, resulting in 67% dead plants in the control without biocontrol application (Table S5). Although single BCA application reduced *D. radicum* damage - *S. feltiae* RS5 resulted in 15% fewer dead plants and increased healthy plants by 25%, whilst *P. chlororaphis* PCLRT03 increased healthy plants by 8% and reduced dead plants by 25% - these effects were not significantly different to the no BCA control. The high efficacy of *P. chlororaphis* PCLRT03 and *S. feltiae* RS5 that was observed under screening conditions, was not observed in the upscaling to the semi-field trial. The combination of BCAs however did significantly reduce *D. radicum* damage (Fig. 2). The combination of the two biocontrol agents resulted in a synergistic interaction (Table S4) and decreased the number of dead radishes by 42% and increased healthy radish bulbs by 34%. The biocontrol effect of the combination was consistent and even the synergistic relationship was preserved

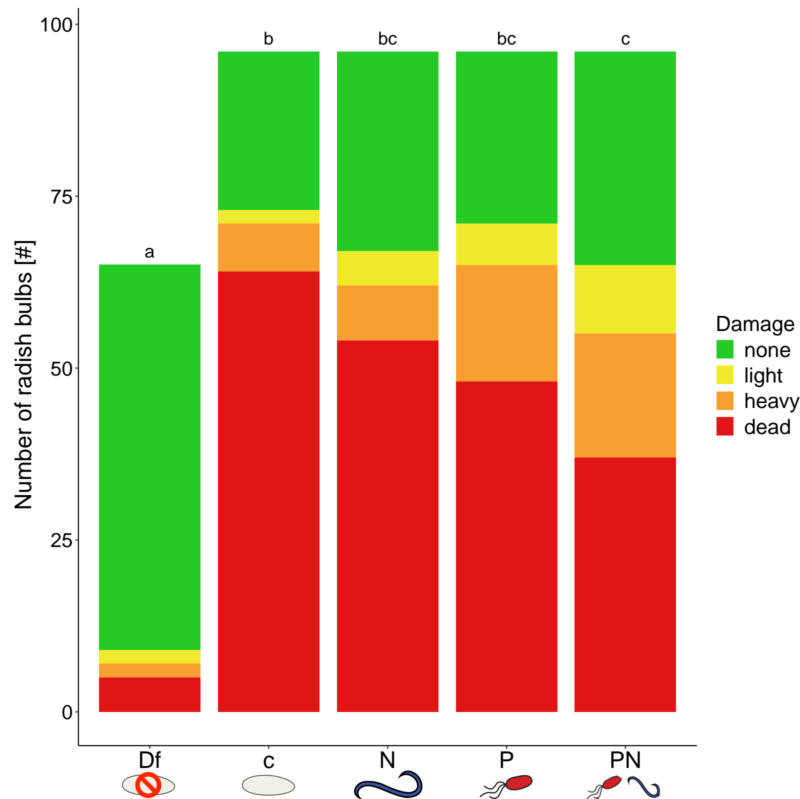


Fig. 2. **Impact of insecticidal *Pseudomonas* and entomopathogenic nematode applied alone and in combination on *Delia radicum* damage on radish bulbs under semi-field conditions.**

D. radicum damage was recorded on radish bulbs grown in pots placed outside under natural weather conditions. In the *Delia*-free (Df) treatment, there was no *D. radicum* inoculation and no BCA application. The remaining treatments were all artificially inoculated with *D. radicum* eggs; c = control with no BCA application; N = EPN population *S. feltiae* RS5; P = EPP strain *P. chlororaphis* PCLRT03; PN = double combination with P and N application. *D. radicum* mining damage on a radish bulb was recorded on the following scale: none = no damage; light = small damage, 1 mining; heavy = large damages, $\geq$ 2 minings; dead = plant dead. Different letters on top of the barplot refer to significant differences among treatments ($P < 0.05$) according to an ordinal regression model.

when upscaling from the screening to a semi-field trial.

To the best of our knowledge, this is the first study to demonstrate a synergistic interaction in a combined application of EPN with entomopathogenic pseudomonads against insects under lab and semi-field conditions. Previous studies have focused predominantly on EPN and *Bacillus thuringiensis* (Bt) combinations. The combination of *H. bacteriophora* and Bt resulted in additive or synergistic effects when controlling grubs in laboratory, greenhouse, and field studies [101, 102]. Li et al. [103] studied a similar pathosystem and discovered additive effects when EPN were added zero or six days after Bt inoculation, and synergistic effects when EPN were added two or four days after Bt under greenhouse conditions. In a subsequent field trial, they applied EPN

two days after Bt and found additive and synergistic effects depending on the applied BCA concentration. In a lab study, additive and synergistic interactions were discovered when EPN *S. feltiae* and *H. bacteriophora* were combined with *B. thuringiensis* subsp. *kurstaki* against the large cabbage white [104]. In a Dipteran pest, *Tipula paludosa*, the additive effects that were observed in the laboratory were not observed in the field; there, the combination of *S. feltiae* and *B. thuringiensis* subsp. *israelensis* was only as good as the EPB alone [105]. The general conclusion from these studies is that biocontrol efficacy is increased when EPN and EPB are applied together, which is more pronounced in laboratory studies compared to field studies. Our results support this conclusion although insecticidal pseudomonads and Bt have different modes of action.

So far, little is known about the interaction between EPN and EPP. Cambon et al. [106] discovered pseudomonads in EPN-infected cadavers. Ogier et al. [107] isolated *P. protegens* and *P. chlororaphis* from infective juveniles of different EPN species and proposed that EPP belong to the EPN pathobiome. The insecticidal toxins produced by EPP and nematode-associated bacteria (NB) show sequence similarity, suggesting a common origin [94]. These findings suggest frequent interactions between EPP and EPN in nature. In summary, *P. chlororaphis* and *S. feltiae* seem to be well compatible when applied together against an insect pest. Their different modes of action might be a reason for the synergistic effects we have observed. Combining these two organisms is a promising approach for developing biological control methods, not only for *D. radicum* but also for other soil pests.

Compatibility of entomopathogenic pseudomonads and fungi in screening assays

A further aim of this study was to extend promising dual combinations of BCA organisms for *D. radicum* control to triple combinations. From several studies screening various entomopathogenic fungi against *D. radicum*, it emerged that isolates of *Beauveria bassiana* and *Metarhizium anisopliae* (certain *M. anisopliae* strains have recently been re-assigned to the species *M. brunneum*) and among the latter especially *M. brunneum* strain BIPESCO 5 / F52 (Bip5) have promising activity against the cabbage maggot [108, 109, 110, 111]. Bip5 has also the advantage that it is already registered as BCA against insect pests in the US [108], the EU [112, 85], and also in Switzerland [65]. However, at the onset of our study it was unclear whether EPF and specifically Bip5 are compatible with EPP. In an *in vitro* inhibition assay on agar plates, the growth of *M. brunneum* Bip5 was suppressed by *P. chlororaphis* PCL1391 and PCLRT03, whereas two *B. bassiana* strains were not or only slightly affected by the bacteria (A. Spescha, unpublished data). Due to these differences between species, two EPF strains, i.e. *M. brunneum* Bip5 and *B. bassiana* ART2587, were selected to study their compatibility

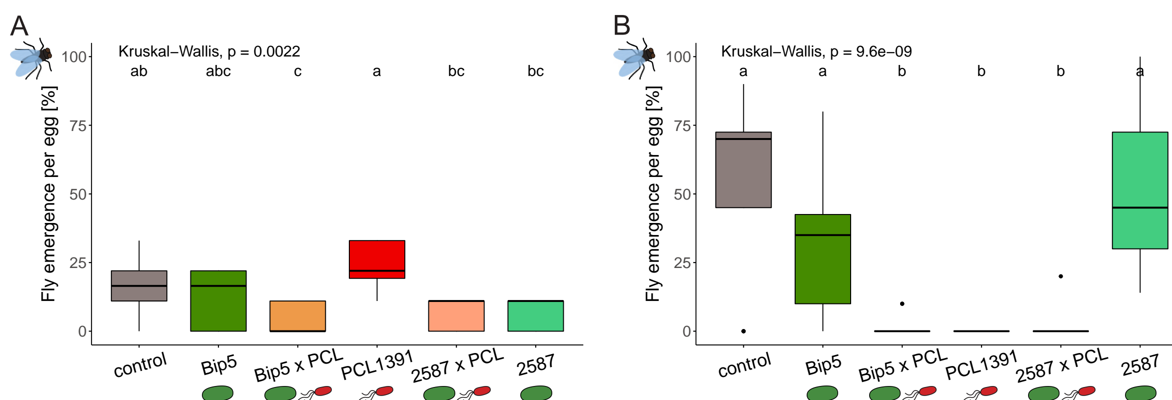


Fig. 3. **Effect of fluorescent pseudomonads and entomopathogenic fungi applied alone and in combination on *D. radicum* development under screening conditions.**

Figures show *D. radicum* fly emergence rates per egg obtained in a radish-sand screening assay after applying combinations of *P. chlororaphis* PCL1391 with *M. brunneum* Bip5 and *B. bassiana* 2587, respectively. Panels A and B) are two independent repetitions. A Kruskal-Wallis and post-hoc Dunn-test with bh-correction were used to identify significant differences between treatments ($P < 0.05$) as indicated with different letters.

with EPP in our screening system with radish bulbs.

In the screening assay, fly emergence rate in the control varied a lot between experiments; on average 17% in the first and 54% in the second experiment (Fig. 3, Table S6). The two EPF strains showed tendencies to reduce fly emergence rates but the effect was not significant in either experiment (Fig. 3). The performance of EPP PCL1391 was highly variable. In the first experiment, the bacteria had no impact at all on fly emergence, in the second, however, all the insects died following PCL1391 application. Therefore, it was not possible to monitor potential effects of combinations in the second experiment. In contrast, the combination of Bip5 and PCL1391 significantly reduced mean fly emergence by 75% compared to the control in the first experiment (vs. 25% and 0% reduction for respective individual strains), thus resulting in a synergistic effect (Table S4).

It was more surprising to find a synergistic effect rather than the inhibitory effects observed in *in vitro* assays between EPF and EPP. Fluorescent pseudomonads are well-known for controlling fungal plant diseases and producing a vast array of antimicrobial exoproducts [28, 74]. EPF, on the other hand, also produce antimicrobial compounds that inhibit bacterial growth [113, 114]. Such inhibitory effects that were visible on agar plates were not observed in the screening assays. This might be explained by 1) the possibility that the cocktail of exoproducts produced by the two BCAs might differ between culture medium and our screening system and 2) by spatial separation. In the screening system, the fungal spores were mixed in the top sand, while the bacteria were on the radish bulb. EPF have already successfully been combined with fluores-

cent pseudomonads for simultaneous pest and disease control, e.g. of leaf miners and collar rot disease in groundnut [115, 116] and leaf folder pest and sheath blight disease of rice [117]. All these findings indicate that the interactions between EPP and EPF can be beneficial in combined applications and therefore promising to evaluate combinations for pest control. We thus included *M. brunneum* Bip5 in our triple combination.

Upscaling the application of a tripartite biocontrol consortium from the greenhouse to the field

Our next step was to compare single, dual and triple combinations of EPP, EPN and EPF in greenhouse trials. All three BCAs significantly reduced fly emergence rates compared to the control (Fig. 4, Table S7). *S. feltiae* RS5 reduced the mean emergence by 44% compared to the control, thereby showing a similar efficacy in the greenhouse as in the screening assay (Table S1). *P. chlororaphis* PCLRT03 reduced fly emergence in the greenhouse by 59%, which was lower than in the screening assay. *M. brunneum* Bip5 significantly impacted *D. radicum* and lowered mean fly emergence by 54%, thus performing better than in the screening assay (Table S6). Both dual combinations (EPP x EPN and EPP x EPF) significantly reduced *D. radicum* survival compared to the control, lowering the mean fly emergence rate by 60%, but did not differ significantly to the single treatments. The same was observed for the triple combination. The synergistic effects observed for EPP combined with EPN and EPF in the screening and the semi-field trial could not be verified in the greenhouse. This indicates that synergistic effects are dependent on different conditions as we discuss below. The effects of both dual combinations were very consistent over both experiments. Even though EPP and EPF as well as EPP and EPN combinations did not exhibit a higher efficacy than the single applications, the mean and median values were less variable between experiments (Table S8). The stabilizing effect of combining EPP either with EPF or with EPN was already observed in the screening assays (Figs. 1C, D, 3, Tables S3, S6). To verify the biocontrol effect of the consortium under natural conditions, the single BCAs and the triple combination were applied in a field trial.

In the field trial, *D. radicum* damage was significantly reduced compared to the untreated control when BCAs were applied alone and in a triple combination (Fig. 5, Table S9). However, there was no difference between the triple combination and any of the single applications. The number of plants with no damage was highest in EPP, and second highest in the triple combination. Application of either EPF or EPN increased the percentage of plants without *D. radicum* damage (category ‘none’ in Fig. 5) by one third, whereas application of EPP and the triple combination increased this by 50% (Table S9). Plants with no damage can be sold to wholesale at standard prices, thus the observed effects of BCA application (either single or in combination) would translate into

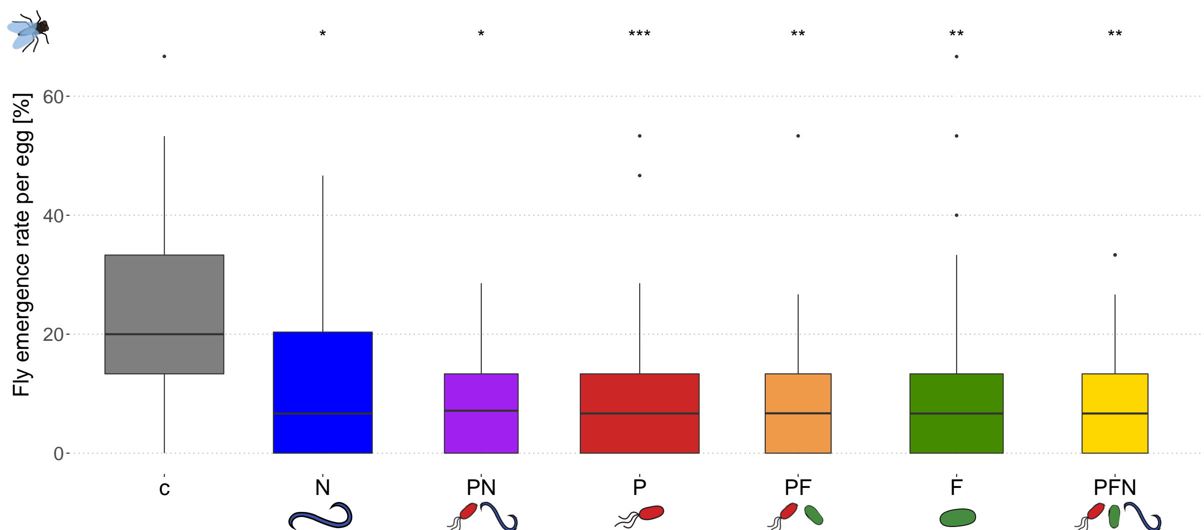


Fig. 4. **Effect of single and combined biocontrol agent applications on *D. radicum* development under greenhouse conditions.**

The figure shows *D. radicum* fly emergence rate per egg data pooled across six independent greenhouse trials combining EPP, EPN and EPF (two trials for each combination). Treatments: c = control with no BCA application; N = EPN population *S. feltiae* RS5; P = EPP strain *P. chlororaphis* PCLRT03; F = EPF strain *M. brunneum* Bip5; PN, PF and PFN = respective double and triple combinations. Boxplot width indicates the number of experiments in which a treatment was applied in (two trials for each combination, four for F and N, six for P and the control). A lmer model was used to analyse the data; asterisks refer to significant differences compared to the control with *P*-values * = <0.05, ** <0.01, *** <0.001.

an increase in marketable plants by 30-50%. The percentage of lightly damaged plants did not differ between treatments, which are plants that can be sold on the farmers market at a lower price. BCA application reduced the proportion of heavily damaged plants, which are plants that cannot be sold (categories 'heavy' and 'severe' in Fig. 5). Again, the most efficient treatment was EPP, which reduced heavy damage by 73%. The combination reduced heavy damage by 66% and both EPF and EPN by around 40%. Our results are promising for *D. radicum* control, but additional studies are needed to determine whether the triple combination is more stable over the long term compared to single applications.

Few published studies on *D. radicum* biocontrol in the field have shown effective control of this pest. For example, Vänninen et al. [72] applied different EPF species as well as *B. thuringiensis* and *S. feltiae* in several field trials, but biocontrol effects were scarce and usually observed in one season only, and Chandler and Davidson [118] reported that *M. anisopliae* reduced *D. radicum* survival under greenhouse conditions, but not in the field. Two recent field studies with application of *M. brunneum* and *B. bassiana* detected a non-significant reduction in pest pressure [70, 71]. The positive exception was a study by Beck et al. [95] who observed significantly reduced cauliflower mortality after application of *S. feltiae* in a field trial with a high natural pest pressure. We achieved

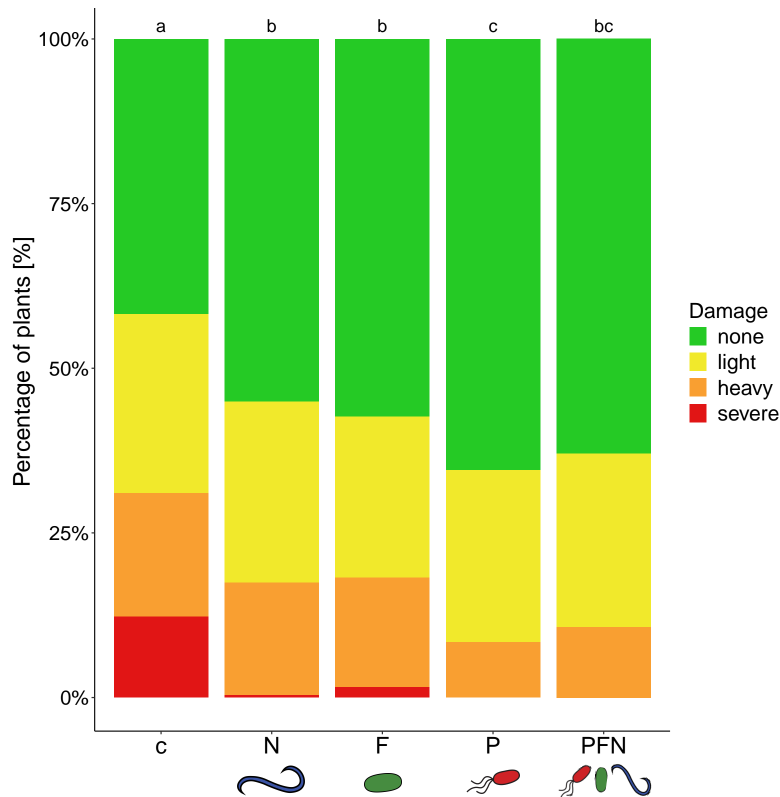


Fig. 5. **Reduction of *Delia radicum* damages on radish bulbs by single and combined BCA application observed under field conditions.**

Treatments: c = control with no BCA application; N = EPN population *S. feltiae* RS5; F = EPF strain *M. brunneum* Bip5; P = EPP strain *P. chlororaphis* PCLRT03; PFN = triple combination with all three BCAs. Bulb damage was scored with the following scale: none = no *D. radicum* damage; light = small damage, 1 larval mining; heavy = large damages, 2-5 minings; severe = severe damages, plant partly or completely destroyed by > 5 minings. Data was analysed using an ordinal regression model. Significant differences between treatments are indicated by the letters above the barplot.

a significant increase in marketable plants upon application of EPF *M. brunneum* and EPN *S. feltiae*. However, the best result was obtained with the entomopathogenic pseudomonads of the species *P. chlororaphis*, which is already used for the biological control of fungal diseases. Although the triple combination did not result in a synergistic effect, our field trial confirmed the results from our greenhouse and semi-field trials: EPP, EPN and EPF are compatible and provide significant *D. radicum* control. Our findings indicate that such a tripartite consortium represents an environmentally friendly method, which could substantially contribute to solve this severe problem in the production of Brassicacean crops in organic and conventional agriculture.

Our study is the first to investigate triple combinations of EPP, EPN and EPF for pest control. In recent studies, EPN and EPP have been combined with arbuscular mycorrhizal fungi (AMF) to improve plant fitness and reduce pest pressure. Imperiali et al. [23] inoculated wheat fields with *P. protegens* CHA0 and *P. chlororaphis* PCL1391 alone and

in combination with different EPN, including *H. bacteriophora* nematop, as well as with different AMF. Under a heavy natural frit fly infestation, the combined application of both *Pseudomonas* strains together with *H. bacteriophora* resulted in the highest yield. Jaffuel et al. [24] treated fields over three years with EPP, EPN and AMF to protect maize from damage caused by the western corn rootworm (WCR), however, the results varied across years. In the first year, EPP (a mixture of *P. protegens* and *P. chlororaphis*), EPN (a mixture of *H. bacteriophora* and *S. feltiae)* as well as the EPP-EPN-AMF combination increased grain yield, however without visible reduction of pest incidence. In the following two years, the applied organisms had no effect on yield, yet negative impacts on WCR were observed, e.g. reduction of larval numbers per plant or reduced pest incidence, though combinations did not result in synergistic effects. Taken together, these two studies in combination with our own indicate that EPN-EPP combinations, alone or together with plant-beneficial fungi, can reduce damage caused by different insects on different crops.

Biocontrol agents do not impact each other's population sizes under semi-field and field conditions

For successful co-application, the biocontrol agents need to persist well together in the soil and on plant roots. We monitored the population levels of EPP, EPF and EPN after single and combined application in two semi-field trials and a field trial. To summarize our results, the EPP, EPN and EPF used in our semi-field and field trials established and persisted well in the soil and on the roots. Population sizes of individual BCAs were not altered in dual or triple combinations.

In the second semi-field and the field trial, the mean EPN population levels at harvest ranged between 50-100 IJ/100 g soil dry weight, and in the first semi-field trial around 350 IJ/100 g soil fresh weight (Tables S11-S13). In the field trial, EPN numbers decreased slightly but significantly over time (Fig. 6B). The combination with other BCAs did not impact EPN population size neither in field nor semi-field trials.

Mean EPF root colonization was very similar in the semi-field and the field trial, ranging from 7 x 10^3 to 1 x 10^4 cfu/g fresh weight (Fig. 6, Tables S12, S13). EPF populations established in the soil were about one order of magnitude higher and means ranged from 1-2 x 10^5 cfu/g dry weight. In the field trial, similar to the EPN populations, EPF levels decreased slightly over the period of the field trial, which was significant in the soil but not on the roots (Fig. 6B). Combining EPF with other BCAs did not influence population sizes.

In the semi-field trials, the mean EPP soil colonization ranged between 3 x 10^5 and 1 x 10^6 cfu/g dry weight (Fig. 6A, Tables S11, S12). Pseudomonads were enriched

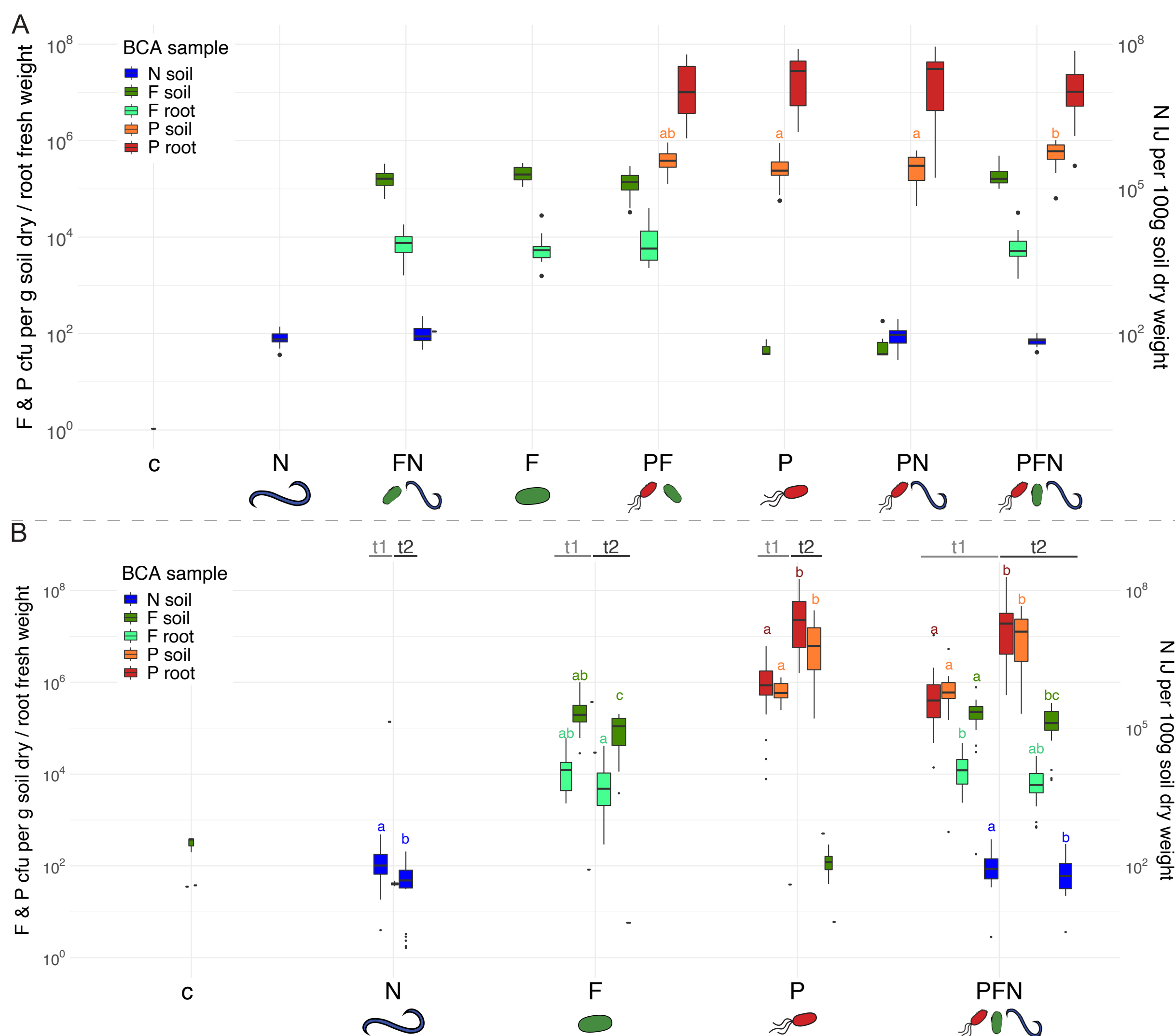


Fig. 6. **Soil and root colonization by BCAs applied alone and in combinations under A) semi-field and B) field conditions.**

A) Soil and root colonization in semi-field trial 2 was assessed for all BCAs during the final evaluation after growing radishes for eight weeks in pots (n=16) placed outside. B) Soil and root samples (n=25) were taken two (t1) and eight weeks (t2) after sowing radishes in a field trial. Treatments: c = control with no BCA application; N = EPN population *S. feltiae* RS5; F = EPF strain *M. brunneum* Bip5; P = EPP strain *P. chlororaphis* PCLRT03; FN, PF, PN and PFN = respective double and triple combinations. Letters refer to significant differences among treatments at $P < 0.05$ according to the Dunn-test and may be compared if written in the same colour. Where no letters are shown, no significant differences were detected.

on the roots and reached population levels between 2-4 x 10^7 in trial 2 and 1-3 x 10^8 cfu/g fresh weight in trial 1. In the first trial, the root colonization was significantly lower in the PN treatment than in P, whereas in the second trial, the soil colonization was significantly higher in the PFN than in the P and PN treatments. In the field trial, the soil and root colonization levels did not differ much and ranged from 7 x 10^5 to 1 x 10^6 cfu/g at the first sampling (Table S13). In contrast to EPF and EPN, EPP population sizes increased more than ten-fold over time and reached levels ranging between 1 and

4 x 10^7 cfu/g eight weeks after sowing (Fig. 6B, Table S13). It is most likely that the second application after the first sampling has boosted populations. This population boost strongly supports the utility of a second EPP application. As already observed for EPN and EPF, combining EPP with other BCAs did not substantially affect EPP root or soil colonization. BCA colonization was also observed in several greenhouse trials, and neither of the three BCAs had an impact on population sizes of the others in combinations (data not shown).

The soil and root colonization levels established in the semi-field and the field trials exceeded the recommended thresholds for biocontrol activity that were established for all three biocontrol organisms. Haas and Défago [28] suggest that 10^5-10^6 cfu/g root are necessary for *Pseudomonas* spp. to exhibit plant-beneficial effects. In leaf feeding assays with *P. chlororaphis* PCL1391, already 10-30 cfu/cm^2 leaf significantly reduced larval survival of two Lepidopteran pests [31]. For EPF, around 10^5-10^6 cfu/g soil are necessary to control insect pests [119]. In their semi-field pot experiment, an application of 10^{15} conidia/ha led to a soil colonisation of approx. 10^5 cfu/g and significantly increased wireworm mortality. The suggested application rate for effective insect biocontrol by EPN is 50 IJ/cm^2 corresponding to 10^5 IJ/m^2 resp. 10^9 IJ/ha [79]. The EPP and EPN colonisation levels as well as the lack of negative interactions were comparable to those obtained by Imperiali et al. [23] and Jaffuel et al. [24]. However, it is noteworthy that especially EPP established very well on radish roots even though *Brassicaceae* produce several antibacterial substances that suppress the growth of various bacteria including pseudomonads [120, 121, 122, 123]. The radish root colonisation was relatively high in the semi-field and field trial, which implies that *P. chlororaphis* PCLRT03 was not inhibited by potentially antibacterial root exudates. Besides their antibacterial activity, *Brassicaceae* are also reported to produce substances with antifungal activity [124, 125]. These might contribute to the lower root than soil colonization by EPF. However, EPF do not need to colonize the rhizosphere for biocontrol activity, yet they can express plant beneficial activities as endophytes [126, 127, 128]. EPP, on the other hand, need to establish in large numbers on the roots in order to have oral effects on root-feeding insects. In our case, potential antifungal and antibacterial substances produced by radishes do not seem to prevent the biocontrol organisms from establishing population sizes relevant for biocontrol.

Conclusions

This study is the first to thoroughly explore the biocontrol potential of *P. chlororaphis* against a root-feeding pest insect using a range of experiments from the greenhouse to the field. The most successful strain from our screening assay strongly inhibited survival of the cabbage maggot *Delia radicum* under controlled conditions and increased the marketable produce significantly in a field trial. It is one of the first field studies that shows the efficacy of EPP for controlling an insect pest. So far, *P. chlororaphis* are only marketed for their plant-growth promoting and disease suppressive traits, but our results indicate that new *P. chlororaphis* products or existing ones could also be developed for use against insects. A novel bacterial and multifactorial BCA for pest control would be most welcome in a future where the use of chemicals is greatly reduced or prohibited.

To our knowledge, this is the first time that a combination of entomopathogenic pseudomonads, nematodes and fungi was successfully used to fight a below-ground insect pest. Taken together, our results from all experiments performed under screening, greenhouse, semi-field and field conditions indicate that a combination of compatible BCAs with different modes of action, such as those studied here, can potentially improve *D. radicum* control. The sequential upscaling and the close monitoring of the applied biocontrol agents were essential to identify and evaluate a consortium of biocontrol agents that are compatible and efficient for pest control. When applying our combinations, we observed synergistic effects under semi-field conditions and more stable results in the greenhouse. We propose that unreliable biocontrol effects obtained when applying single BCAs may be overcome by the application of multiple BCAs. A successful consortium does not necessarily have to display pronounced synergisms, but should perform better under variable conditions. In case the performance of an individual BCA is hampered by adverse environmental conditions, the other consortium members could compensate and provide effective control. It is also possible that consortia might have a broader activity spectrum than single-organism-based products. This especially applies to the consortium evaluated here, because all its members have demonstrated activity against several insect species. An added value of including *P. chlororaphis* is that these bacteria can also control fungal root pathogens. We presume that further testing of the double and triple combinations described in our study will lead to promising results against other root-damaging insects and show further plant beneficial effects.

Monitoring the three BCA in pot, semi-field and field studies showed clearly that EPN, EPF and EPP do not impact each other's soil and root colonization capacity. This is very promising for the development of combined biocontrol products. However, the interaction between EPP, EPN and EPF needs to be studied more closely, especially regarding the formulation and application. Any negative interactions must be excluded

if all three BCAs were applied together (e.g. in one tank mixture) or even included in one single product. It is clear that costs for production, registration, and application will be higher in products containing BCA consortia than in single BCA products. This impairs the uptake of combined application strategies of BCAs by farmers for the time being. However, we believe that with improved registration processes, cheaper large-scale production, and more pressure to reduce pesticide application in many countries worldwide, these hurdles can be overcome in the future.

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Declaration of Interests

The authors declare no conflict of interests.

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Supplementary Material

Supplementary Tables

Table S1. **Effect of insecticidal fluorescent pseudomonads and entomopathogenic nematodes on *D. radicum* development under screening conditions: summary of all experiments.**

Species	Strain	Experiment	Fly emergence test strain (%)	Fly emergence control (%)	Reduction compared to control (%)
Pseudomonas sp.	PILAR01	2,3	35.75	28.4	-
P. protegens	PPRAR03	2,3	19.95	28.4	29.7%
	PPRAR04	1-5	27.1	39.6	31.6%
	CHA0	1,4,5	29.2	47.1	38%
	PF	1	23.4	18.8	-
P. chlororaphis	PCLRT03	2-5	7.4	44.8	83.5%
	PCLAR03	1-5	19.2	39.6	51.5%
	PCL1391	1,4,5	16.0	47.1	65.9%
H. bacteriophora	618b	7-10	26.9	48.75	44.8%
	nematop	1-10	34.0	36.5	9.9%
S. affine	656	7-10	39.1	48.75	19.7%
S. carpocapsae	596a	7-10	47.2	48.75	3.3%
	nemastar	9-10	40.9	48.75	16.1%
S. feltiae	nemaplus	1-8	31.1	38.1	18.4%
	594	3-6, 9-10	21.7	36.6	40.7%
	608	3-6	27.8	32.1	13.4%
	RS5	3-10	22.0	40.4	45.5%
S. poinarii	617	9	38.9	61.1	36.33%
	646	9-10	39.5	45.7	13.6%

Fly emergence test strain: mean fly emergence rate per egg of all experiments a *Pseudomonas* or nematode strain was tested in. Fly emergence control: mean of untreated controls of the specific experiments a strain was tested in. Reduction compared to control: The reduction of fly emergence compared to the control was calculated as follows: 1 – mean strain/mean control. Results and statistical analyses are displayed in Figs. 1A and B.

Table S2. **Results from individual screening assays with EPP and EPN against *D. radicum.***

***Pseudomonas* screening assays**

strain	exp	mean	reduction	SD	SE	min	median	max
control	1	18.8	NA	16.4	5.8	0	18.8	50
control	2	26.8	NA	18.4	6.5	0	25	63
control	3	30	NA	16	5.7	10	30	50
control	4	66.2	NA	7.4	2.6	60	65	80
control	5	56.2	NA	10.6	3.8	40	55	70
PILAR01	2	31.5	-17.54%	20.1	7.1	0	31.5	63
PILAR01	3	40	-33.33%	14.1	5	20	40	60
PPRAR03	2	17.4	35.07%	16.2	5.7	0	13	50
PPRAR03	3	22.5	25.00%	14.9	5.3	10	20	50
PPRAR04	1	6.2	67.02%	9.4	3.3	0	0	25
PPRAR04	2	22.2	17.16%	17.3	6.1	13	13	63
PPRAR04	3	22.5	25.00%	26.6	9.4	0	15	70
PPRAR04	4	38.1	42.45%	25.3	9	0	45	70
PPRAR04	5	46.2	17.79%	13	4.6	30	50	70
PCLRT03	2	15.8	41.04%	17.5	6.2	0	12.5	38
PCLRT03	3	6.2	79.33%	9.2	3.2	0	0	20
PCLRT03	4	7.5	88.67%	8.9	3.1	0	5	20
PCLRT03	5	15	73.31%	NA	NA	NA	NA	NA
PCLAR03	1	17.2	8.51%	18.8	6.7	0	12.5	50
PCLAR03	2	0	100.00%	0	0	0	0	0
PCLAR03	3	22.5	25.00%	20.5	7.3	0	20	60
PCLAR03	4	23.8	64.05%	26.7	9.4	0	15	70
PCLAR03	5	32.5	42.17%	13.9	4.9	10	30	50
CHA0	1	6.2	67.02%	9.4	3.3	0	0	25
CHA0	4	57.5	13.14%	20.5	7.3	20	60	80
CHA0	5	23.8	57.65%	14.1	5	0	25	40
PCL1391	1	3.1	83.51%	5.8	2	0	0	12.5
PCL1391	4	28.8	56.50%	18.9	6.7	0	30	60
PCL1391	5	16.2	71.17%	16.9	6	0	15	40
PF	1	23.4	-24.47%	19.4	6.9	0	25	50

Nematode screening assays

Table S2. **Results from individual screening assays with EPP and EPN against *D. radicum.***

strain	exp	mean	reduction	SD	SE	min	median	max
control	1	42.5	NA	11.6	4.1	30	45	60
control	2	30	NA	26.2	9.3	10	20	70
control	3	29.7	NA	14.8	5.2	12.5	25	50
control	4	26.2	NA	19.2	6.8	0	30	50
control	5	38.8	NA	24.2	8.5	10	40	70
control	6	33.8	NA	19.2	6.8	0	30	60
control	7	67.5	NA	13.9	4.9	50	70	90
control	8	36.2	NA	14.1	5	10	40	60
control	9	61.1	NA	22.4	7.9	33	56	100
control	10	30.2	NA	11.4	4	11	33	44
Hb 618b	7	31.2	53.78%	15.5	5.5	10	30	60
Hb 618b	8	21.2	41.44%	14.6	5.2	0	25	40
Hb 618b	9	27.6	54.83%	19.7	7	0	27.5	56
Hb 618b	10	27.6	8.61%	19.8	7	11	22	67
Hb nematop	1	46.2	-8.71%	17.1	4.3	20	50	80
Hb nematop	2	30.6	-2.00%	18.1	4.5	10	30	80
Hb nematop	3	42.2	-42.09%	14.8	5.2	25	37.5	75
Hb nematop	4	21.2	19.08%	9.9	3.5	10	20	40
Hb nematop	5	35	9.79%	17.7	6.3	20	30	70
Hb nematop	6	30	11.24%	14.1	5	0	35	40
Hb nematop	9	41.5	32.08%	20.6	7.3	22	33	78
Hb nematop	10	16.5	45.36%	16.6	5.9	0	16.5	44
Sa 656	7	40	40.74%	20	7.1	10	40	70
Sa 656	8	27.5	24.03%	15.8	5.6	0	35	40
Sa 656	9	44.6	27.00%	20	7.1	11	56	67
Sa 656	10	44.4	-47.02%	24.6	8.7	0	44	78
Sc 596a	7	41.2	38.96%	24.2	8.5	10	40	90
Sc 596a	8	36.2	0.00%	20	7.1	10	30	70
Sc 596a	9	66.9	-9.49%	15.8	5.6	44	67	89
Sc 596a	10	44.2	-46.36%	18.1	6.4	33	33	78
Sc nemastar	9	44.4	27.33%	23.9	8.5	0	44	78
Sc nemastar	10	37.4	-23.84%	15.8	5.6	11	38.5	56

Table S2. **Results from individual screening assays with EPP and EPN against *D. radicum.***

strain	exp	mean	reduction	SD	SE	min	median	max
Sf nemaplus	1	47.5	-11.76%	14.8	3.7	10	50	70
Sf nemaplus	2	18.1	39.67%	15.2	3.8	0	15	40
Sf nemaplus	3	40.6	-36.70%	23.9	8.4	12.5	37.5	87.5
Sf nemaplus	4	7.5	71.37%	8.9	3.1	0	5	20
Sf nemaplus	5	42.5	-9.54%	11.6	4.1	30	40	60
Sf nemaplus	6	31.2	7.69%	27	9.5	10	20	80
Sf nemaplus	7	42.5	37.04%	20.5	7.3	0	45	60
Sf nemaplus	8	15	58.56%	12	4.2	0	10	40
Sf 594	3	32.8	-10.44%	9.3	3.3	25	31.2	50
Sf 594	4	12.5	52.29%	13.9	4.9	0	10	40
Sf 594	5	35	9.79%	17.7	6.3	10	40	60
Sf 594	6	23.8	29.59%	10.6	3.8	10	25	40
Sf 594	9	16.5	73.00%	13.1	4.6	0	11	44
Sf 594	10	9.6	68.21%	9.2	3.2	0	11	22
Sf 608	3	31.2	-5.05%	22.2	7.8	0	31.2	75
Sf 608	4	15	42.75%	13.1	4.6	0	15	40
Sf 608	5	37.5	3.35%	15.8	5.6	20	30	70
Sf 608	6	27.5	18.64%	13.9	4.9	0	30	40
Sf RS5	3	34.4	-15.82%	11.1	3.9	25	31.2	50
Sf RS5	4	12.5	52.29%	16.7	5.9	0	10	50
Sf RS5	5	26.2	32.47%	13	4.6	10	30	50
Sf RS5	6	31.2	7.69%	27	9.5	0	35	70
Sf RS5	7	21.2	68.59%	23.6	8.3	0	15	60
Sf RS5	8	7.5	79.28%	11.6	4.1	0	0	30
Sf RS5	9	26.4	56.79%	20.7	7.3	0	22	56
Sf RS5	10	16.5	45.36%	15.6	5.5	0	11	44
Sp 617	9	38.9	36.33%	21.6	7.6	0	38.5	67
Sp 646	9	50	18.17%	21.6	7.7	11	50	78
Sp 646	10	29	3.97%	13.4	4.7	11	27.5	56

Table S2. **Results from individual screening assays with EPP and EPN against *D. radicum.***

For each *Pseudomonas* and nematode strain, results from each experiment in which a strain was tested are displayed as: mean fly emergence rate per egg (%), reduction of fly emergence rate compared to the control (in %), standard deviation (SD) and error (SE), as well as minimum, median and maximum fly emergence rate per egg. This table contains the detailed data of Fig. 1 and Table S1

Table S3. **Effect of EPP and EPN combinations on *D. radicum* development under screening conditions.**

Experiment 1

Strain	control	PCL1391	Sf RS5	Sf 594	PCL x RS5	PCL x 594
Pupation rate (%)	42.5	19.2	16.7	32.5	4.2	3.3
Reduction	-	54.8%	60.7%	23.5%	90.1%	92.2%
Fly emergence rate (%)	35.8	12.5	12.5	25.0	1.7	0.8
Reduction	-	65.1%	65.1%	30.2%	95.2%	97.8%

Experiment 2

Strain	control	PCLRT03	Sf RS5	Sf nemaplus	PCL x RS5	PCL x nema.
Pupation rate (%)	57.8	0	32.5	36.7	0	0
Reduction	-	100%	43.7%	36.5%	100%	100%

Data shown are mean pupation and fly emergence rate per egg (%) and reduction compared to the control (%). Detailed results and statistical analyses are displayed in Figs. 1C and D. PCL = *P. chlororaphis*, Sf = *S. feltiae*.

Table S4. **Evaluation of interactions for BCA combinations.**

Combination	Variable	Bliss Calculation	Interaction
EPP x EPN combination experiments under screening conditions			
RS5 x PCL1391	Pupation	0.607 + 0.548 – (0.607x0.548) = 0.822 < 0.901	synergism
594 x PCL1391	Pupation	0.235 + 0.548 – (0.235x0.548) = 0.654 < 0.922	synergism
RS5 x PCL1391	Fly emerg.	0.651 + 0.651 – (0.651x0.651) = 0.878 < 0.952	synergism
594 x PCL1391	Fly emerg.	0.302 + 0.651 – (0.302x0.651) = 0.756 < 0.978	synergism
RS5 x PCLRT03	Pupation	0.437 + 1 – (0.437x1) = 1 = 1	additivity
nema x PCLRT03	Pupation	0.365 + 1 – (0.365x1) = 1 = 1	additivity
EPP x EPN combination experiment under semi-field conditions			
RS5 x PCLRT03	Dead pl.	0.156+0.25 – (0.156*0.25) = 0.367 < 0.423	synergism
RS5 x PCLRT03	Healthy pl.	0.25+0.08 – (0.25*0.08) = 0.31 < 0.323	synergism
EPP x EPF combination experiments under screening conditions			
Bip5 x PCL1391	Fly emerg.	-0.418 + 0.248 – (-0.418x0.248) = -0.07 < 0.752	synergism
2587 x PCL1391	Fly emerg.	-0.418 + 0.582 – (-0.418x0.582) = 0.407 < 0.582	synergism
Bip5 x PCL1391	Fly emerg.	1 + 0.4 – (-1x0.4) = 1 > 0.969	antagonism
2587 x PCL1391	Fly emerg.	1 + 0.087 – (1x0.087) = 1 > 0.969	antagonism

Data shown are calculations according to the Bliss Independence formula ($E_{12} = E_1 + E_2 - (E_1 x E_2)$ with $E_{12} > E_{combo}$ = antagonism, $E_{12} = E_{combo}$ = basic additivity and $E_{12} < E_{combo}$ = synergism) and reduction values as displayed in Tables S3 and S6 and as described in chapters 3.2 and 3.3. Variables: Pupation = reduction in pupation rate compared to the control, Fly emerge. = reduction in fly emergence rate compared to the control, Dead pl. = reduction in dead plants compared to the control, healthy pl. = increase in healthy plants compared to the control.

Table S5. **Results from semi-field trial 1 with EPP and EPN application against *D. radicum.***

Treatment	0 [#]	1 [#]	2 [#]	D [#]	0 [%]	1 [%]	2 [%]	D [%]
Delia-free	56	2	2	5	86.15	3.08	3.08	7.69
Control	23	2	7	64	23.96	2.08	7.29	66.67
N	29	5	8	54	30.21	5.21	8.33	56.25
P	25	6	17	48	26.04	6.25	17.71	50.00
PN	31	10	18	37	32.29	10.42	18.75	38.54

Number and percentage of radish bulbs per damage category for each treatment with 4 plants per pot (n=24; *Delia*-free n=16). 0 = no *D. radicum* specific damage; 1 = small damage, 1 mining; 2 = large damages, $\geq$ 2 minings; D = plant dead. N = EPN population *S. feltiae* RS5, P = EPP strain *P. chlororaphis* PCLRT03.

Table S6. **Effect of EPP and EPF combinations on *D. radicum* development under screening conditions.**

Experiment 1						
strain	control	PCL1391	Mb Bip5	Bb 2587	PCL x Bip5	PCL x 2587
Fly emergence rate (%)	16.5	23.4	12.4	6.9	4.1	6.9
Reduction	-	NA	24.8%	58.2%	75.2%	58.2%
Experiment 2						
strain	control	PCL1391	Mb Bip5	Bb 2587	PCL x Bip5	PCL x 2587
Fly emergence rate (%)	54.2	0	32.5	49.5	1.7	1.7
Reduction	-	100%	40%	8.7%	96.9%	96.9%

Data shown are mean fly emergence rates per egg (%) and reduction compared to the control (%). Detailed results and statistical analyses are displayed in Figs. 3A and B. PCL = *P. chlororaphis;* Mb = *M. brunneum*, Bb = *B. bassiana*.

Table S7. **Effect of combined biocontrol agent applications on *D. radicum* development under greenhouse conditions: summary of all experiments.**

Treatment	Experiment	Fly emergence test strain (%)	Fly emergence control (%)	Reduction compared to control
P	1-6	9.25	22.66	59.2%
N	1-2, 5-6	12.31	22.02	44.1%
F	3-6	10.73	23.235	53.8%
PN	1-2	8.64	21.75	60.3%
PF	3-4	9.59	24.175	60.3%
PFN	5-6	10.21	22.295	54.2%

Fly emergence test strain: mean fly emergence rate per egg of all experiments a strain or combination was used in. Fly emergence control: mean of untreated controls of all specific experiments a treatment was tested in. Reduction compared to control: the reduction of fly emergence compared to the control was calculated as follows: 1 – mean strain/mean control. Detailed results and statistical analyses are displayed in Figure 4. F = EPF strain *M. brunneum* Bip5, N = EPN population *S. feltiae* RS5, P = EPP strain *P. chlororaphis* PCLRT03, PN, PF and PFN = respective combinations of F, N and P.

Table S8. **Results from individual greenhouse trials with BCA against *D. radicum*.**

treatment	exp	mean	reduction	SD	SE	min	median	max
control	5	19.17	NA	9.70	2.42	0.00	20.00	33.33
control	6	25.42	NA	14.03	3.51	0.00	26.67	40.00
control	3	26.68	NA	17.55	4.39	6.70	23.35	66.70
control	4	21.67	NA	9.27	2.32	13.33	20.00	40.00
control	1	21.00	NA	12.48	2.79	0.00	23.34	40.00
control	2	22.50	NA	14.52	3.25	0.00	21.43	50.00
F	5	4.17	78%	4.13	1.03	0.00	6.67	13.33
F	6	20.00	21%	19.32	4.83	0.00	13.33	66.67
F	3	3.76	86%	5.95	1.49	0.00	0.00	20.00
F	4	15.00	31%	10.18	2.55	0.00	13.33	40.00
N	5	2.92	85%	4.19	1.05	0.00	0.00	13.33
N	6	33.33	-31%	11.16	2.79	13.33	36.66	46.67
N	1	5.33	75%	6.70	1.50	0.00	3.34	20.00
N	2	10.00	56%	10.97	2.45	0.00	7.14	28.57
P	5	1.25	93%	2.69	0.67	0.00	0.00	6.67
P	6	11.25	56%	9.34	2.33	0.00	10.00	26.67
P	3	6.25	77%	6.64	1.66	0.00	6.70	20.00
P	4	15.00	31%	14.09	3.52	0.00	13.33	53.33
P	1	9.67	54%	11.54	2.58	0.00	6.67	46.67
P	2	11.43	49%	9.39	2.10	0.00	10.71	28.57
PF	3	7.09	73%	8.94	2.24	0.00	6.70	26.70
PF	4	12.08	44%	13.38	3.35	0.00	10.00	53.33
PFN	5	4.17	78%	5.90	1.48	0.00	0.00	20.00
PFN	6	16.25	36%	8.77	2.19	6.67	13.33	33.33
PN	1	8.00	62%	7.37	1.65	0.00	6.67	26.67
PN	2	9.29	59%	8.06	1.80	0.00	7.14	28.57

Data shown are mean fly emergence rate per egg (%), reduction of fly emergence rate compared to the control (in %), standard deviation (SD) and error (SE), as well as minimum, median and maximum fly emergence rate per egg. F = EPF strain *M. brunneum* Bip5, N = EPN population *S. feltiae* RS5, P = EPP strain *P. chlororaphis* PCLRT03. This table contains the detailed data of Fig. 4 and Table S7.

Table S9. **Impact of BCA application on *Delia radicum* damages in radish bulbs under field conditions.**

Treatment	c	F	N	P	PFN
Percentage undamaged plants (cat. 0)	41.7	57.32	55.02	65.42	62.95
Increase in undamaged plants		37%	32%	57%	50%
Percentage light damaged plants (cat. 1)	27.23	24.39	27.51	26.17	26.34
Reduction of light damage		10%	NA	4%	3%
Percentage heavy damaged plants (cat. 2+3)	31.06	18.3	17.47	8.41	10.71
Reduction of heavy damage		41%	44%	73%	66%

Reductions resp. increases are given compared to the control. Detailed results and statistical analyses are displayed in Fig 5. c = negative control treatment, F = EPF strain *M. brunneum* Bip5, N = EPN population *S. feltiae* RS5, P = EPP strain *P. chlororaphis* PLCRT03, PFN = combination of P, F and N.

Table S10. **Detailed results of the field trial with BCA application against *D. radicum*.**

treat	plot	0 [#]	1 [#]	2 [#]	3 [#]	NA [#]	0 [%]	1 [%]	2 [%]	3 [%]
c	1	15	25	12	6	2	25.86	43.10	20.69	10.34
c	2	22	14	14	2	11	42.31	26.92	26.92	3.85
c	3	32	8	14	14	3	47.06	11.76	20.59	20.59
c	4	29	17	4	7	6	50.88	29.82	7.02	12.28
F	1	32	15	6	1	12	59.26	27.78	11.11	1.85
F	2	32	17	15	0	3	50.00	26.56	23.44	0
F	3	40	14	10	2	1	60.61	21.21	15.15	3.03
F	5	37	14	10	1	2	59.68	22.58	16.13	1.61
N	2	33	15	10	0	15	56.90	25.86	17.24	0
N	3	36	12	4	0	10	69.23	23.08	7.69	0
N	4	30	20	15	0	0	46.15	30.77	23.08	0
N	5	27	16	10	1	1	50.00	29.63	18.52	1.85
P	1	41	16	3	0	9	68.33	26.67	5.00	0
P	3	35	12	1	0	12	72.92	25.00	2.08	0
P	4	25	15	11	0	5	49.02	29.41	21.57	0
P	5	39	13	3	0	7	70.91	23.64	5.45	0
PFN	1	32	14	3	0	4	65.31	28.57	6.12	0
PFN	2	44	3	2	0	13	89.80	6.12	4.08	0
PFN	4	41	24	13	0	6	52.56	30.77	16.67	0
PFN	5	24	18	6	0	4	50.00	37.50	12.50	0

Number and percentage of radish bulbs per damage category, for each treatment and each plot per treatment in the field trial. 0 = no damage; 1 = small damage, 1 mining; 2 = large damages, 2-5 minings; 3 = severe damages, bulbs destroyed by $>$ 5 minings; NA = rating not possible, bulbs rotten due to wetness. c = control, F = EPF strain *M. brunneum* Bip5, N = EPN population *S. feltiae* RS5, P = EPP strain *P. chlororaphis* PCLRT03, PFN = combination of P, F and N.

Table S11. **Colonization values from semi-field trial 1 with EPP and EPN application against *D. radicum*.**

treat	P root	P soil	N soil
c	BD	BD	3.30
N	NA	NA	3.46×10^2 a
P	2.78×10^8 a	7.12×10^5 a	NA
PN	1.19×10^8 b	1.02×10^6 a	3.94×10^2 a

Mean colonization values at harvest. One soil sample per pot (n = 12) was taken resp. all roots per pot collected. P was determined by plating on selective medium and N using qPCR. Values for P root and soil are per g fresh weight and values for N soil per 100 g fresh weight. c = control, N = EPN population *S. feltiae* RS5, P = EPP strain *P. chlororaphis* PCLRT03, PN = combination of P and N. BD = below detection limit, NA = not available, measurement not conducted. Different letters after values within one column indicate statistically significant differences according to Kruskal-Wallis test.

Table S12. **Colonization values from semi-field trial 2 with EPP, EPN and EPF application against *D. radicum*.**

treat	P root	P soil	F root	F soil	N soil
c	BD	BD	BD	BD	0.5
F	BD	BD	6.80×10^3 a	2.15×10^5 a	NA
N	NA	NA	NA	NA	6.79×10^1 a
P	4.10×10^7 a	2.98×10^5 a	BD	9.40	NA
PF	2.03×10^7 a	4.40×10^5 ab	1.05×10^4 a	1.48×10^5 a	NA
FN	BD	6.90	8.18×10^3 a	1.69×10^5 a	1.02×10^2 a
PN	3.03×10^7 a	3.07×10^5 a	BD	2.71×10^5 a	9.42×10^1 a
PFN	1.97×10^7 a	5.85×10^5 b	7.69×10^3 a	2.11×10^5 a	7.00×10^1 a

Mean colonization values at harvest. One soil sample per pot (n = 16) was taken resp. all roots per pot collected and processed. P and F were determined by plating on selective medium, and N via qPCR. P & F root samples are per g fresh weight, P & F soil samples per g dry weight and N soil per 100 g dry weight. c = control, F = EPF strain *M. brunneum* Bip5, N = EPN population *S. feltiae* RS5, P = EPP strain *P. chlororaphis* PCLRT03, PF, PN, FN and PFN = respective combinations of P, F and N. BD = below detection limit, NA = not available, measurement not conducted. Different letters after values within one column indicate statistically significant differences according to Kruskal-Wallis and post-hoc Dunn test.

Table S13. **Colonization values from the field trial with BCA application against *D. radicum*.**

Time-point 1 (2 weeks after sowing)

treat	P root	P soil	F root	F soil	N soil
c	BD	BD	BD	2.35	0.08
F	BD	BD	1.11×10^4 ab	2.44×10^5 ab	5.60
N	BD	BD	BD	BD	1.28×10^2 a
P	1.33×10^6 a	6.97×10^5 a	BD	1.57	0.13
PFN	9.02×10^5 a	8.35×10^5 a	1.35×10^4 a	2.44×10^5 a	1.25×10^2 a

Time-point 2 (8 weeks after sowing)

treat	P root	P soil	F root	F soil	N soil
c	BD	BD	BD	6.48×10^1	2.91
F	1.48×10^4	1.17×10^3	7.30×10^3 b	1.08×10^5 c	0.96
N	9.15×10^3	BD	BD	1.64×10^1	5.68×10^1 b
P	3.91×10^7 b	1.00×10^7 b	2.03×10^1	3.77×10^1	0.59
PFN	3.24×10^7 b	1.53×10^7 b	8.13×10^3 ab	1.54×10^5 bc	6.70×10^1 b

Mean colonization values from the first and second sampling after two resp. eight weeks, 3-5 soil and root samples were taken per plot. P and F were determined by plating on selective medium, and N via qPCR. P & F root samples are per g fresh weight, P & F soil samples per g dry weight and N soil per 100 g dry weight. c = control, F = EPF strain *M. brunneum* Bip5, N = EPN population *S. feltiae* RS5, P = EPP strain *P. chlororaphis* PCLRT03, PFN = combination of P, F and N. BD = below detection limit. Different letters after values within one column (both time-points) indicate statistically significant differences according to Kruskal-Wallis and post-hoc Dunn test.

Supplementary Figures

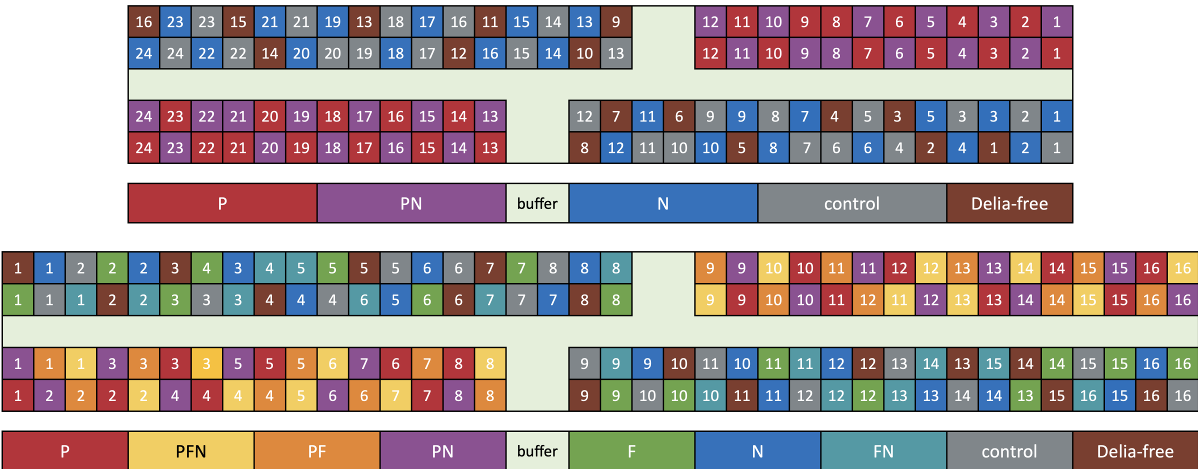

Fig. S1. **Set-up of semi-field trials with combined application of EPN, EPF and EPP.**

Upper part: Semi-field trial 1 with single and combined application of EPP strain *P. chlororaphis* PCLRT03 (P) and EPN population *S. feltiae* RS5 (N). Lower part: Semi-field trial 2 with EPP, EPN and EPF strain *M. brunneum* Bip5 (F) single application as well as all double and triple combinations. All pots except for the Delia-free treatment were artificially inoculated with *D. radicum* eggs. Pots were distributed over the whole length of seedbeds at Agroscope, Zurich, Switzerland. Treatments with and without bacteria were kept in separate blocks in order to prevent bacteria that readily move in the soil from migrating to pots of bacteria-free treatments. Within the blocks, treatments were randomized. Barley was sown into the space between the EPP and EPP-free blocks.

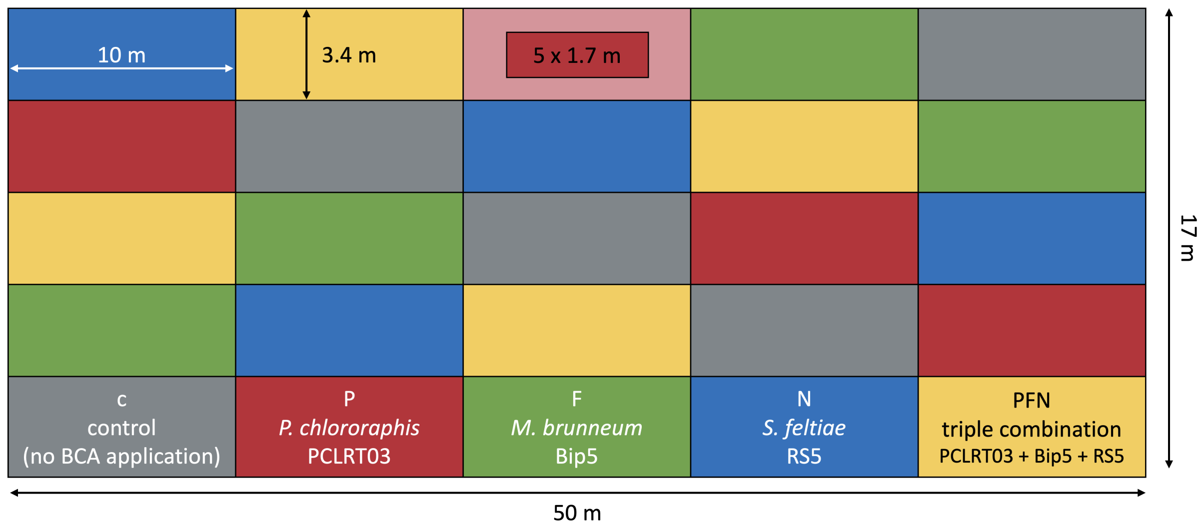

Fig. S2. **Set-up of the field trial with combined application of EPN, EPF and EPP.**

In the field trial, EPP strain *P. chlororaphis* PCLRT03 (P), EPN population *S. feltiae* RS5 (N) and EPF strain *M. brunneum* Bip5 (F) were applied alone and in a triple combination (PFN). In the control, no biocontrol agents were applied. The field was split into 5 x 5 plots and treatments distributed according to a Latin square design. Each plot contains 8 rows of radish *Raphanus sativus* L. cultivar 'Andes F1' plants. Biocontrol agents were applied in the inner rectangle representing 4 plant rows of 5 m length (5 x 1.7 m).

Chapter 3

When competitors join forces: Consortia of entomopathogenic microorganisms increase killing speed and mortality in leaf- and root-feeding insect hosts

This chapter was submitted to Microbial Ecology by:

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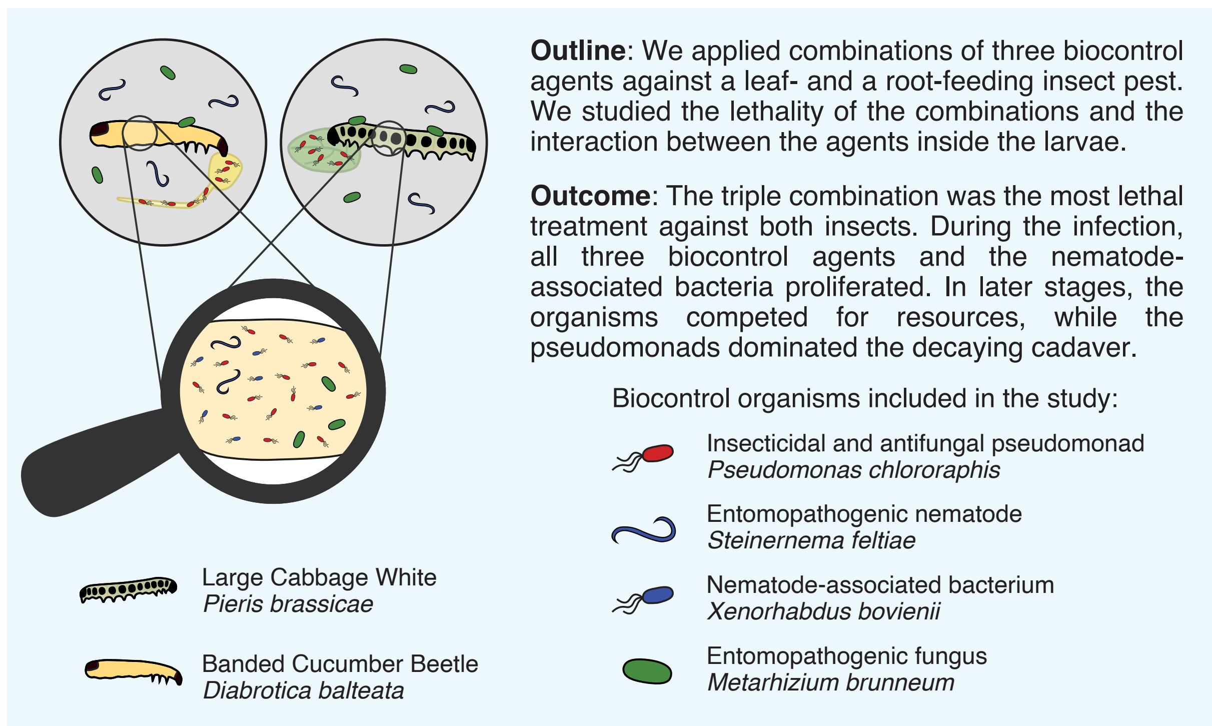
Abstract

Combining different biocontrol agents (BCA) is an approach to increase efficacy and reliability of biological control. If several BCA are applied together, they have to be compatible and ideally work together. We studied the interaction of a previously selected BCA consortium of entomopathogenic pseudomonads (*P. chlororaphis*), nematodes (*Steinernema feltiae* associated with *Xenorhabdus bovienii*) and fungi (*Metarhizium brunneum*). We monitored the infection course in a leaf- (*Pieris brassicae*) and a root-feeding (*Diabrotica balteata*) pest insect after simultaneous application of the three BCA as well as their interactions inside the larvae in a laboratory setting. The triple combination caused the highest mortality and increased killing speed compared to single applications against both pests. Improved efficacy against *P. brassicae* was mainly caused by the pseudomonad-nematode combination, whereas the nematode-fungus combination accelerated killing of *D. balteata*. Co-monitoring of the three BCA and the nematode-associated *Xenorhabdus* symbionts revealed that the four organisms are able to co-infect the same larva. However, with advancing decay of the cadaver there is increasing competition and cadaver colonisation is clearly dominated by the pseudomonads, which are known for their high competitivity in the plant rhizosphere. Altogether, the combination of the three BCA increased killing efficacy against a Coleopteran and a Lepidopteran pest which indicates that this consortium could be applied successfully against a variety of insect pests.

Graphical Abstract



Introduction

Global food production relies heavily on synthetic pesticides to protect crops from pathogens and pests [1]. The pressure to limit pesticide use and the demand for alternative control solutions are increasing [2]. One alternative is biological control, which is the «use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be«[3]. For some diseases, biocontrol solutions are widely utilized, yet for many pathogens and pests efficient biocontrol products are not available [4]. Despite numerous success stories [2, 5, 6], the unstable performance of biocontrol agents (BCA) is a great challenge for reliable biocontrol solutions [7]. One approach to increase biocontrol efficacy is to combine different biocontrol agents with different modes of action and ecological niches [8]. Several studies found improved biocontrol success when applying BCA consortia [9, 10, 11]. However, other studies have reported antagonistic interactions when applying combinations of microbial BCA [12]. It is crucial to assess compatibility of selected organisms in order to develop an efficient BCA consortium.

Interactions between BCA can increase the efficacy of the consortium, but BCA can also negatively impact the other consortium members. Competition for nutrients and space could lead to inhibition; toxins and antimicrobial compounds produced by microbial BCA may affect the consortium partners or the defence reaction of the host. In this study, we explored the interactions within a consortium of three biocontrol agents, namely entomopathogenic pseudomonads (EPP), nematodes (EPN) and fungi (EPF). EPP from the species *Pseudomonas chlororaphis* are root-colonizing bacteria with plant-growth promoting, antifungal and insecticidal properties [13, 14, 15]. Their oral insecticidal activity largely relies on multiple toxins, enzymes and antimicrobial exoproducts [16, 17]. The studied EPN species *Steinernema feltiae* is associated with entomopathogenic *Xenorhabdus bovienii* (nematode-associated bacteria, NB) [18]. The bacteria are released in the insect haemocoel by the nematodes where they multiply and kill the insect with toxins and antimicrobials, though the nematodes themselves also contribute with own toxins [19, 20]. When resources are used up after several cycles of nematode reproduction, the nematodes take up bacteria and form a free-living infective juvenile (IJ) stage to hunt for a new host [18]. Finally, the EPF member of the consortium is *Metarhizium brunneum*, a common organism in agricultural soils especially in temperate regions [21]. *M. brunneum* infects and kills insects by attaching to and breaching through the cuticle, colonizing the insect haemolymph and producing different proteases, toxins and exoproducts during the whole process [22, 23]. According to earlier studies, biocontrol combinations of EPF and EPN can have additive, synergistic and, rarely, also antagonistic effects [24]. For EPP and EPN, recent publications report frequent interactions

during EPN infections, and it was proposed that EPP belong to the EPN pathobiome [25, 26].

In a previous study, we have investigated the biocontrol effect of a *P. chlororaphis-S. feltiae-M. brunneum* consortium against the cabbage maggot *Delia radicum* in pot and field experiments. Our results indicated that these BCA do not impede each other's survival in the soil or in the rhizosphere [27]. However, we know little about how they interact with and affect each other while infecting the same host. Therefore, our aim in this study was to explore the interactions between these EPP, EPF and EPN in mixed infections, and to examine the effect of different combinations on the host insect and the BCA themselves. We used single BCA and different combinations thereof to infect larvae of the large cabbage white (LCW) *Pieris brassicae* (Lepidoptera: Pieridae), an important pest feeding on above-ground plant parts of Brassicacean crops, and the root-feeding banded cucumber beetle (BCB) *Diabrotica balteata* LeConte (Coleoptera: Chrysomelidae), a sister species of the highly devastating western corn rootworm *D. virgifera virgifera*. While monitoring larval mortality over time, our main focus was to observe performance and proliferation of the three BCA and the EPN-associated *Xenorhabdus* bacteria (NB), i.e. four entomopathogens, inside their insect hosts. We hypothesise that the combined BCA application is more efficient in killing insects compared to single infections but that the BCA compete in the cadaver for resources and might hinder each other's proliferation. This study allowed us to gain new insight into the interaction dynamics between a nematode, a bacterial and a fungal biocontrol agent.

Methods

Rearing of organisms

Eggs of the large cabbage white (LCW) *Pieris brassicae* were obtained from the Biocommunication Group (ETH Zurich, Switzerland). Larvae were fed on Savoy cabbage and kept at 25 °C (16 h, 12 kLux), 20 °C (8 h, dark) and 60% rH during rearing and experiments (see Supplementary Methods). Eggs of the banded cucumber beetle (BCB) *Diabrotica balteata* were received from Syngenta AG (Stein, Switzerland) and reared on maize seedlings (variety Damaun KS, sativa, Switzerland) at 28 °C. Experiments were conducted at 25 °C in the dark at 60% rH (see Supplementary Methods).

EPP *P. chlororaphis* PCLRT03-gfp and PCLRT03-mturq (Table 1) were stored in 44% glycerol at -80 °C and grown for 3 days on King's B agar [28] supplemented with cycloheximide 100 mg/l, chloramphenicol 13 mg/l and gentamycin 10 mg/l at 24 °C. Bacteria were incubated overnight in Lysogeny broth (LB) [29] at 24 °C and 180 rpm for LCW experiments, but harvested directly from King's B plates for BCB experiments [27]. Bacteria were washed with ddH_2O and the concentration adjusted measuring optical density

at 600 nm (OD_{600}) (Genesys150, Thermo Fisher Scientific, MA, USA) with an OD_{600} of 0.1 corresponding to 10^8 cfu/ml. Approx. 300 ml suspension were prepared in a glass beaker with 2.5 x 10^8 cfu/ml and 5 x 10^8 cfu/ml for experiments with LCW and BCB, respectively.

EPF *M. brunneum* Bip5 (wildtype) and Bip5-gfp (Table 1) were grown on selective medium (SM) agar [30] for ten days at 24 °C in the dark. Conidiospores were scraped off plates using a Drigalski glass spatula, dissolved in Tween 80 0.01%, and washed once in ddH_2O. For LCW experiments, 20 ml of 10^7 spores/ml were prepared in a 50 ml beaker with ddH_2O. For BCB experiments, 10 ml of 2 x 10^8 spores/ml were prepared in a 25 ml beaker.

EPN *S. feltiae* RS5 (wildtype, Table 1) were multiplied in *Galleria mellonella* (Lepidoptera: Pyralidae) larvae (Hebeisen fisher store, Zurich, Switzerland) at 22 °C using the White trap method [31, 32]. From *G. mellonella* cadaver infested with RS5, *X. bovienii* SM5 was isolated and tagged with mcherry and a kanamycin resistance cassette. SM5-mcherry was re-associated with *Steinernema feltiae* RS5 by injecting SM5-mcherry and kanamycin into *G. mellonella* larvae infected by RS5, and the emerging IJ population was called RS5-mche (Table 1, see Supplementary Methods). For LCW experiments, 30 ml of 1000 IJ/ml tap water were prepared, whereas 2000 IJ/ml were prepared for the BCB experiments.

Table 1. **Entomopathogens used in this study.**

Species	Strain	Origin	Reference	Experiment
Pseudomonas chlororaphis	PCLRT03	Potato root, Switzerland	Vesga et al. [33]	-
P. chlororaphis	PCLRT03-gfp	Derivative of PCLRT03, PCLRT03::miniTn7-gfp2; Gm^R	Spescha et al. [27]	time-shift 1-3, LCW 1-4
P. chlororaphis	PCLRT03-mturq	Derivative of PCLRT03, PCLRT03::miniTn7-mturquoise2; Gm^R	This study; provided by Jordan Vacheron (University of Lausanne, Switzerland)	LCW 5, BCB 1-4
Steinernema feltiae	RS5 (RS-5, wildtype)	Soil, wheat field, Switzerland	Jaffuel et al. [34]	time-shift 1-3, LCW 1-4

Table 1. **Entomopathogens used in this study.**

Xenorhabdus bovienii	SM5	*Steinernema feltiae* RS5	Provided by Ricardo Machado (University of Neuchâtel, Switzerland)	-
X. bovienii	SM5-mcherry	Derivative of SM5, SM5::16S-mcherry; Kan^R	Provided by Alice Regaiolo (Johannes Gutenberg-University, Mainz, Germany)	-
S. feltiae	RS5-mche	RS5 re-associated with SM5-mcherry	This study	LCW 5, BCB 1-4
Metarhizium brunneum	Bip5 (BIPESCO5 /F52, wildtype)	*Cydia pomonella*, Austria	European Food Safety Authority [35]	time-shift 1-3, LCW 1-4
M. brunneum	Bip5-gfp	Derivative of BIPESCO5, Bip5::pK2-BAR-egfp; glufosinate^R	Provided by Jürg Enkerli	LCW 5, BCB 1-4

Experiment indicates in which experiments and repetition a strain was used. Time-shift refers to experiments in which EPP x EPN were applied individually and in combination with a time-shift; LCW refers to experiments in which all three biocontrol agents (EPP, EPN, EPF) were applied single and in combination against the large cabbage white *P. brassicae*; BCB refers to likewise experiments conducted with the banded cucumber beetle *D. balteata*; the numbers refer to the repetition of the respective experiment. Details about monitoring BCA in different experiments are provided in Fig. 1, the Supplementary Methods and Table S1.

Experimental set-up

Larvae (3rd instar LCW and 2nd instar BCB) were starved for 6 h before use. LCW larvae were placed individually onto one ø 32 mm filter paper disk (Whatman, Huberlab, Switzerland) per well of a 6-well plate (CELLSTAR®, Greiner Bio-One, Austria). BCB larvae were placed onto two ø 20 mm filter paper disks (Whatman, Huberlab, Switzerland) per well of a 12-well plate (CELLSTAR®) (Fig. 1). Plates with BCB were sealed with a lid and 2 layers of Breathe-Easy sealing membrane (Diversified Biotech, MA, USA) to avoid escapes.

For triple infection experiments with LCW, larvae were submerged for 5 s in EPF suspension (ddH$_2$O as control), 50 µl EPN suspension was pipetted on the filter paper (tap

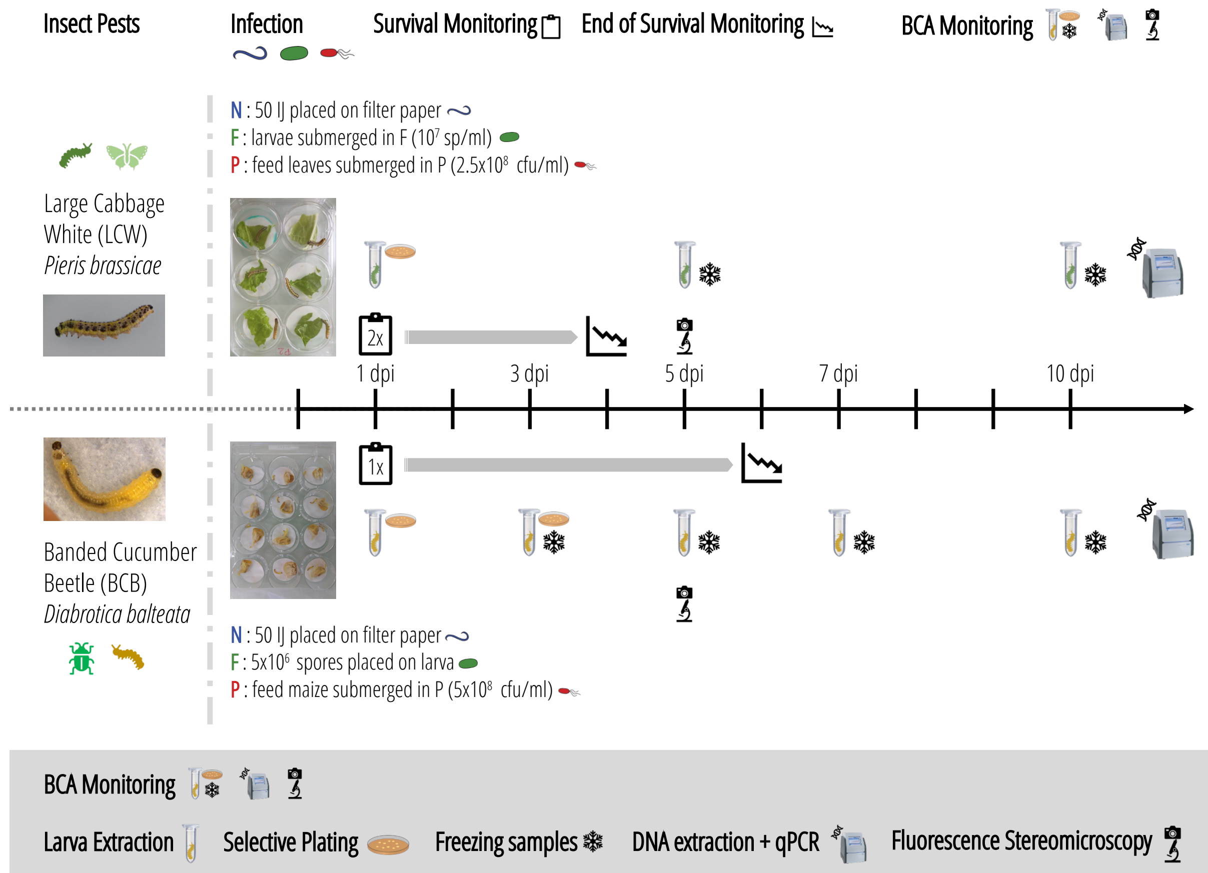


Fig. 1. **Experimental procedure for co-infection experiments with EPN, EPF and EPP in *P. brassicae* (LCW) and *D. balteata* (BCB) larvae.**

LCW and BCB larvae were infected with different BCA and BCA proliferation was monitored in repetition 5 of the LCW experiment and in repetition 2 of the BCB experiment. Larvae were infected with infective juveniles (IJ) of the EPN *S. feltiae* RS5-mche (N), EPF *M. brunneum* Bip5-gfp (F) and EPP *P. chlororaphis* PCLRT03-mturq (P). Survival of LCW larvae was monitored twice a day for 4 days and once daily during 6 days for BCB larvae. For the LCW experiment, six larvae per treatment (control n=3) were extracted alive at 1 day post inoculation (dpi) and dead at 5 and 10 dpi. For the BCB experiment, three alive larvae were extracted at 1 dpi, eight larvae (4 alive, 4 dead; control n=4) at 3 dpi, and six (control n=3) dead larvae at 5, 7 and 10 dpi. Larval extracts were plated on selective medium at 1 and 3 dpi. At 3, 5, 7, and 10 dpi, larvae were frozen for subsequent DNA extraction. Pictures of six dead larvae per treatment were taken at 5 dpi in both insect species using a fluorescence stereomicroscope. For a detailed description, see the Supplementary Methods and Table S1.

water as control), and larvae were fed with Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) leaf discs previously submerged for 30 min in EPP suspension (in ddH_2O as control). For EPP-EPN time-shift application experiments, EPN were added 6 h before (t-6h), simultaneously (t0) or 6 h after (t+6h) EPP infection.

For BCB infection experiments, 25 µl EPF suspension (or ddH_2O) and 25 µl EPN suspension (or tap water) were pipetted on the filter paper and larvae were fed with maize

seedlings submerged for 60 min in EPP suspension (or in ddH_2O).

Survival was monitored twice a day for 3 days for LCW and once a day for 6 days for BCB. For EPP-EPN-time-shift experiments, EPP colonisation was determined by selective plating at 1 day post infection (dpi) and EPN proliferation was estimated by the White trap method [32] at the end of the experiment in repetitions 2 and 3. For LCW, colonisation was assessed by selective plating at 1 dpi and by qPCR at 5 and 10 dpi in repetition 5. For BCB, BCA colonisation was assessed by selective plating at 1 and 3 dpi and by qPCR at 3, 5, 7 and 10 dpi in repetition 2. At 5 dpi, deceased larvae were photographed under a fluorescence stereomicroscope filtering for the fluorophores of the respective strain-tag. For survival and BCA monitoring, 18 (time-shift), 24 (LCW) and 72 (BCB) larvae per treatment and repetition were prepared (see Fig. 1 and the Supplementary Methods for more details).

Statistical Analysis

Data were analysed with Rstudio (version 1.4.1717) using R (version 4.1.2). Pooled data on larval survival was analysed using a cox model controlling for repetition effects and emmeans was used for post-hoc pairwise testing (packages coxme and emmeans). Larval survival was additionally analysed for each repetition individually using log-rank and pairwise survival difference tests (packages survival and survminer). Larval colonisation by BCA was compared using ANOVA and TukeyHSD tests.

Results

Combinations of EPP, EPN and EPF killed larvae faster and increased mortality

In a first experiment, we tested the effect of application timing for EPP-EPN combinations on LCW larvae. Survival curves based on pooled data of three repetitions (time-shift 1-3) are shown in Fig. 2A and data of individual repetitions in Table S1. Mortality was higher in all treatments compared to the control. Over all repetitions, the EPP-EPN combinations were significantly more lethal than EPP regardless of application timing, and significantly different to EPN when EPN were added 6 h before EPP (Fig. 2A). In the individual repetitions, the simultaneous application of EPP and EPN reduced the mean survival time compared to single applications (EPN: 6-7 h, EPP: 9-20 h), and consistently resulted in a higher mortality (94-100%), whereas mortality of single applications was more variable (EPN: 70-100%, EPP: 50-80%; Table S1). Larva colonization by EPP 1 day after infection was not affected by the presence of EPN at any application time-point (Figs. 3A, C, Table S2). EPN reproduction (= emergence of infective juveniles) took place in the presence of EPP, but only in half of the larvae when EPN were added 6 h after EPP (Figs. 3B, D, Table S2).

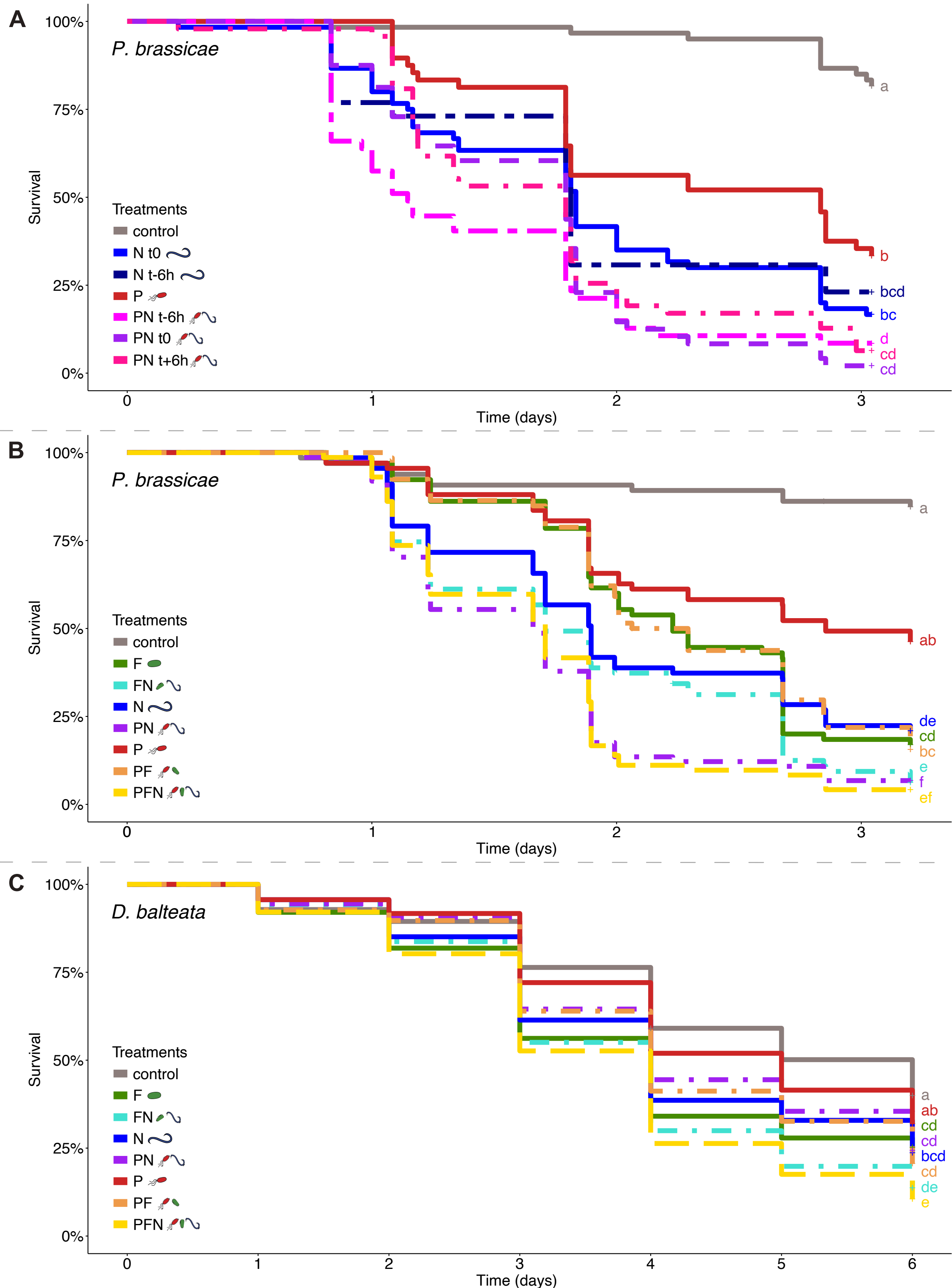


Fig. 2. **Survival of *P. brassicae* (LCW) and *D. balteata* (BCB) larvae after infection with single and combined applications of EPP, EPF and EPN.**

A) EPP x EPN time-shift (ts) application experiment: t-6h = EPN applied 6 h before EPP, t0 simultaneous application of EPP and EPN, t+6h EPN applied 6 h after EPP. B) LCW experiment with single and combined simultaneous EPP, EPF, and EPN applications. C) BCB experiment with single and combined simultaneous EPP, EPF and EPN applications. Treatments: control

= no BCA application, P = EPP *P. chlororaphis* PCLRT03-gfp or PCLRT03-mturq, N = EPN *S. feltiae* RS5 or RS5-mche, F = EPF *M. brunneum* Bip5 or Bip5-gfp, FN, PN, PF and PFN = double and triple combinations of respective BCA. Survival curves represent pooled data from three (ts 1-3) or four (LCW 1-4 and BCB 1-4) independent repetitions with 18 LCW and 60 BCB larvae per treatment and repetition. Different letters to the right of the survival curves indicate significant differences among treatments at $P < 0.05$ among pooled data. Data of individual repetitions of the three experiments (mean survival, final mortality and statistical analysis of the survival curves) are displayed in Tables S1, S3-S4.

In a second series of experiments, all three BCA were added simultaneously to LCW and BCB larvae. Survival curves based on pooled data (LCW 1-4 and BCB 1-4) are shown in Figs. 2B and 2C and data on individual repetitions of the experiments in Tables S3 and S4. In these experiments, EPF *M. brunneum* Bip5 and EPN *S. feltiae* RS5 alone were generally faster at killing larvae and caused higher mortality than EPP *P. chlororaphis* PCLRT03, which was significant for EPN and EPF in LCW and for EPF in BCB (Fig. 2B, C). The triple combination was the deadliest and fastest killing treatment against both insects and was significantly different to all single applications except for EPN in LCW. In individual repetitions, the triple combination caused 90-100% mortality in LCW and 80-95% in BCB (Tables S3, S4). In LCW, the EPP-EPN double combination was significantly more lethal compared to all other single and double applications (Fig. 2B). The EPN-EPF combination reached more consistently a high mortality compared to EPN and EPF, while the EPF-EPP combination behaved similarly to EPF (Table S3). In BCB, the EPN-EPF combination was the most lethal double combination, yet it was only significantly different to EPP (Fig. 2C). Both double combinations with EPP were only as lethal as the EPN or EPF partner alone, though significantly more effective than the EPP treatment.

Taken together, the triple combination was the most lethal treatment against both insect species. Faster and higher mortality was mainly caused by the combination of EPP with EPN for LCW, and of EPN with EPF for BCB.

Proliferation and competition of BCA after co-infection of *P. brassicae* and *D. balteata*

The development of the BCA populations inside their insect hosts was monitored in repetition 5 of the LCW and repetition 2 of the BCB experiment by selective plating (cfu/larva) and by qPCR (units/larva) and in addition pictures of dead larvae were taken with a fluorescence stereomicroscope (Fig. 1). In several pictures signals of two or even three fluorophores were detected, which indicated that BCA can co-exist in larvae at 5 dpi where we expected high BCA proliferation (Fig. 4).

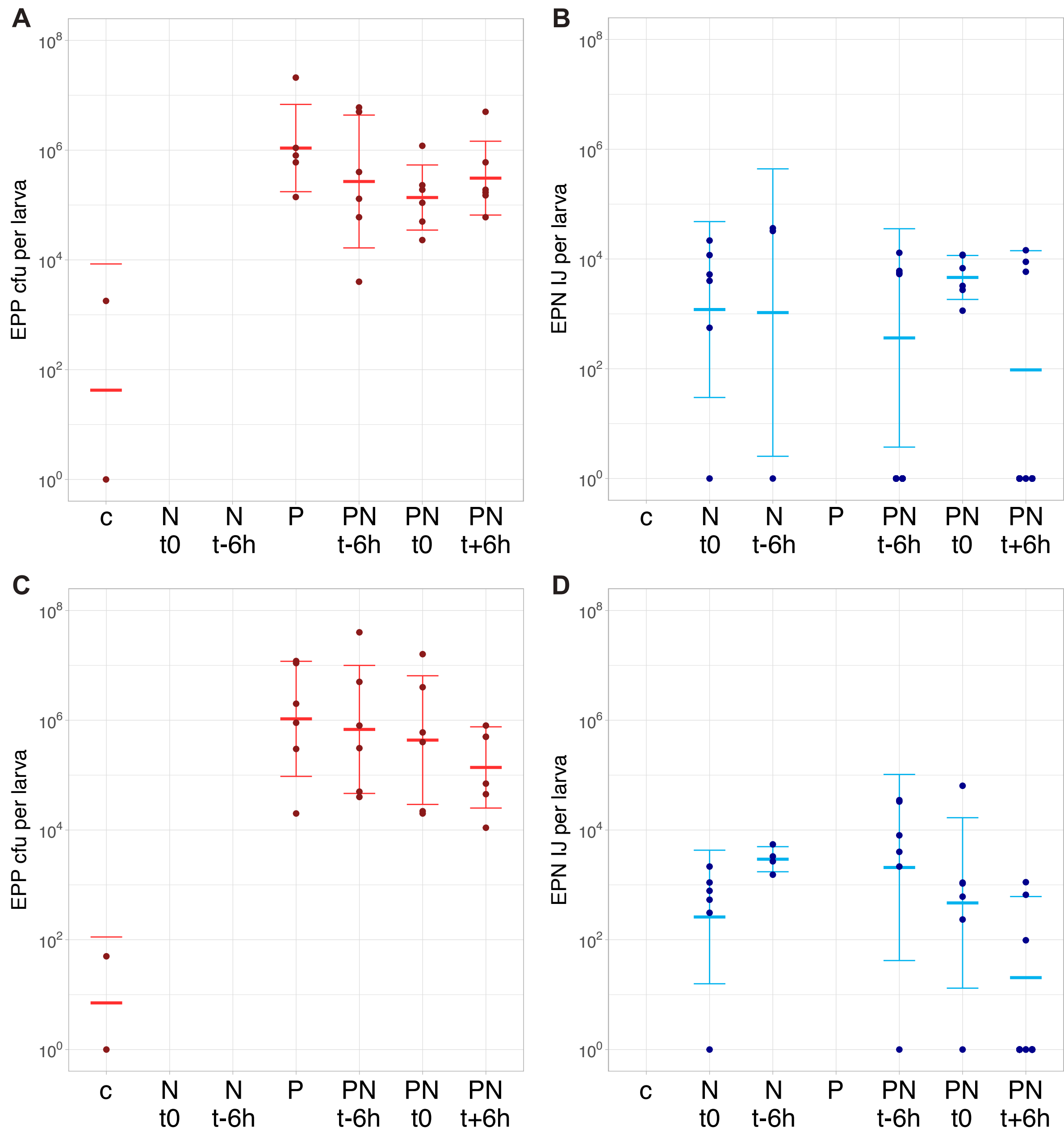


Fig. 3. **Proliferation of EPN and EPP in *P. brassicae* (LCW) larvae.**

Infective juvenile emergence (IJ per larva) and *Pseudomonas* colonization (cfu per larva) were assessed in the EPP x EPN time-shift application experiment for repetition 2 (A, B) and repetition 3 (C, D). Treatments: c = control with no BCA application, N t0 = EPN *S. feltiae* RS5, N t-6h = EPN applied 6 hours earlier, P = EPP *P. chlororaphis* PCLRT03-gfp, PN t-6h = EPN applied 6 hours before EPP, PN t0 = EPN and EPP applied simultaneously, PN t+6h = EPN applied 6 hours after EPP. Left (A, C): six alive larvae were homogenized at 1 dpi and plated on selective medium and values are displayed as colony forming units (cfu) per larva. Right (B, D): six dead larvae were transferred on White traps for infective juvenile (IJ) emergence and values are displayed as IJ per larva. Each dot represents one larva and crossbars show mean and standard deviation; no dot = not assessed. Mean colonisation density and statistical evaluation are shown in Table S2 and data on larval survival in respective experiments can be found in Table S1.

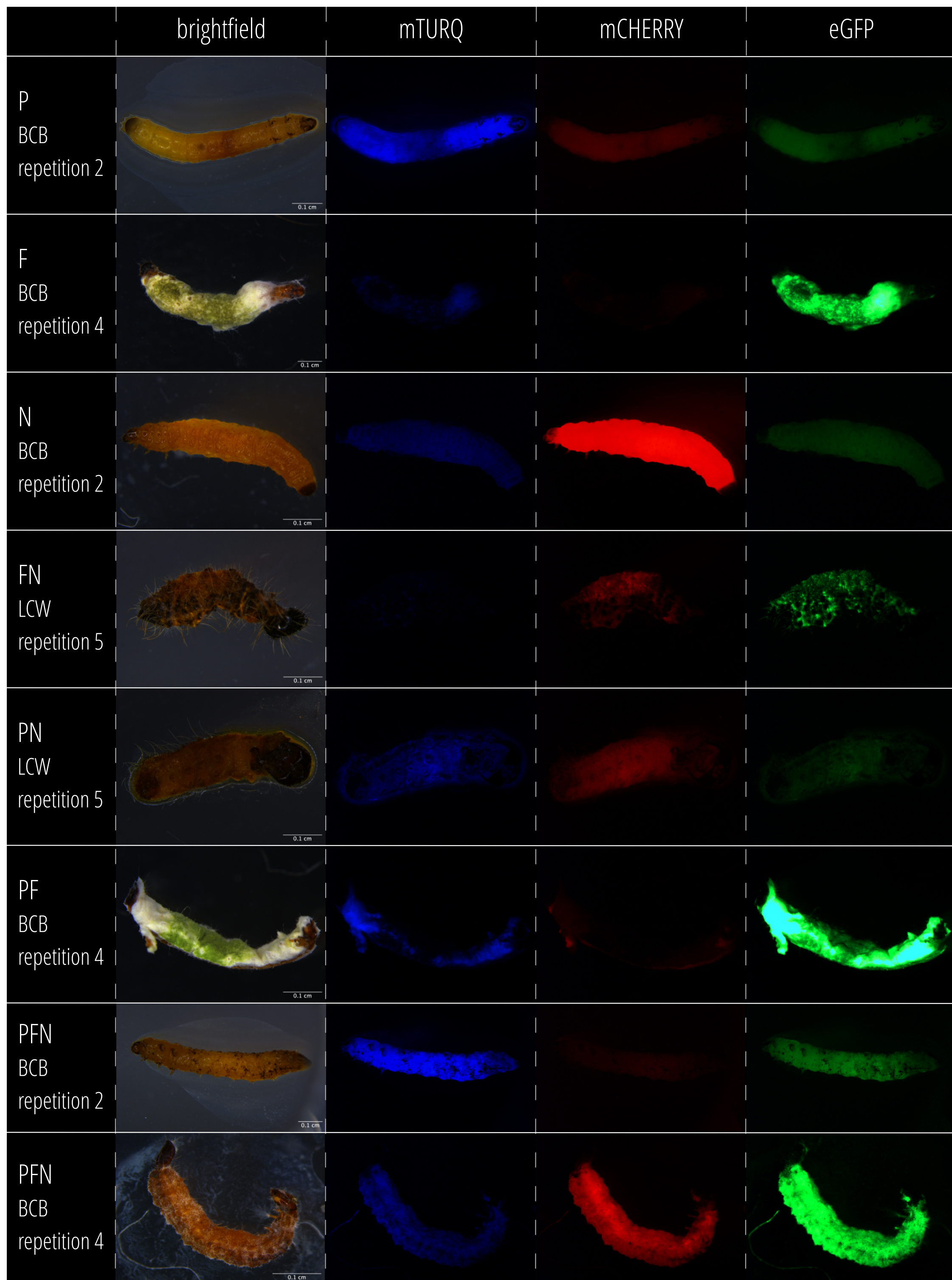


Fig. 4. **Pictures of *P. brassicae* (LCW) and *D. balteata* (BCB) larvae infected with EPP, EPN and EPF under brightfield and fluorescence filters.**

Each row shows pictures of the same larva at 5 dpi acquired under a stereomicroscope using different filters: brightfield, ET CFP (mTURQ), ET mCHER (mCHERRY) and ET GFP (eGFP). The first column states the treatment and the experiment of the larva. Treatments: P = EPP *P. chlororaphis* PCLRT03-mturq, N = EPN *S. feltiae* RS5-mche, F = EPF *M. brunneum* Bip5-gfp, FN, PN, PF and PFN = double and triple applications of respective BCA. Information on fluorescence imaging is given in the Supplementary Methods.

EPP *P. chlororaphis* PCLRT03 were detected at 10^4-10^5 (LCW, 1 dpi) and 10^2-10^5 (BCB, 3 dpi) cfu/larva at the onset of the infection and population sizes reached 10^7-10^9 units/larva at later stages in cadavers (Figs. 5, 6, Tables S5, S6). Whereas EPP populations in BCB were not affected by co-inoculation with other BCA, mean EPP populations in LCW were elevated in cadavers at 10 dpi following applications of double and triple combinations.

EPF *M. brunneum* Bip5 was detected at 50-400 cfu/larva at 1 and 3 dpi and colonisation levels in the single treatment were on average 10^7-10^8 units/larva at 5 dpi (Figs. 5, 6, Tables S5, S6). EPF were clearly impacted by co-inoculation: in LCW, EPF colonisation levels were lower in the combinations at 5 dpi and in BCB, EPF were detected less frequently in double and triple combinations at 3 dpi and 5 dpi.

EPN *S. feltiae* RS5 were present in both insects at 40-600 units/larva at 5 dpi but were detected in fewer larvae in the triple combination compared to the single EPN application (Figs. 5, 6, Tables S5, S6). EPN populations in the single treatment increased on average 500-fold from 5 to 7 dpi in BCB, and decreased in both insects at 10 dpi. In the combination treatments, however, EPN were scarcely detected in BCB or LCW larvae at 7 and 10 dpi. Interestingly, in the EPP-EPN-combination at 7 dpi, half of the BCB larvae were occupied by EPN (400-800 units/larva) and EPP (10^6-10^8 units/larva), but not by nematode-associated bacteria (NB).

The NB *X. bovienii* SM5 was monitored additionally to the EPN. NB were detected in all LCW larvae in the EPN single treatment and in around half the larvae in combinations of EPN with other BCA at 1 dpi (Fig. 5) but only in a few dead BCB larvae at 3 dpi (Fig. 6). At 5 dpi, NB were present in almost all larvae on average at 10^7 units/larva (Tables S5, S6). However, similar to EPN, NB were detected less frequently in the triple combination compared to all other EPN treatments, although not in lower numbers if present (Figs. 5, 6). In BCB, NB population size decreased with progressing cadaver decay from 5 to 7 dpi (Fig. 6, Table S6). In LCW, NB population size did not decrease from 5 to 10 dpi, yet NB disappeared in the triple treatment in two thirds of the larvae (Fig. 5, Table S5).

Four entomopathogens can co-exist inside the same larva

To further investigate co-existence, we looked at population sizes of the four entomopathogens inside six individual larvae for each combination treatment at 5 dpi when larvae had died, but cadavers were not yet decayed (Fig. 7). In the EPF-EPN combination, NB were present in all BCB and LCW larvae together with either EPN or EPF, except for one LCW and two BCB larvae where all three organisms were detected. EPF were present in five out of twelve larvae. In the EPP-EPN combination, both bacteria

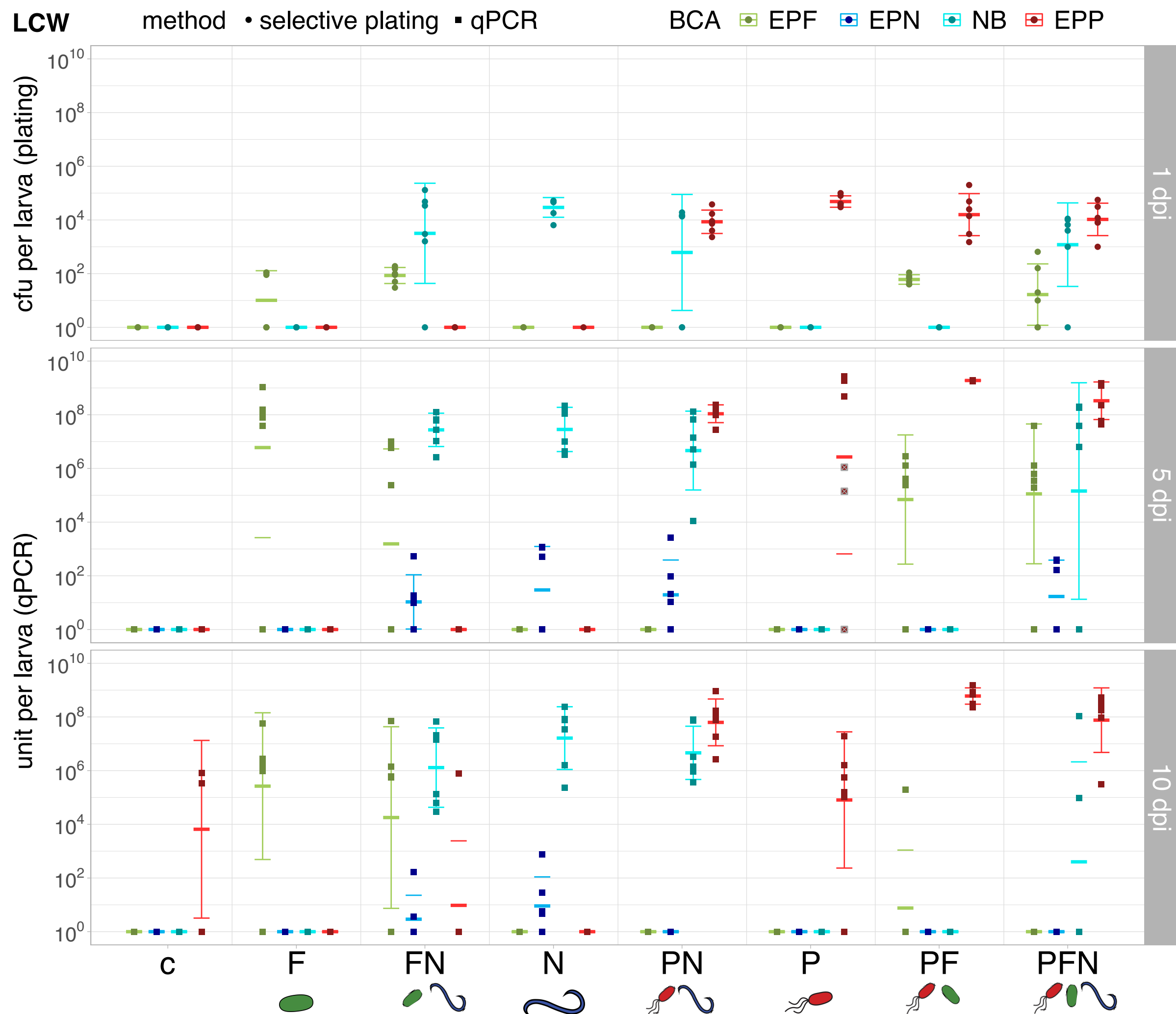


Fig. 5. **Colonisation of *P. brassicae* (LCW) larvae by EPF, EPP, EPN and their associated NB.**

BCA counts in cfu and units per larva were assessed by selective plating (1 dpi) and qPCR (5 and 10 dpi) in LCW repetition 5. Treatments: c = control with no BCA application, P = EPP *P. chlororaphis* PCLRT03-mturq, N = EPN *S. feltiae* RS5-mche (associated with NB *X. bovienii* SM5-mcherry), F = EPF *M. brunneum* Bip5-gfp, FN, PN, PF and PFN = double and triple combinations of respective BCA. BCA: EPF = Bip5-gfp, EPN = RS5-mche, NB = SM5-mcherry, EPP = PCLRT03-mturq. At 1 dpi, six alive larvae, and at 5 and 10 dpi six dead larvae (control n=3) were selected for homogenization. At 5 dpi, three alive larvae had to be taken in treatment P because not enough dead larvae were available. At 1 dpi, colonisation was assessed by selective plating and values are displayed as colony forming units (cfu) per larva. At 5 and 10 dpi, colonisation was assessed by qPCR and colonisation values are displayed in units per larva (relative to bacteria cells, fungal spores and nematode IJ). Each dot or square represents one larva and crossbars show mean and standard deviation; outlined squares marked with a cross indicate which larvae were still alive before homogenization at 5 dpi in treatment P. Mean colonisation densities and statistical analyses are shown in Table S7, and the survival curves and corresponding data are displayed in Fig. S3 and Table S3.

colonised nearly all larvae and EPN were also found in the majority of the larvae. In the EPP-EPF combination, EPP were always present in high numbers whereas EPF

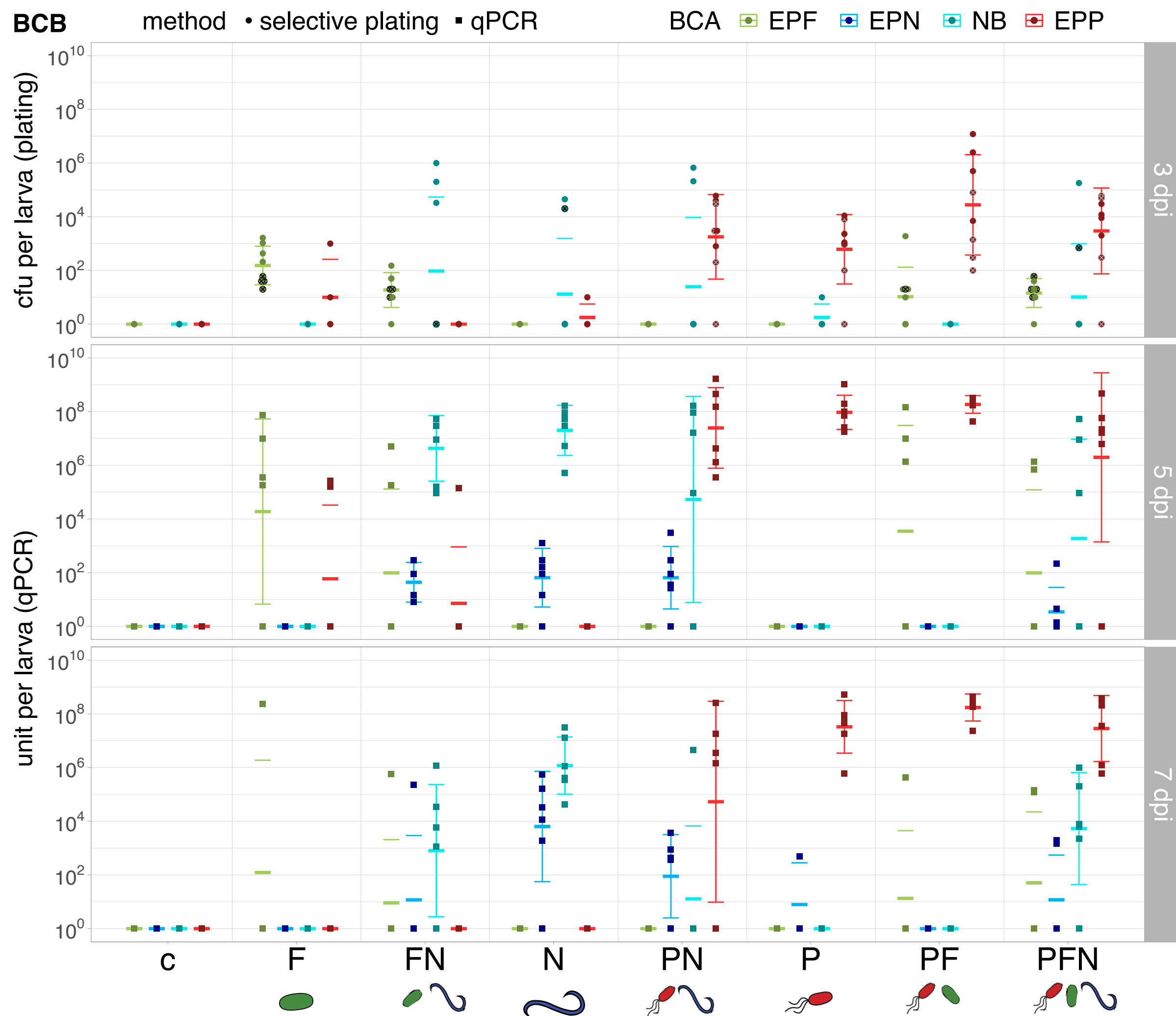


Fig. 6. **Colonisation of *D. balteata* (BCB) larvae by EPF, EPP, EPN and their associated NB.**

BCA counts in cfu and units per larva were assessed by selective plating (3 dpi) and qPCR (5 and 7 dpi) in BCB repetition 2. Treatments: c = control with no BCA application, P = EPP *P. chlororaphis* PCLRT03-mturq, N = EPN *S. feltiae* RS5-mche (associated with NB *X. bovienii* SM5-mcherry), F = EPF *M. brunneum* Bip5-gfp, FN, PN, PF and PFN = double and triple combinations of respective BCA. BCA: EPF = Bip5-gfp, EPN = RS5-mche, NB = SM5-mcherry, EPP = PCLRT03-mturq. At 3 dpi, four alive and four dead larvae (control n=4) and at 5 and 7 dpi six dead larvae (control n = 3) were selected for homogenization. At 3 dpi, colonisation was assessed by selective plating and values are displayed as colony forming units (cfu) per larva. At 5 and 7 dpi, colonisation was assessed by qPCR and colonisation values are displayed in units per larva (relative to bacteria cells, fungal spores and nematode IJ). Each dot or square represents one larva and crossbars show mean and standard deviation; outlined dots marked with a cross (in black or grey) indicate that larvae were alive before homogenization at 3 dpi. Mean colonisation density and statistical evaluation are shown in Table S8, and the survival curve and corresponding data are displayed in Fig. S4 and Table S4.

had propagated in most LCW larvae but only half of the BCB. In the triple combination, EPP colonised all larvae at high population sizes, except for one BCB larva that was not

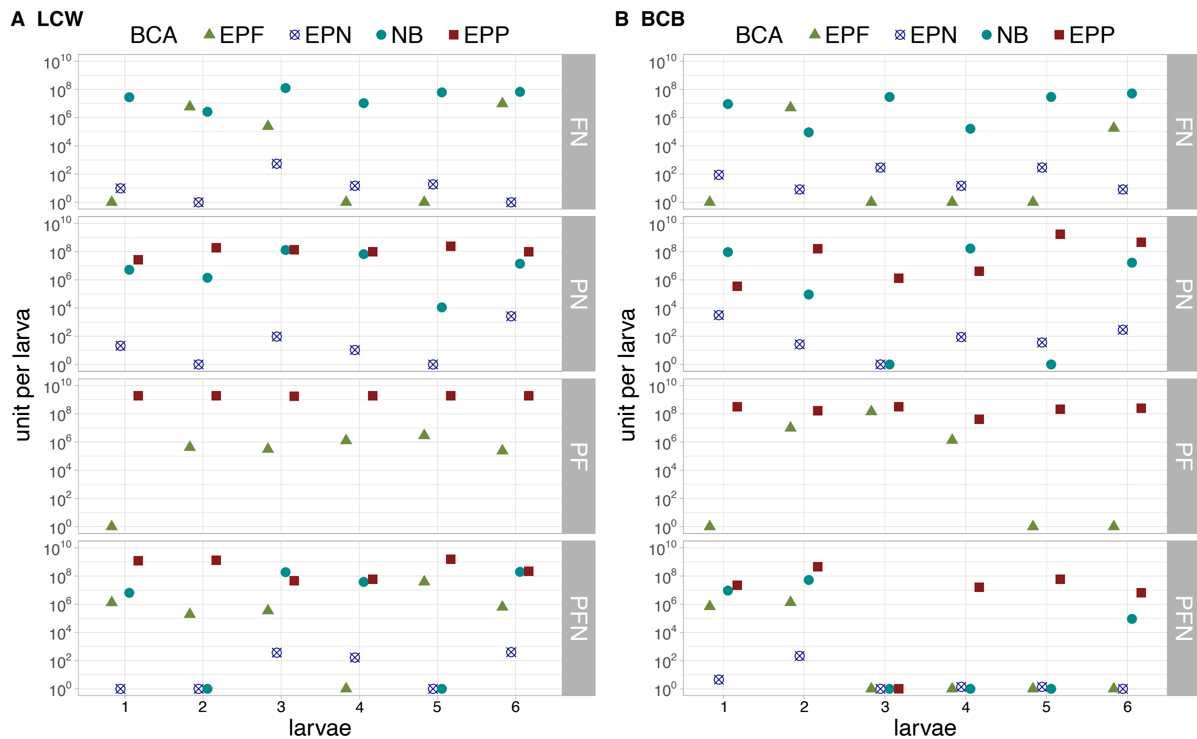


Fig. 7. **Colonisation of individual *P. brassicae* (LCW) and *D. balteata* (BCB) larvae by EPF, EPP, EPN and their associated NB.**

Colonisation of six individual larvae by BCA after simultaneous application at 5 dpi in A) LCW repetition 5 and B) BCB repetition 2. BCA: EPF = *M. brunneum* Bip5-gfp, EPN = *S. feltiae* RS5-mche, NB = *X. bovienii* SM5-mcherry, EPP = *P. chlororaphis* PCLRT03-mturq. Treatments: FN = combined application of EPF and EPN (associated with NB), PN = EPP and EPN (with NB), PF = EPP and EPF, PFN = EPP, EPF and EPN (with NB). Colonisation was assessed by qPCR and colonisation values are displayed in units per larva (relative to bacteria cells, fungal spores and nematode IJ). For improved readability, BCA that are not part of a double combination were removed in this plot; all datapoints are shown in Figs. 5 and 6.

colonized by any BCA. In BCB, all four entomopathogens were present in two larvae, EPP alone in two and both bacteria in one. In LCW, two larvae were colonised by all four entomopathogens, two by EPP and EPF, and two by both bacteria and either EPN or EPF. The co-colonisation of individual larvae by multiple BCA was also observed by fluorescence microscopy (Fig. 4).

In summary, the single larva analysis revealed that EPP and NB can co-colonise insect larvae together with EPN or EPF. EPF are mostly not impacted by EPP, but inhibited in co-infections with EPN. The application of the triple combination leads in most cases to the final establishment of only two entomopathogens with EPP always among these. Yet, in spite of the observed exclusion, in some cases, all four organisms can co-exist and grow together in the same cadaver (Figs. 4, 7).

Discussion

In this study, we investigated the impact of a BCA consortium on larval mortality, killing speed and BCA proliferation in two taxonomically distant insect pests living in different habitats. As we predicted, BCA combinations were generally more deadly and faster in killing larvae than single applications and the triple combination was the most lethal treatment in both insects. In the BCB experiments, the results of the repetitions 1, 3 and 4 have to be considered with care since the mortality in the control was high because experimental conditions were unfavourable for the animals. Still, the same tendencies as for repetition 2 were observed, i.e. faster killing and higher mortality in the triple combination compared to single applications. In previous greenhouse and field trials, the same consortium decreased insect survival and damage on plants attacked by a Dipteran pest, the cabbage maggot *D. radicum*, by 50% [27]. In previous laboratory assays, the EPP were the most effective agent when applied alone, and double combinations of EPP with either EPN or EPF had synergistic effects [27]. In this study, EPF and EPN were more effective than EPP in single applications. The best double combinations were EPN-EPP against the Lepidopteran LCW and EPN-EPF against the Coleopteran BCB. These findings indicate that the performance of the BCA and synergisms between individual consortium members vary depending on the host insect. Despite the variability in efficacy observed for single and double applications, the triple combination was effective against all three insect pests targeted across our two studies. Similar results of BCA combinations have been observed in other studies. For example, Jabbour et al. [36] found a linear increase in mortality with increasing pathogen species richness when infecting Colorado potato beetles with combinations of three EPN (*Heterorhabditis megidis*, *S. feltiae*, *S. carpocapsae*) and one EPF (*Beauveria bassiana*). The combination of one EPN with EPF had the highest impact on mortality and resulted in synergistic effects. Bueno-Pallero et al. [24] used different inoculation methods for EPF (*B. bassiana*) which affected insect mortality yet combinations of EPF and EPN (*S. feltiae*) additively increased mortality in nearly all settings.

During the infection, the BCA have to overcome the insect's immune defence and compete with the insect microflora or scavengers [37, 38, 39]. The three BCA have different infection pathways: EPN enter through natural openings and release the NB into the haemolymph, EPF penetrate the cuticle and EPP need to be ingested [15, 18, 22]. Furthermore, the four entomopathogens (the three BCA and the nematode-associated NB) all produce a cocktail of insecticidal and antimicrobial toxins [23, 40, 41]. EPF and NB both produce compounds that modulate and suppress the insect's immune response [42, 43]. We assume that the different infection pathways and modes of action of the consortium members contribute to the overall activity of the consortium against

different insect pests. An insect is more likely to succumb to infection and might do so faster when challenged with different physical damages and a larger variety of toxic compounds. Potentially, our consortium of three potent BCA has a greater range of target species than the single BCA or even the double combinations. This indicates that it may be applied against various agronomic pests from the families Lepidoptera, Coleoptera and Diptera.

We further hypothesized that the BCA might hinder each other's proliferation in the cadaver due to a competition for nutrients or antimicrobial interactions. Studies showing that both bacteria and the fungus inhibit each other *in vitro* [27, 44, 45] indicate that the susceptibility towards the opponent's antimicrobials is given. Thus, one of our major aims was to co-monitor all organisms after simultaneous host attack. To the best of our knowledge, our study is the first to observe the co-occurrence of four entomopathogens associated with biocontrol during the course of an infection and provides novel insights to understanding their interactions within the host. The proliferation of EPF and EPN is clearly affected by the presence of other BCA, especially in the triple combination, while EPP proliferate equally well or even better in combinations compared to single applications. Possibly, EPP profit in co-infections from EPF or EPN entering the insect and damaging the tissue. EPP could then reach the haemolymph more easily where they can multiply and reach high numbers. In triple combinations, EPP always prevailed after 5 days while one, two or even three of the other entomopathogens had vanished in most larvae (Fig. 7). It is remarkable that EPP colonize insects to such high densities, since insects were only recently discovered as an ecological niche of EPP [33]. Even though EPP are highly competitive in the rhizosphere [13, 14, 15], they do not seem to outcompete the other entomopathogens in double combinations neither during infection nor during colonization of the cadaver. EPN could reproduce in larvae co-infected with EPP and IJ emergence was not reduced in infections with simultaneous application (Fig. 4). Blanco-Pérez et al. [38] observed that high competition in the cadaver affected IJ fitness, but this was not assessed in our study. Ogier et al. [25] discovered EPP in the 'frequently associated microbiome' of *Steinernema* IJ from lab and natural environments, suggesting a close link between EPP and EPN. In several BCB cadavers only EPP and EPN but no NB were detected (Fig. 6). Possibly, IJ carrying few NB had formed at this time-point and NB were below detection limit, or EPN could reproduce without the presence of NB.

In comparison to what we observe with EPP, EPF and EPN were unable to proliferate in the same cadaver for a long time. Tarasco et al. [44] observed a strong competition for space and nutrients between EPF and EPN. EPF and EPN spread from their primary infection site and usually one outcompeted the other, though in some cases, both EPF and EPN symptoms were observed on different parts of individual cadavers. In our

study, EPF and NB were able to co-colonise cadavers at relatively high densities, yet EPF and EPN were rarely detected in the same cadaver. Probably, once EPF have established, they presumably suppress EPN reproduction but not that of their symbionts.

Interestingly, a third of the cadavers were colonised by all four BCA at 5 dpi (Fig. 7). The entomopathogens seem to be sufficiently tolerant to each other's antimicrobial substances to proliferate in the same cadaver, and competition for resources might be more limiting for co-colonisation than direct antimicrobial interactions. We assume that the competition inside the cadaver, i.e. inhibition of EPF sporulation and EPN reproduction, does most likely not lower biocontrol efficacy itself, at least in inundative biocontrol approaches. Biopesticide strategies are mainly based on repeated BCA treatments at intervals depending on field persistence of BCA and the pest pressure and do not rely on the performance of subsequent generations of the BCA.

In conclusion, the combination of three BCA might increase biocontrol efficacy. The co-infections resulted in increased killing speed and mortality against two agricultural insect pests. When comparing the two insect species, different BCA double combinations showed similar colonisation dynamics but distinct insect killing effects. The competition between the entomopathogens increased with advancing decay of the cadaver and limitation of nutrients, and EPP finally dominated the cadaver in all combinations. Our findings indicate that the studied entomopathogenic pseudomonads, nematodes including their symbionts and fungi are compatible, can jointly infect insect larvae and can potentially be used to control a range of insect pests.

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Declaration of Interests

The authors declare no conflict of interests.

Author Contributions

AS, MZ and MM designed the study with input from PB, JE, RCH and GG. AS, MH and AM conducted the experiments. AS and MZ analysed the data. AS wrote the first draft of the manuscript. MZ and MM thoroughly revised the manuscript and PB, JE, RCH and GG revised later versions of the manuscript. All authors approve of the final version.

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Supplementary Information

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Supplementary Material and Methods

Insect Rearing

Eggs of the large cabbage white (LCW) *Pieris brassicae* (Lepidoptera: Pieridae) were obtained from the Biocommunication Group (Institute of Agricultural Sciences, ETH Zurich, Switzerland) and incubated in petri dishes (ø 150 mm) with Savoy cabbage (*Brassica oleracea* var. *sabauda* L.) leaves. Freshly hatched larvae were transferred into 720 ml BugDorms (BugDorm, Taiwan) and fed with Savoy cabbage. In the evening before experiments started, sufficient larvae, mainly early 3rd instar, were collected in petri dishes (ø 150 mm) with a moistened filter paper (ø 150 mm, Conatex, Germany) and a Savoy cabbage leaf. In the morning before an experiment started, larvae were starved for approx. 6 h. Eggs and larvae were incubated during their entire lifespan in a phytotron with 60% rH and a day-night cycle with 16 h at 25 °C and 12 kLux followed by 8 h at 20 °C and darkness. Savoy and Chinese cabbage used for feeding was bought at supermarkets (Coop or Spar, Switzerland).

Eggs of the banded cucumber beetle (BCB) *Diabrotica balteata* LeConte (Coleoptera: Chrysomelidae) were provided by Syngenta Crop Protection (Stein, Switzerland). Eggs were stored in ø 150 mm petri dishes with a moistened filter paper (ø 150 mm) until hatching. For larval rearing, plastic containers (24 x 16 x 10 cm, Topline, Migros, Switzerland) were prepared by removing a rectangle from the lid and sealing it with a 125 µm mesh (03-125/45, Sefar, Switzerland). Freshly hatched larvae were transferred into these rearing boxes and covered with 5-day-old germinated maize seedlings (*Zea mays mays* variety Damaun KS, sativa, Switzerland) and peat substrate (Jiffy Products International, Moerdijk, the Netherlands). In the morning of an experiment, sufficient larvae, mainly early 2nd instar (approximately one week after hatching), were collected in petri dishes (ø 150 mm) with a moistened filter paper (ø 150 mm) and starved for 6 h. Eggs and larvae were incubated in the dark at 27 °C for rearing and at 25 °C with 70% relative humidity during experiments.

Reassociation of *Steinernema feltiae* with modified *Xenorhabdus bovienii*

Xenorhabdus bovienii SM5-mcherry was stored in 20% glycerol at -80 °C and grown on Lysogeny broth (LB) agar supplemented with ampicillin 40 mg/l and kanamycin 50 mg/l for two days at 28 °C. LB liquid cultures were incubated over night at 28 °C and 180 rpm. Subsequently, 6th instar *Galleria mellonella* larvae (Hebeisen fisher store, Zurich, Switzerland) were injected with 10 µl of a 1:1 mix of kanamycin (100 mg/ml) and SM5 overnight culture following the injection protocol described in Flury et al. [46]. The larvae were transferred to a petri dish (ø 60 mm) containing a filter paper (ø 55 mm, Conatex, Germany) and 400 µl *Steinernema feltiae* RS5 wildtype (1000 IJ/ml) were added onto the paper. Cadavers and freshly emerging nematodes were examined under a fluores-

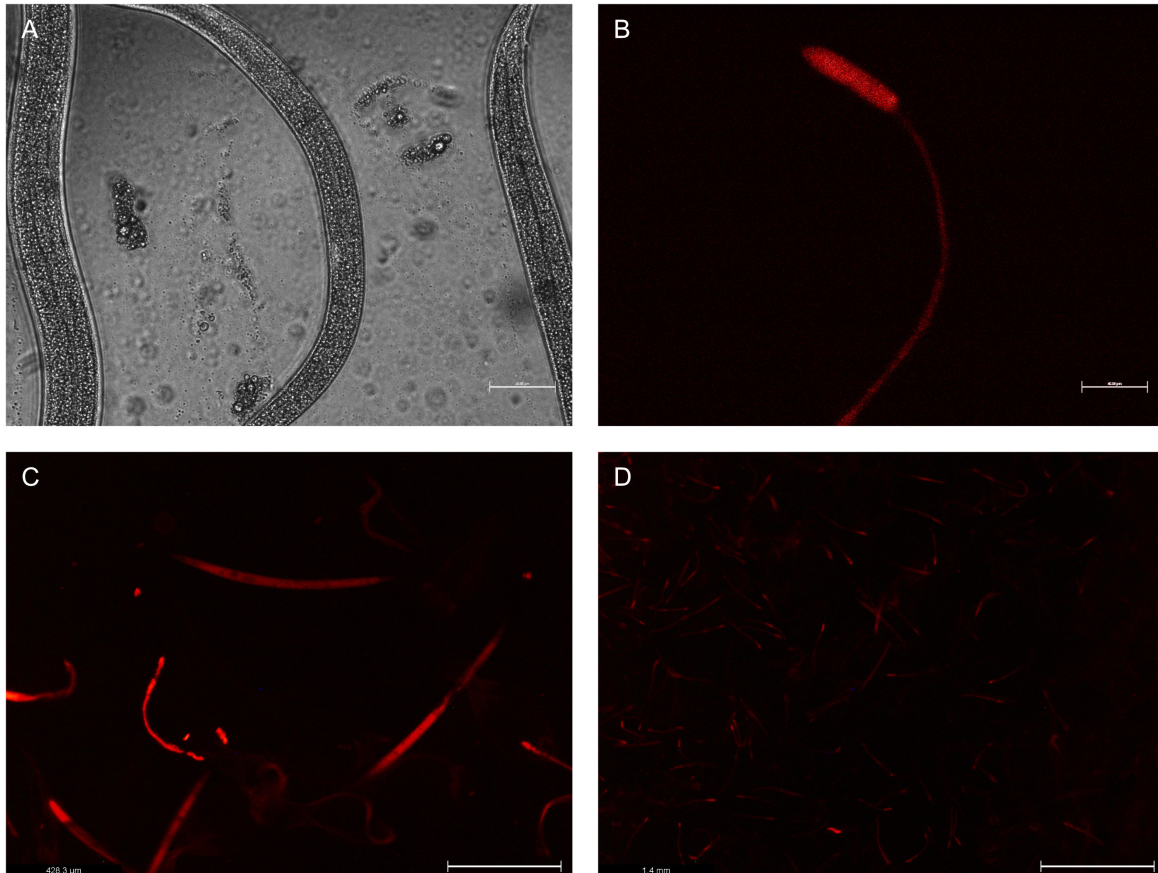

Fig. A1. ***Xenorhabdus bovienii*** **SM5-mcherry re-associated with *Steinernema feltiae* RS5.**

Pictures A and B were taken using a microscope and show the same IJs, A with brightfield and B with an mcherry-filter. Pictures C and D were taken using a stereomicroscope and an mcherry-filter. Pictures were taken three days after IJ started to emerge from *G. mellonella* cadavers. Scale bars represent 40.56 µm in A and B, 428.3 µm in C and 1.4 mm in D.

cence microscope (Leica DM2500, Leica Microsystems, Germany) and a fluorescence stereomicroscope (LEICA M205FCA, Leica Microsystems, Germany) for an mcherry signal (Fig. A1). The emerging population was collected using the White-Trap method [31] and called RS5-mche. To uphold selection pressure, *G. mellonella* were injected with 10 µl kanamycin (10 mg/ml) before infection with RS5-mche for further multiplication. Based on stereomicroscope observations, we estimated that >90% of RS5-mche IJ carry SM5-mcherry bacteria. To test the infectivity of the new population, *P. brassicae* larvae were infected with RS5-mche or RS5 wildtype and the survival of 18 larvae per strain was monitored as described in the main text. The survival was very similar and statistically not significantly different for RS5-mche and RS5 wildtype (Fig. A2).

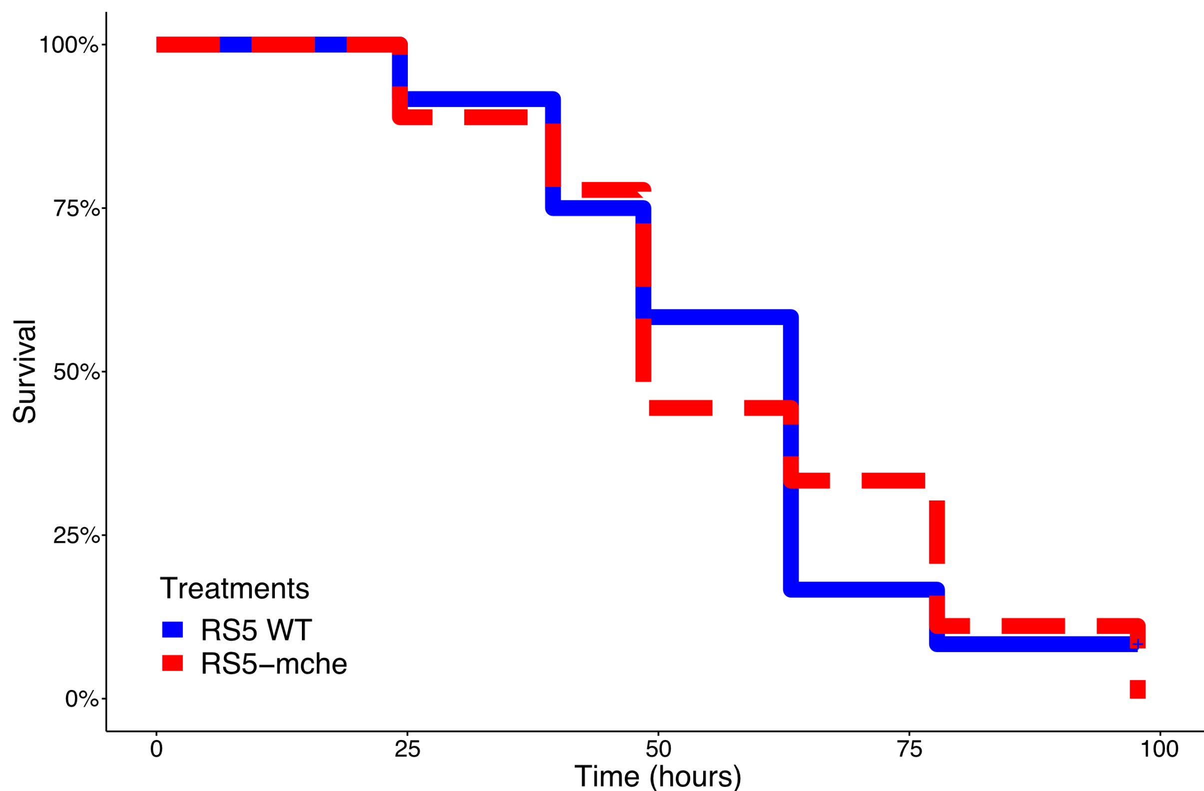

Fig. A2. **Survival of *P. brassicae* (LCW) larvae after infection with EPN**

The figure shows the larval survival of *P. brassicae* to compare the infectivity of *S. feltiae* RS5 wildtype (WT) and RS5-mche. No significant differences ($P < 0.05$) were detected according to a log-rank test and a pairwise survival difference.

Determining larval colonisation with BCA

Larvae were surface disinfected by submerging them for 20 s each, first in 70% (v/v) EtOH, subsequently in 0.05% (w/v) SDS, 70% (v/v) EtOH and finally in ddH_2O before homogenization.

– Selective Plating

Two different homogenization protocols were applied. In EPP x EPN time-shift experiments, larvae were homogenized in 2 ml Eppendorf tubes containing 1 ml 0.9% NaCl with the Polytron RT-MR2100 blender, 500W (Kinematica, Switzerland) as described by Flury et al. [47]. For each one LCW (repetition 5) and BCB (repetition 2) experiment, larvae were disrupted in 2 ml Eppendorf tubes containing 100 µl 0.9% NaCl using one sterilized ø 5 mm bead and the MM300 TissueLyser (Retsch, Germany) for 2 x 45 s at 30/s. After bead disruption, 900 µl 0.9% NaCl was added to each tube. Larval homogenates were plated on King's B agar supplemented with cycloheximide 100 mg/l, chloramphenicol 13 mg/l and gentamycin 10 mg/l (KB^{++G}) to detect EPP, and in LCW and BCB experiments additionally on selective medium (SM) agar to detect EPF and on LB agar supplemented with ampicillin 40 mg/l and kanamycin 50 mg/l (LB^{AK}) to detect

NB. For treatments containing EPF, for each larva 100 µl of the undiluted homogenate was plated. For treatments containing EPP or EPN, 10 µl droplets of 10-fold serial dilutions (10^0 – 10^{-5}) were spotted on the respective medium per larva. To check for cross-contaminations, 100 µl of the undiluted homogenate was plated on all the media using Drigalski spatula for half of the extracted larvae. The detection limits were 100 cfu/larva for EPP and NB and 10 cfu/larva for EPF.

– qPCR

At 3 dpi, 600 µl of larval homogenate was pelleted in 1.5 ml Eppendorf tubes centrifuged at 3500 rcf for 30 s and the pellet was frozen at -20 °C. For later time points, dead LCW and BCB larvae were collected at 2 dpi and at 4 dpi, respectively, surface disinfected, transferred into 2 ml tubes containing 10 µl 0.9% NaCl and further incubated under experimental conditions. At 5, 7 and 10 dpi, previously surface disinfected larvae (n=6, control n=3) were frozen at -20 °C. After thawing, beads were added and larvae were disrupted with the TissueLyser as described above. DNA was extracted using the QIAGEN Blood & Tissue kit according to the manufacturer's instructions with the adaptions for insect samples regarding sample lysis. The samples were lysed by adding 180 µl buffer ATL and 20 µl proteinase K and incubated at 56 °C and 300 rpm overnight (approx. 16 h) for larvae collected at 5, 7 and 10 dpi and for 4 h for pellets collected at 3 dpi. For DNA elution, 100 µl elution buffer was used. DNA concentration was measured using NanoDrop2000 (Thermo Fisher Scientific, MA, USA). qPCR was performed using EvaGreen®, a Lightcycler® 480 (Roche, Switzerland) and a Mosquito® HV pipetting robot (SPT Labtech, UK) at the Genetic Diversity Center (GDC, Zurich, Switzerland). For the master mix, 2.375 µl miliQ, 0.25 µl BSA, each 0.25 µl forward and reverse primer (10 nM) and 2 µl EvaGreen® 5x were mixed per sample, and 1 µl undiluted sample DNA added. Samples were run in duplicates with 12 min initial activation at 95 °C, 35 cycles of 15 s denaturation at 95 °C, 30 s annealing at 60 °C, 30 s extension at 72 °C, and a stepwise melting curve with 15 s at 95 °C, 1 min at 55 °C and a continuous increase to 95 °C. Published species-specific primers were used for *S. feltiae* (Campos-Herrera et al. [48]) and *M. brunneum* (Ma1763 and Ma2079, Schneider et al. [49]) and primers targeting the *phzF* gene involved in phenazine biosynthesis for *P. chlororaphis* (Imperiali et al. [50]). Primers targeting the *rpoD* gene in *X. bovienii* SM5 were designed and kindly provided by Tabea Patt (ETH Zurich) (SM5_F: TTT CAC CGC TAC ACG TGG AAT, SM5_R: AGC GTA AAT AGC GCT GTT GAT TGA). For separate standard curves, DNA of 300 IJ RS5, 10^8 spores Bip5, 10^9 cells SM5-mcherry and PCLRT03-mturq was extracted and 10-fold dilution series with 5 steps prepared. Colonisation values relative to cells, spores and IJ were calculated and displayed as unit per larva. These values need to be interpreted with caution because EPN and EPF are not in the same state in the larvae as in the standard curve, i.e. EPN reproduce in larvae and are present in

juvenile, adult and egg stages, while EPF grow mainly as hyphae inside the cadaver and only form spores when the larva is overgrown with mycelium. The qPCR data was cut-off at a Cp value of 28, resulting in approximate detection limits (per larva) of 1 IJ for *S. feltiae*, 10^5 spores for *M. brunneum*, 10^4 cells for *X. bovienii* and 10^5 cells for *P. chlororaphis*, with small variations between qPCR runs.

– White trap

In EPP x EPN time-shift experiments, freshly deceased LCW larvae were transferred on a filter paper (ø 30 mm, Whatman, Huberlab, Switzerland) on the lid of a ø 30 mm petri dish within a ø 60 mm petri dish filled with approx. 8 ml tap water. After four weeks at 22 °C in the dark, the emerged IJ in the tap water were counted under a stereomicroscope and IJ emergence per larva was calculated.

– Fluorescence stereomicroscopy

LCW larvae were transferred from 6-well into 12-well plates and BCB larvae from 12-well into 24-well (CELLSTAR®, Greiner Bio-One, Austria) plates. Pictures were acquired using a LEICA M205FCA stereomicroscope equipped with a Leica DFC 7000T CCD colour camera and the software Leica ApplicationSuite X (Leica Microsystems). Serial images were captured in the brightfield as well as using the filters ET CFP (10447409, A: 436/20 E: 480/40), ET mCHER (10450195, A: 560/40 E: 630/75) and ET GFP (10447408, A: 470/40 E: 525/50). For the brightfield conditions, 40 s exposure, 2x gain and 20% lamp intensity were used whereas for the fluorescence filters, 10x gain and no lamp was used, with 950 s exposure for LCW and 400 s for BCB experiments. Images were processed using the Fiji package of ImageJ (https://fiji.sc).

Supplementary Results

Supplementary Tables

longtable

Table S1. **Effect of EPP and EPN time-shift application on *P. brassicae* (LCW) larvae.**

Treat	Repetition 1			Repetition 2			Repetition 3		
	mean	mort.	stats	mean	mort.	stats	mean	mort.	stats
c	71.9	31.2	a	68.4	13.6	a	72.5	13.6	a
N t0	49.9	81.2	bcd	47.5	68.2	bcd	40.2	100.0	b
N t-6h	NA	NA	NA	53.8	71.4	bc	37.0	83.3	b
P	64.9	50.0	ab	60.9	61.1	b	43.9	83.3	b
PN t-6h	36.4	91.7	cd	34.8	88.8	d	33.7	94.1	b
PN t0	44.1	100.0	d	40.4	94.4	cd	34.8	100.0	b
PN t+6h	59.2	83.3	bc	35.5	94.1	d	34.0	100.0	b

Results from the EPP x EPN time-shift experiment. Mean = mean survival time (in hours). Mort. = final larval mortality (in percent) at the end of the experiment with n = 18 larvae per treatment. Stats = pairwise comparison of survival curves; different letters indicate statistically significant differences at $P < 0.05$. Treatments: c = control with no BCA application, N t0 = EPN *S. feltiae* RS5, N t-6h = EPN applied 6 h earlier, P = EPP *P. chlororaphis* PCLRT03-gfp, PN t-6h = EPN applied 6 h before EPP, PN t0 = EPN and EPP applied simultaneously, PN t+6h = EPN applied 6 h after EPP.

Table S2. **Proliferation of EPP and EPN in *P. brassicae* (LCW) larvae in the time shift experiment.**

Treat	control	N t0	N t-6h	P	PN t-6h	PN t0	PN t+6h
Repetition 2							
EPP	9.0×10^2	4.5×10^1	ND	2.6×10^7	1.9×10^6	3.0×10^5	1.0×10^6
EPN	ND	7.2×10^3	2.3×10^4	ND	5.0×10^3	6.3×10^3	4.9×10^3
Repetition 3							
EPP	2.5×10^1	BD	ND	4.4×10^6	7.7×10^6	3.5×10^6	3.2×10^5
EPN	ND	8.2×10^2	3.2×10^3	ND	1.4×10^4	1.1×10^4	3.1×10^2

Data shown are mean colonisation by EPP *P. chlororaphis* PCLRT03-gfp in cfu per larva as determined at 1 dpi by selective plating (n = 6) and mean proliferation of EPN *S. feltiae* RS5 as emerging IJ per larva as determined with white traps (n = 6), respectively. Treatments: c = control with no BCA application, N t0 = EPN *S. feltiae* RS5, N t-6h = EPN applied 6 h earlier, P = EPP *P. chlororaphis* PCLRT03-gfp, PN t-6h = EPN applied 6 h before EPP, PN t0 = EPN and EPP applied simultaneously, PN t+6h = EPN applied 6 h after EPP. BD = below detection; ND = not determined. No significant differences among treatments ($P < 0.05$) were detected according to an Anova and TukeyHSD test.

Table S3. **Effect of EPP, EPF and EPN applied alone and in combinations on *P. brassicae* (LCW) larvae**

Treat.	Repetition 1			Repetition 2			Repetition 3			Repetition 4			Repetition 5		
	mean	mort.	stats	mean	mort.	stats	mean	mort.	stats	mean	mort.	stats	mean	mort.	stats
control	75.2	18.8	a	59.2	37.5	a	73.3	5.9	a	76.8	0.0	a	114.0	3.7	a
F	64.1	93.8	c	62.7	56.3	a	47.6	82.3	cd	43.2	100.0	d	50.0	100.0	c
N	55.4	76.5	bc	44.6	93.8	bc	35.7	94.4	de	63.8	50.0	b	34.8	95.8	b
P	66.9	40.0	a	53.1	52.9	ab	68.5	41.2	b	54.5	77.8	bc	111.0	20.8	a
FN	52.9	100.0	b	46	75.0	abc	37.0	94.4	de	46.6	100.0	bc	31.8	100.0	b
PN	36.2	100.0	d	37.8	100.0	c	32.4	100.0	e	52.3	73.7	bcd	33.9	95.8	b
PF	59.1	92.3	bc	64.1	58.8	a	51.0	83.3	c	47.2	100.0	cd	42.8	100.0	c
PFN	39.0	100.0	d	41.9	94.1	bc	32.4	100.0	e	46.9	89.5	cd	35.3	100.0	b

Data shown are results from LCW repetition 1-5 with single and combined simultaneous application of EPP, EPF and EPN. Mean = mean survival time (in h). Mort. = final larval mortality (in percent) at the end of an experiment with n = 18 larvae per treatment. Stats = pairwise comparison of survival curves; different letters indicate statistically significant differences at $P < 0.05$. Treatments: control = no BCA application, P = EPP *P. chlororaphis* PCLRT03-gfp or PCLRT03-mturq (repetition 5), N = EPN *S. feltiae* RS5 or RS5-mche (repetition 5), F = EPF *M. brunneum* Bip5 or Bip5-gfp (repetition 5), FN, PN, PF and PFN = double and triple combinations of respective BCA.

Table S4. **Effect of EPP, EPF and EPN applied alone and in combinations on *D. balteata* (BCB) larvae.**

Treat.	Repetition 1			Repetition 2			Repetition 3			Repetition 4		
	mean	mort.	stats	mean	mort.	stats	mean	mort.	stats	mean	mort.	stats
control	3.5	65.9	ab	5.4	41.5	a	4.7	65.6	ab	4.7	68.7	a
F	2.7	87.2	bc	3.7	83.6	cd	4.8	52.4	a	3.9	80.9	ab
N	2.9	92.5	bc	4.3	70.8	bc	4.7	70.7	ab	4.2	76.9	ab
P	4.0	65.9	a	4.8	62.9	b	4.8	68.9	a	4.3	73.9	ab
FN	3.3	94.9	bc	3.5	90.3	d	4.5	78.3	ab	3.8	84.9	b
PN	3.0	87.8	bc	4.7	74.6	b	4.6	70.8	ab	4.4	73.9	ab
PF	2.9	82.9	abc	4.4	95.2	c	4.7	69.2	ab	4.4	72.3	ab
PFN	2.2	97.3	c	3.7	95.2	d	4.2	87.7	b	4.0	80.9	ab

Data shown are results from BCB repetition 1-4 with simultaneous EPP, EPF and EPN single and combined applications. Mean = mean survival time (in days). Mort = final larval mortality (in percent) at the end of an experiment with n = 60 larvae per treatment. Stats = pairwise comparison of survival curves; different letters indicate statistically significant differences at $P < 0.05$. Treatments: control = no BCA application, P = EPP *P. chlororaphis* PCLRT03-mturq, N = EPN *S. feltiae* RS5-mche, F = EPF *M. brunneum* Bip5-gfp, FN, PN, PF and PFN = double and triple application of respective BCA.

Table S5. **Colonisation of *P. brassicae* (LCW) larvae by EPF, EPN, NB and EPP.**

Treat	control	F	N	P	FN	PN	PF	PFN
1 dpi, selective plating								
EPF	BD	5.2×10^1	BD	BD	1.0×10^2	6.5×10^1	BD	1.4×10^2
NB	BD	BD	3.6×10^4	BD	3.6×10^4	BD	1.0×10^4	5.5×10^3
EPP	BD	BD	BD	5.4×10^4	BD	4.9×10^4	1.3×10^4	1.9×10^4
5 dpi, qPCR								
EPF	BD	2.4×10^8	BD	BD	2.7×10^6	BD	8.5×10^5	6.8×10^6
EPN	BD	BD	4.8×10^2	BD	9.8×10^1	4.6×10^2	BD	1.6×10^2
NB	BD	BD	8.3×10^7	BD	5.0×10^7	3.6×10^7	BD	7.1×10^7
EPP	BD	BD	BD	8.3×10^8 ab	BD	1.3×10^8 a	1.9×10^9 b	7.2×10^8 a
10 dpi, qPCR								
EPF	BD	1.1×10^7 a	BD	BD	1.2×10^7 ab	BD	3.2×10^4 bc	BD c
EPN	BD	BD	1.3×10^2	BD	2.9×10^1	BD	BD	BD
NB	BD	BD	7.2×10^7 a	BD	1.7×10^7 a	2.6×10^7 a	BD	2.2×10^7 b
EPP	3.9×10^5	BD	BD	3.6×10^6 a	1.3×10^5	2.1×10^8 b	7.3×10^8 b	2.3×10^8 b

Data shown are the mean colonisation of LCW larvae by EPF *M. brunneum* Bip5-gfp, EPN *S. feltiae* RS5-mche, NB *Xenorhabdus* sp. SM5-mcherry and EPP *P. chlororaphis* PCLRT03-mturq as determined at 1, 5, and 10 dpi (n = 6 per time-point) by selective plating (in cfu per larva) and qPCR (in units per larva), respectively, in LCW repetition 5. Treatments: control = no BCA application, P = EPP *P. chlororaphis* PCLRT03-mturq, N = EPN *S. feltiae* RS5-mche, F = EPF *M. brunneum* Bip5-gfp, FN, PN, PF and PFN = double and triple combinations of respective BCA. BD = below detection. Letters in the second line refer to significant differences at $P < 0.05$ according to a TukeyHSD test.

Table S6. **Colonisation of *D. balteata* (BCB) larvae by EPF, EPN, NB and EPP.**

Treat	control	F	N	P	FN	PN	PF	PFN
1 dpi, selective plating								
EPF	BD	1.6×10^{2}	BD	BD	3.0	BD	3.3×10^{1}	5.7×10^{1}
NB	BD	BD	BD	BD	2.0×10^{2}	BD	BD	BD
EPP	BD	BD	BD	7.0×10^{2}	BD	9.7×10^{2}	6.7×10^{1}	1.7×10^{4}
3 dpi, selective plating (EPF, NB, EPP) or qPCR (EPN)								
EPF	BD	4.4×10^{2} a	BD	BD	3.9×10^{1} ab	BD	2.5×10^{2} b	2.2×10^{1} ab
EPN	BD	NA	2.6×10^{3}	1.6×10^{3}	1.8×10^{3}	4.6×10^{3}	BD	2.7×10^{2}
NB	BD	BD	8.1×10^{3}	2.0	1.5×10^{5}	1.1×10^{5}	BD	2.3×10^{4}
EPP	BD	2.5×10^{2}	2.0	3.1×10^{3}	BD	1.7×10^{4}	1.9×10^{6}	2.0×10^{4}
5 dpi, qPCR								
EPF	BD	1.4×10^{7}	BD	BD	8.8×10^{5}	BD	2.6×10^{7}	3.4×10^{5}
EPN	BD	BD	3.1×10^{2}	BD	1.2×10^{2}	6.0×10^{2}	BD	3.7×10^{1}
NB	BD	BD	5.8×10^{7}	BD	2.0×10^{7}	4.6×10^{7}	BD	1.0×10^{7}
EPP	BD	7.1×10^{4}	BD	2.4×10^{8}	2.4×10^{4}	3.8×10^{8}	2.2×10^{8}	9.5×10^{7}
7 dpi, qPCR								
EPF	BD	5.8×10^{7}	BD	BD	9.8×10^{4}	BD	8.7×10^{4}	4.4×10^{4}
EPN	BD	BD	1.3×10^{5}	1.7×10^{2}	4.6×10^{4}	8.9×10^{2}	BD	5.7×10^{2}
NB	BD	BD	7.7×10^{6} a	BD	2.0×10^{5} ab	7.5×10^{5} b	BD	2.0×10^{5} ab
EPP	BD	BD	BD	1.2×10^{8}	BD	4.6×10^{7}	2.4×10^{8}	1.5×10^{8}
10 dpi, qPCR								
EPF	BD	BD	BD	BD	BD	BD	5.9×10^{4}	1.1×10^{5}
EPN	BD	BD	7.0×10^{2}	BD	9.0	2.8×10^{2}	2.0	9.0
NB	BD	BD	2.8×10^{7}	BD	1.4×10^{6}	3.7×10^{7}	1.5×10^{4}	3.2×10^{7}
EPP	BD	1.7×10^{5}	BD	2.0×10^{7}	BD	9.0×10^{7}	2.7×10^{7}	2.4×10^{8}

Data shown are mean colonisation of BCB larvae by EPF *M. brunneum* Bip5-gfp, EPN *S. feltiae* RS5-mche, NB *Xenorhabdus* sp. SM5-mcherry and EPP *P. chlororaphis* PCLRT03-mturq as determined at 1 dpi (n = 3), 3 dpi (n = 8), 5, 7 and 10 dpi (n=6) by selective plating (in cfu per larva) and qPCR (in units per larva), respectively, in BCB repetition 2. Treatments: control = no BCA application, P = EPP *P. chlororaphis* PCLRT03-mturq, N = EPN *S. feltiae* RS5-mche, F = EPF *M. brunneum* Bip5-gfp, FN, PN, PF and PFN = double and triple combinations of respective BCA. BD = below detection. Letters in the second line refer to significant differences at $P < 0.05$ according to a TukeyHSD test.

Supplementary Figures

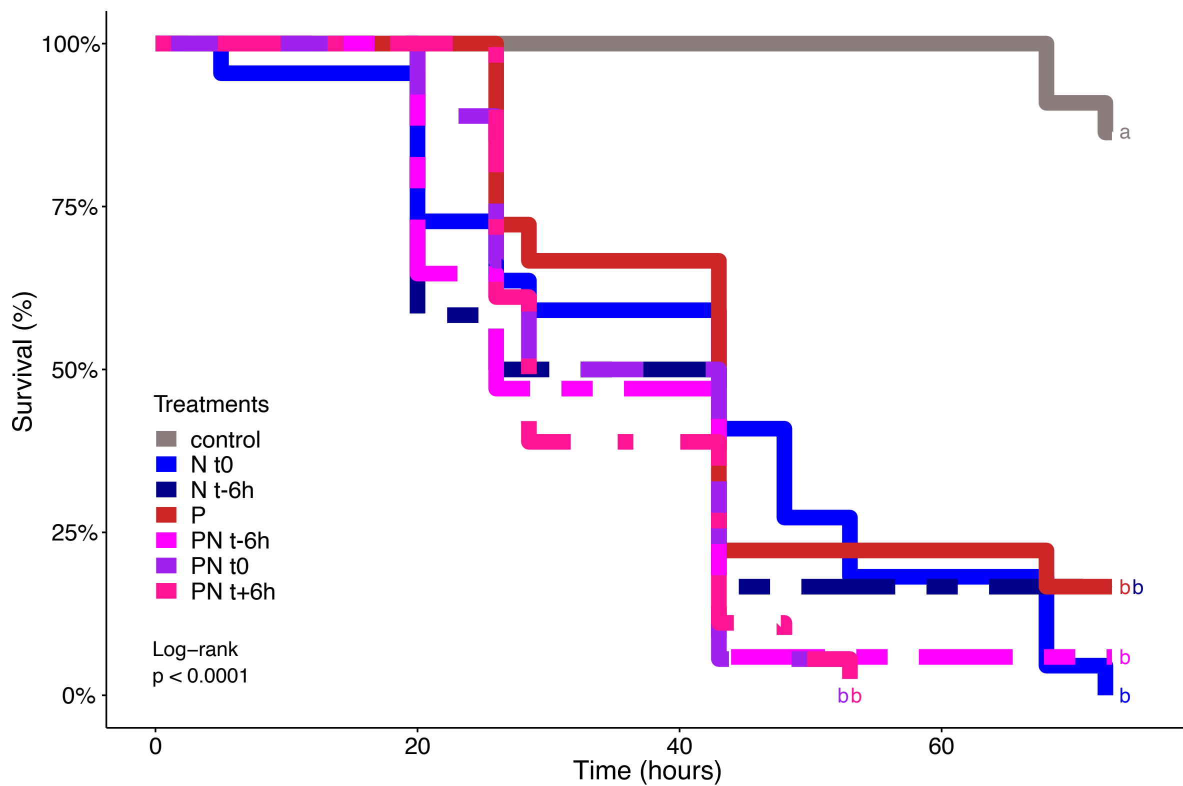

Fig. S1. **Survival of *P. brassicae* (LCW) larvae after time-shift applications of EPP and EPN in repetition 3.**

Treatments: control = no BCA application, N = EPN *S. feltiae* RS5, N t-6h = EPN applied 6 h earlier, P = EPP *P. chlororaphis* PCLRT03-gfp, PN t-6h = EPN applied 6 h before EPP, PN t0 = EPN and EPP applied simultaneously, PN t+6h = EPN applied 6 h after EPP. Different letters indicate significant differences at $P < 0.05$ according to a pairwise survival difference. Mean survival time and final mortality of this experiment are displayed in Table S1.

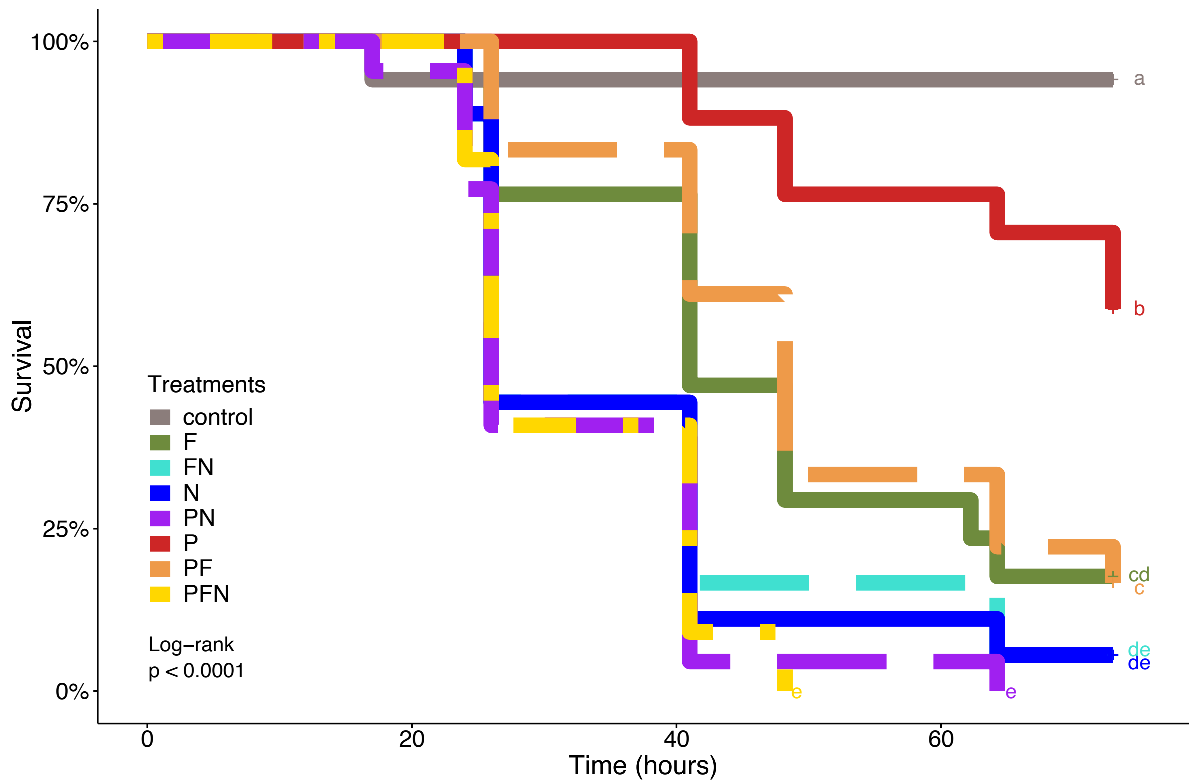

Fig. S2. **Survival of *P. brassicae* (LCW) larvae after infection with single and combined applications of EPP, EPF and EPN in LCW repetition 3.**

Treatments: control = no BCA application, P = EPP *P. chlororaphis* PCLRT03-gfp, N = EPN *S. feltiae* RS5, F = EPF *M. brunneum* Bip5, FN, PN, PF and PFN = double and triple combinations of respective BCA. Different letters indicate significant differences at $P < 0.05$ according to a pairwise survival difference. Mean survival time and final mortality of this experiment are displayed in Table S3.

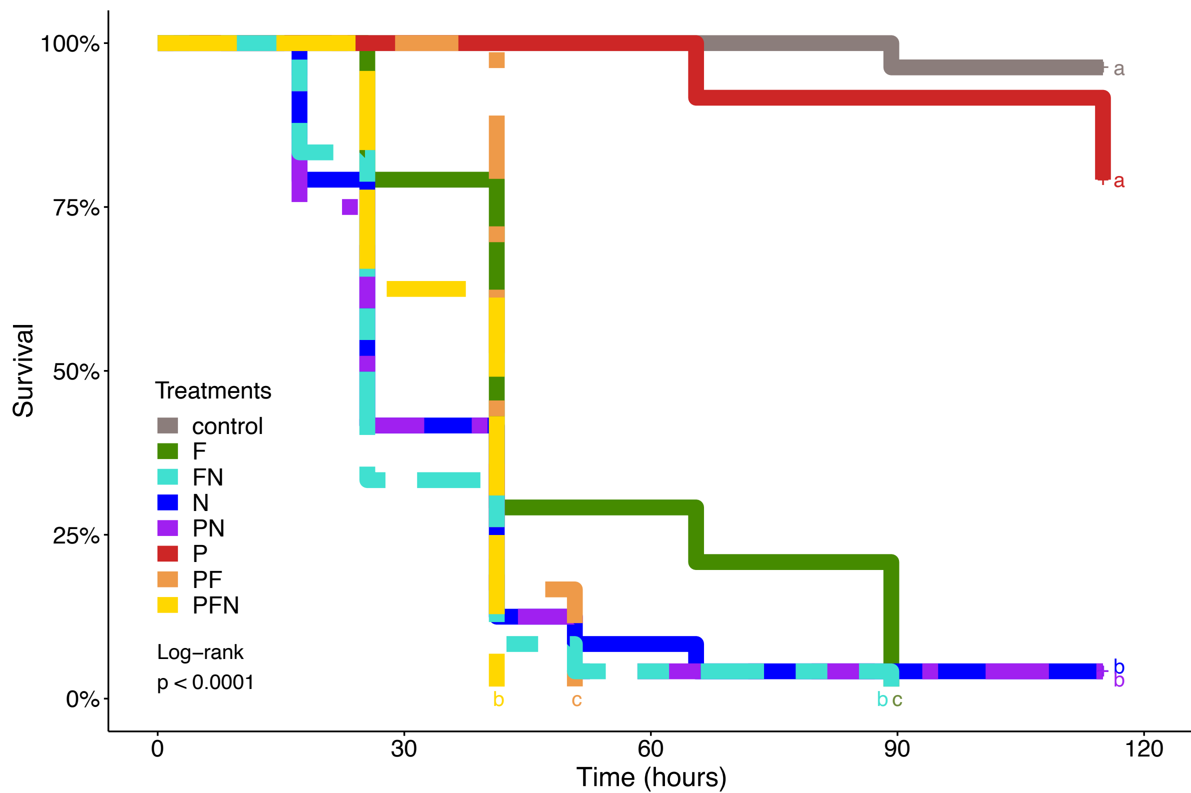

Fig. S3. **Survival of *P. brassicae* (LCW) larvae after infection with single and combined applications of EPP, EPF and EPN in LCW repetition 5.**

Treatments: control = no BCA application, P = EPP *P. chlororaphis* PCLRT03-mturq, N = EPN *S. feltiae* RS5-mche, F = EPF *M. brunneum* Bip5-gfp, FN, PN, PF and PFN = double and triple combinations of respective BCA. Different letters indicate significant differences at $P < 0.05$ according to a pairwise survival difference. Mean survival time and final mortality of this experiment are displayed in Table S3.

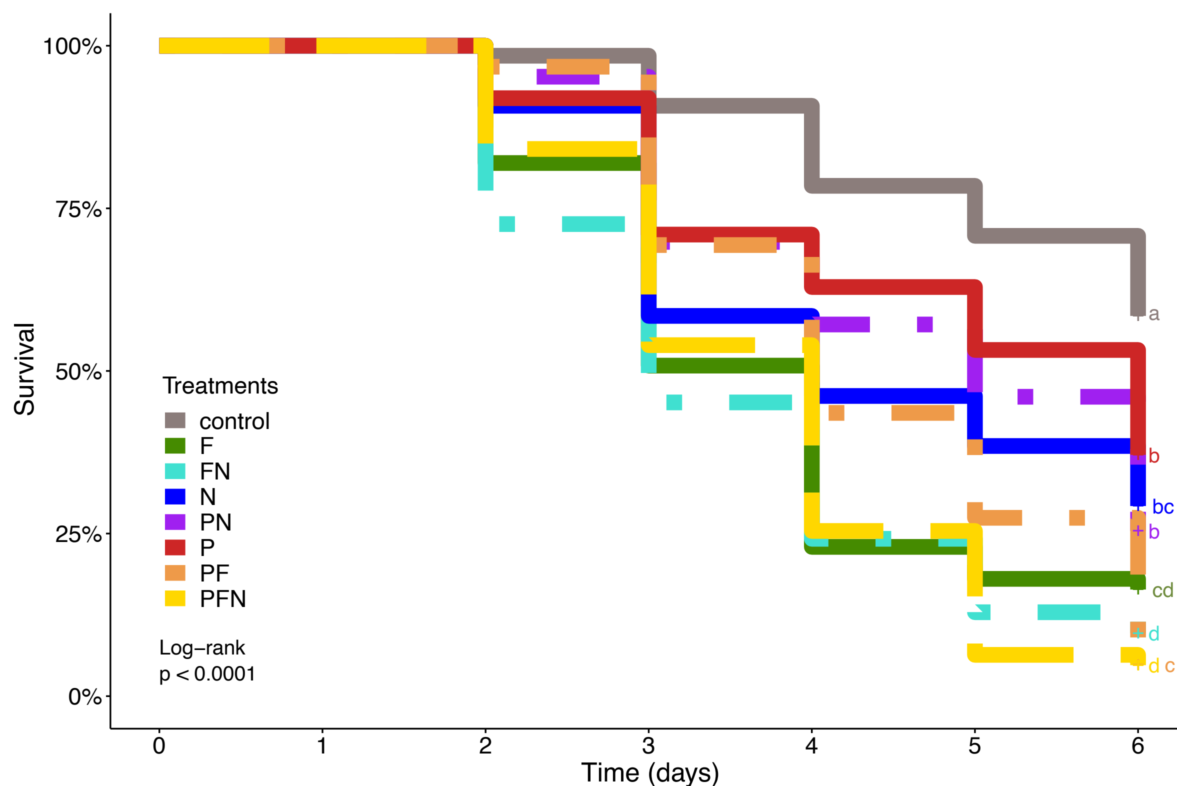

Fig. S4. **Survival of *D. balteata* (BCB) larvae after infection with single and combined applications of EPP, EPF and EPN in BCB repetition 2.**

Treatments: control = no BCA application, P = EPP *P. chlororaphis* PCLRT03-mturq, N = EPN *S. feltiae* RS5-mche, F = EPF *M. brunneum* Bip5-gfp, FN, PN, PF and PFN = double and triple combinations of respective BCA. Different letters indicate significant differences at $P < 0.05$ according to a pairwise survival difference. Mean survival time and final mortality of this experiment are displayed in Table S4.

Chapter 4

Exploring the biocontrol potential of fluorescent pseudomonads isolated from the phyllosphere

A version of this chapter is in preparation for publication in BioControl by:

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Abstract

Entomopathogenic pseudomonads belonging to the phylogenetic subgroups of *P. chlororaphis* and *P. protegens* are promising biocontrol agents against soil-borne pathogens and pests. However, their application against foliar diseases is challenging due to the poor persistence of our most potent entomopathogenic strains in the phyllosphere. To overcome this limitation, we isolated fluorescent pseudomonads from radish leaves. From the 18 leaf isolates for which housekeeping genes were sequenced, 15 belong to the *P. fluorescens* and two to the *P. koreensis* subgroups while one belongs to the *P. putida* group, i.e. to (sub)groups which have so far shown no or weak oral insecticidal activity. Interestingly, two *P. fluorescens* subgroup leaf isolates exhibited potent oral insecticidal activity with similar killing speed and mortality as *P. chlororaphis* and *P. protegens* reference strains. In plant assays, the four tested *P. fluorescens* subgroup isolates could protect cucumber plants against an oomycete pathogen but they did not show any antifungal activity *in vitro*. Furthermore, these four leaf isolates showed a higher persistence on wheat leaves than the included *P. protegens*, *P. chlororaphis* and *P. brassicacearum* strains. Since the new isolates did not show any antifungal activity *in vitro*, but a *P. protegens* strain included in the study did, especially against the foliar wheat pathogen *Zymoseptoria tritici*, this strain was chosen for an *in planta* disease suppression assay. *P. protegens* was able to reduce pycnidia production of *Z. tritici* after 21 days despite its poor persistence on wheat leaves. These results indicate that both, biocontrol strains isolated from roots and the new leaf isolates, might have the potential to control foliar pathogens and pests.

Introduction

Every year, substantial yield losses occur worldwide due to insects feeding on plant leaves and fungi infecting leaf tissue [1]. Foliar pests and pathogens can be targeted by spray application of chemical pesticides [2]. In some production systems, 20 spray applications per season are common, for example in intense apple or cherry production [3]. However, negative impacts on the environment and emergence of resistant pathogens put pressure towards reduction of chemical pesticides [4]. The application of biocontrol agents is an environment-friendly alternative to pesticide use [5]. For several foliar insect pests, *Bacillus thuringiensis* (Bt) is applied as biocontrol agent [6]. However, Bt persists only for a few days on the leaf surface and insects have been shown to develop resistances towards its toxins [6, 7]. Foliar application of entomopathogenic nematodes to control the diamondback moth worked only with 80% relative humidity, otherwise nematodes would desiccate quickly [8]. Spraying entomopathogenic fungi in 7-day intervals in cherry orchards reduced cherry fruit fly infestation even though more than 70% of the inoculum was degraded within the first 24 hours after application [9]. These outcomes indicate that survival in the phyllosphere is challenging for biocontrol agents, especially for microorganisms. The extreme variability in water and nutrient availability, the solar radiation and the large temperature changes are the main abiotic stress factors [10]. Furthermore, biotic factors such as plant genotype, leaf age and the present microbial community strongly impact BCA persistence on the leaves [10]. As a result of the hostile environment on the leaves, microorganisms that are adapted to life in the phyllosphere share several traits, like protection mechanisms against reactive oxygen stress and metabolic adaptations to available nutrient sources [11]. In order to protect themselves from desiccation, leaf microorganisms are observed to form larger aggregates [10]. Additionally, some microorganisms can alter the leaf habitat to increase nutrient availability [11]. Overall, the species richness is generally high in the phyllosphere, yet the diversity is lower than in the rhizosphere [10].

Pseudomonas are among the most abundant bacterial phyla in the phyllosphere [10, 12]. Helfrich et al. screened more than 200 *Arabidopsis thaliana* leaf isolates *in vitro* in pairwise combinations and found that four out of the ten most suppressive strains belong to the genus *Pseudomonas* and at least one of these belongs to the *P. fluorescens* group [13]. However, most studies using pseudomonads for biocontrol focus on soil-borne diseases since *Pseudomonas* are a predominant phyla in the rhizosphere and are well-known for their association with disease-suppressive soils, their plant-growth promoting effects and their antagonism against soil-borne fungal and oomycete pathogens [14, 15]. So far, there are only a few examples where fluorescent pseudomonads were successfully applied against above-ground diseases. *P. fluorescens*

strain A506 is commercially available in the product BlightBan A506 and is applied against fire blight caused by the bacterium *Erwinia amylovora* in pear and apple trees during bloom [16, 17]. Another *P. fluorescens* strain that persisted on apple flowers for up to two months under field conditions was also able to suppress fire blight in apples [18, 19]. More recently, a *Pseudomonas graminis* strain isolated from the apple phyllosphere was shown to suppress fire blight as efficiently as A506 in apple and pear production and colonise blossoms during the entire bloom period [20]. *P. fluorescens* Pf1 was able to suppress blister blight in tea under field conditions in two seasons [21]. A *P. putida* strain isolated from Amazonian suppressive soils was able to reduce foliar blight severity on signal grass upon foliar application [22]. An endophytic *P. putida* strain isolated from healthy beans reduced common bean rust severity under greenhouse conditions [23]. *P. protegens* Pf-5 and a *P. synxantha* strain were able to inhibit cannabis pathogens *in vitro* and Pf-5 was able to lower disease severity slightly but significantly when applied against gray mold on leaves *in planta* [24]. It was not evaluated whether the rhizosphere strain Pf-5 persisted on the leaves and whether the low efficacy *in planta* is linked to a low leaf colonisation. In several studies, pseudomonads were isolated from the plant organ on which they were applied to control a disease. Another approach was to screen strains e.g. isolated from the rhizosphere with described biocontrol properties against foliar diseases. Both approaches have advantages and disadvantages and can be successful to identify a potent biocontrol agent against a specific pest or disease. Testing well described strains increases the chances that the strain inhibits the target disease, yet it might have a lower persistence on the target plant organ, e.g. the leaf, compared to a strain isolated from the leaf.

Entomopathogenic pseudomonads (EPP) are promising biocontrol agents not only against fungal pathogens, but also against insect pests [25]. However, their use in biocontrol is so far limited to application against soil-borne pathogens [26]. Spray application of *P. protegens* CHA0 and *P. chlororaphis* PCL1391 on leaves and subsequent feeding of three Lepidopteran insect pests on detached leaves led to a high mortality in all three species [27]. Both EPP strains were isolated from plant roots and persist well in the rhizosphere under greenhouse and field conditions [28] but do not persist more than 2 days on cabbage leaves (Beat Ruffner and Monika Maurhofer, personal communication). Similarly, in an experiment performed at the onset of this study, *P. chlororaphis* PCLRT03 sprayed on Chinese cabbage leaves persisted poorly (Fig. S3). When *P. brassicae* larvae were placed on the inoculated plantlets larval mortality did not exceed 15% after 3 days (Fig. S3). In order to reliably control foliar diseases and pests, pseudomonads need to be able to persist several days on the leaves. For our quest for strains with good foliar persistence we chose to isolate strains from radish leaves during the field trial described in chapter 2. The goal was to identify fluorescent pseu-

domonads with strong phyllosphere persistence and activity against leaf feeding insect pests and against foliar pathogens. Ideally, a *Pseudomonas* biocontrol agent with dual use against foliar pests and pathogens would be identified.

Material and Methods

Bacteria isolation and handling

During the field trial in Windisch (47.476110 N, 8.227799 E; Aargau, Switzerland) described in Chapter 2, leaf samples were taken from control plots. From each plot, two samples consisting of three radish *Raphanus sativus* L. cult. 'Andes' leaves were taken as shown in Fig. S1. Samples were immediately placed into 50 ml falcon tubes, stored on ice at the field and over night at 3°C. The next morning, 40 ml NaCl 0.9% were added and samples shaken for 30 min at 3 °C and 300 rpm. 100 µl of the undiluted, the 1:10 and 1:100 dilution were plated on KB^{+++} medium (see Supplementary Methods for media recipes) and grown at 24 °C. All grown colonies were examined for fluorescence under a universal UV-lamp (Camag, Muttenz, Switzerland) and fluorescing colonies picked and streaked on a new plate to receive single colonies that were again checked for fluorescence. A total of 78 colonies were transferred in a 96-well plate with 200 µl LB liquid and incubated over night at 24 °C and 180 rpm (referred to as LB ON culture from now on). From this plate, a 1:10 dilution was prepared for colony PCR as well as three plates with glycerol (1:1 LB ON culture : glycerol 87%) for storage at -80 °C.

For identifying possible *P. protegens* and *P. chlororaphis* strains, *Pseudomonas* spp. specific 16S, a FitD, and specific *P. protegens* and *P. chlororaphis* primers were used to perform colony PCR (Table S1-S3). 18 isolates were selected for further analysis (Table S4) and LB ON cultures were grown to prepare new glycerol stocks and for DNA extraction using approx. 4 mL pelleted LB ON culture as starting material. For DNA extraction, the QIAmp DNA mini-Kit (QIAGEN, The Netherlands) was used according to the manufacturer's instructions for DNA purification from tissues with adaptations for gram-negative bacteria. Sequencing PCR were conducted using Phusion polymerase and primers targeting the four housekeeping genes 16S rRNA, *rpoD*, *rpoB* and *gyrB* (Tables S1-S3). PCR products were sent with respective primers for Sanger sequencing to Microsynth (Balgach, Switzerland). Phylogenetic trees were generated with the help of Jordan Vacheron (University of Lausanne, Switzerland) using Unipro UGENE, Seaview, CLC Genomics Workbench 20, MEGA11 and iTol. J. Vacheron aligned the new sequences with his database containing approx. 400 *Pseudomonas* strains, including most available type strains in the *P. fluorescens* group and generated separate phylogenetic trees for *rpoD*, *rpoB* and *gyrB*. The *rpoD* and *gyrB* sequences were concatenated and a tree constructed with Seaview using the maximum likelihood method

(PhyML) with a GTR model and a bootstrap approach with 100 replicates.

Table 1. **List of used *Pseudomonas* reference strains, plant pathogens and insects.**

Species	Strain	Origin	Reference
***Pseudomonas* bacteria**			
P. protegens	CHA0	Tobacco root, Switzerland	[29]
P. protegens	CHA0-gfp2	Derivative of CHA0	[30]
P. protegens	PF	Wheat leaf, USA	[31]
P. protegens	PF-gfp	Derivative of PF	
P. chlororaphis	PCLRT03	Potato root, Switzerland	[32]
P. chlororaphis	PCLRT03-mturq	Derivative of PCLRT03	Chapt. 3
P. chlororaphis	PCL1391	Tomato root, Spain	[33]
P. brassicacearum	TM1A3	Tomato root, Switzerland	[34]
P. brassicacearum	TM1A3-gfp	Derivative of TM1A3	
Plant pathogens			
Fusarium oxysporum	Fo 5176	*Brassica oleracea*, Australia	[35, 36]
F. oxysporum lycopersici	Fol 4287	Tomato, Spain	[37, 38]
Zymoseptoria tritici	ST99CH_3D1	Wheat, Switzerland	[39]
Z. tritici	ST99CH_3D7	Wheat, Switzerland	[39]
Z. tritici	3D7-mcherry	Derivative of ST99CH_3D7	[40]
Pythium ultimum	Pu-11	Soil, Switzerland	[32]
Insects			
Galleria mellonella		Hebeisen Fisher Store	
Plutella xylostella		Syngenta Crop Protection	
Pieris brassicae		Biocommunication group, ETH	

This table displays information about the *Pseudomonas* strains, the plant pathogens and insect pests used in the experiments in this chapter. *Pseudomonas* bacteria: strains were used to compare biocontrol activity of leaf isolates to. The strains were selected according to their insecticidal and disease suppressive abilities and will be referred to as reference strains in this Chapter. Plant pathogens and insect pests were used in different assays to test the biocontrol activity of the leaf isolates.

To set the activity of the leaf isolates into context, several *Pseudomonas* strains with well-described plant protective abilities were chosen and will from now on be referred to as reference strains (Table 1). Strains of both *P. protegens* (CHA0, PF) and *P. chlororaphis* (PCLRT03, PCL1391) were included in most assays because they display different levels of insecticidal activity and disease suppressive ability and additionally, they produce different antimicrobial metabolites [32]. Furthermore, *P. brassicacearum* TM1A3 was used in different assays because it was shown to possess no insecticidal activity, but it produces several antimicrobial substances also produced by *P. protegens*. *P. protegens* PF was specifically included in several assays because it was isolated from wheat leaves [31] but since it was recovered more than three decades ago from a different host than the new leaf isolates, we consequently do not refer to PF when we use

the term leaf isolates in this chapter unless specifically stated so. Bacteria strains from glycerol stocks were grown on KB^{+++} agar at 24 °C for two days or at 18 °C for three days. From these, LB ON cultures were prepared. These were used directly for *G. mellonella*, *P. xylostella* and *in vitro* inhibition assays. For the other experiments, 200 µl LB ON culture were plated on KB (without antibiotics) and grown for 24 h at 24 °C. Bacteria were washed twice in ddH_2O or 0.9% NaCl and the optical density at 600 nm (OD_{600}) (Genesys150, Thermo Fisher Scientific, MA, USA) was measured. Bacteria suspensions were prepared in ddH_2O or 0.9% NaCl with an OD_{600} of 0.1 corresponding to 10^8 colony forming units (cfu) per ml.

Screenings for insecticidal activity

Greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) larvae (Hebeisen Fisher Store, Zurich, Switzerland) were injected with 10 µl bacteria suspension at an OD_{600} of 0.5 or 0.9% NaCl as control. Per treatment, 9-11 larvae were injected and kept in ø 90 mm petri dishes with a filter paper. Larval mortality was monitored twice a day and larvae were considered dead if they did not react to poking. The experiment was repeated three times with a selection of the reference strains *P. brassicacearum* TM1A3, *P. protegens* CHA0 and *P. chlororaphis* PCLRT03 (Table 1) and the new leaf isolates L2, L6, L7, L8, L14, L15, L16 and L18 (Table S4).

Eggs of the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) were provided by Syngenta Crop Protection (Stein, Switzerland). Larvae were reared and experiments conducted as described by Flury et al. [29]. In summary, 1-week old larvae were presented a food pellet with 10 µl bacteria suspension at an OD_{600} of 0.5 or 0.9% NaCl as control. In each repetition, 32 larvae per treatment were kept in single wells in 128 well trays.

Larvae of the large cabbage white *Pieris brassicae* (Lepidoptera: Pieridae) were fed with Chinese cabbage previously submerged for 30 mins in bacteria suspension at an OD_{600} of 0.25 or ddH_2O as control. In each repetition, 18 larvae per treatment were kept in single wells in 6 well plates for survival monitoring and 6 additional larvae for assessment of larval colonisation. Detailed rearing and experimental procedures can be found in Chapter 3.

For larval colonisation, larvae were surface sterilised, homogenised using a blender and plated on selective medium as described in Chapter 3.

Screenings for antifungal activity

Z. tritici strains ST99CH_3D1 (Zt 3D1) and ST99CH_3D7 (Zt 3D7) and 3D7-mcherry (Table 1) were stored in glycerol 20% at -80 °C. Strains were grown in YSB medium

supplemented with kanamycin (50 mg/l) at 18 °C and 125 rpm for six days for *in vitro* and four days for *in planta* assays. *F. oxysporum* strains Fol 4287 and Fo 5176 (Table 1) were grown in 1/2 PDB at 27 °C and 180 rpm for six days from glycerol stocks kindly provided by Susanne Dora (Plant Cell Biology, ETH Zurich).

For *in vitro* assays, fungal cultures were washed in ddH_2O and concentration measured by counting 10 µl of a 1:10 or 1:100 dilution in a KOVA chamber. 10^6 *F. oxysporum* spores and 10^7 *Z. tritici* spores were evenly spread with a Drigalski spatula on five PDA square plates per fungal strain. For each bacterial test strain, two droplets of 5 µl at an OD_{600} of 0.5 were placed on each plate according to a scheme as shown in Fig. S2.

For disease suppressive assays with *Pythium ultimum* on cucumber, *P. ultimum* strain Pu-11 (Table 1) was used as described in Vesga et al. [32]. Briefly, three pre-germinated cucumber seedlings were planted into a pot infected with *P. ultimum* by mixing 0.1g *P. ultimum*-covered millet into the sand-soil-substrate. Then, pots were inoculated with bacterial suspensions by pouring 50 ml at an OD_{600} = 0.25 to each pot containing 200 g substrate. In the first repetition, only three pots per treatment were prepared and no statistical analysis was performed due to the small number of replicates. In the second repetition, five pots per treatment were prepared.

For the *in planta* assay with *Z. tritici*, 3D7-mcherry was used against *P. protegens* PF-gfp. The infection assay was performed as described by Meile et al. [41], yet at 21 °C during the day (16 h) and 18 °C at night in a greenhouse chamber. For each treatment (control, 3D7-mcherry, PF-gfp, 3D7 x PF one application, 3D7 x PF two applications), two pots with 10-12 plants of wheat *Triticum aestivum* L. variety Drifter were grown. 10^6 spores/ml in 0.1% Tween20 were evenly sprayed until runoff on 17-day old wheat plants and plants incubated in plastic bags for three days to ensure moist conditions for fungal infection. The day before the fungal infection, a bacterial suspension at an OD_{600} of 1.1 was sprayed on wheat plants until runoff. For the treatment with two applications, the bacterial suspension was applied a second time 3 days after the fungal infection. Five days after 3D7-mcherry infection, images were taken with the Zeiss 780 laser scanning confocal microscope to see if the bacteria and fungi were present on the leaves. The taken pictures were edited using ImageJ 1.53k / Java 1.8.0_172 (64-bit). 14 and 21 days after the fungal infection, 12 and 16 second leaves, respectively, were harvested, scanned and evaluated for percentage of leaf area covered with lesions (PLACL) and pycnidia density per leaf area and per PLACL as described by Stewart and McDonald [42].

Plant colonisation assays

Wheat plants were grown as described for the *Z. tritici in vivo* inhibition assay. For each treatment, one pot with approx. 12 plants was prepared and all wheat leaves were sprayed with 20 ml bacterial suspensions at OD_{600} = 1.1 or ddH_2O as control. 1, 7 and 14 days after inoculation, six leaves were harvested, weighed, placed in a falcon tube with 20 mL (1 day post inoculation) or 12 mL (7 and 14 dpi) 0.9% NaCl and shaken for 1 hour at 3 °C and 200 rpm. Subsequently, the suspension was serially diluted and plated on selective medium, KB^{+++} for wildtype (leaf isolates) or KB^{++G} for strains tagged with a fluorophore (reference strains). At 1 dpi, for the wildtype strains, 100 µL of the 10^{0}–10^{-2} dilutions were plated on round plates using a Drigalski spatula, while the tagged strains were plated by spotting 10 µL on square plates, thereby increasing the detection limit 10-fold. At 7 and 14 dpi, 1 mL and 100 µL of the undiluted sample and 100 µL of the 1:10 dilution were plated on round plates to have the same detection limit for all strains.

Chinese cabbage *Brassica rapa* subsp. *pekinensis* 'Michilili' seeds were pre-germinated in Jiffy substrate and transplanted into pots filled with Jiffy substrate in a greenhouse chamber with the following conditions: 16 hours light at 21 °C and 8 hours night at 18 °C. Three weeks after germination, bacterial suspension with an OD_{600} of 1.1 or ddH_2O as control were sprayed on all leaves of six plants per treatment. 24 hours after inoculation, six leaves from different plants were collected, weighed, incubated in 30 mL 0.9% NaCl for 30 min at 3 °C and 200 rpm and the undiluted and 1:10 diluted suspensions were plated on KB^{+++} as described above.

Radish *Raphanus sativus* var. Riesenbutter seeds were pre-germinated in Jiffy substrate and transplanted into pots filled with Jiffy substrate in a greenhouse chamber with the same conditions as the Chinese cabbage plants. One and two weeks after germination, 25 ml of bacterial suspensions adjusted to an OD_{600} = 0.1 were spread around the seedlings to reach 10^{7} cfu/g soil. One week after the second inoculation, radish roots were harvested, washed quickly with tap water, incubated in 10 mL 0.9% NaCl for 30 mins at 3 °C and 200 rpm and serially diluted suspensions (10^{0} and 10^{-2}) were plated on KB^{+++} as described above.

Statistical analysis

Statistical analysis was performed in Rstudio (version 1.4.1717) using R (ver. 4.1.2). *P*-values below 0.05 were considered statistically significant and treatments that contain the same letter in their labelling are not significantly different to each other. Standard boxplots, barplots and crossbars as well as survival curves were visualised using ggplot2 (ver. 3.3.5). Larval survival was analysed using log-rank and pairwise survival differences (packages survminer ver. 0.4.9 and survival ver. 3.2-11). For *in vitro* inhi-

bition assays, differences in inhibition zone sizes were analysed using Kruskal-Wallis and post-hoc pairwise Wilcoxon tests (package FSA ver. 0.9.1). The same tests were used to evaluate the results of the *in planta Z. tritici* inhibition assay. An ordinal regression model (function polr, package Mass ver. 7.3-55) was run for the *P. ultimum* disease ratings. Kruskal-Wallis and post-hoc Dunn or Wilcoxon test were performed for log-transformed colonisation data from larvae, leaves and roots since data did not follow a normal distribution according to a Shapiro-Wilk Normality test.

Results and Discussion

Fluorescent pseudomonads isolated from radish leaves

From the 78 fluorescing colonies that were screened using specific primers to identify EPP, 18 were selected for DNA extraction and housekeeping gene sequencing. Unfortunately, none of these isolates belongs to the *P. chlororaphis* or *P. protegens* subgroups. 15 belong to the subgroup *P. fluorescens*, two to the subgroup *P. koreensis* and one to the group *P. putida* (Table 2). For further classification and comparison, a phylogenetic tree was created (Fig. 1). Some isolates cluster closely together, which is sometimes linked to their sampling origin. For example, L4, L5, L6 and L7 were isolated from plot m3 sample 1 and L5, L6 and L7 are very closely related and might therefore be clones (Table S4). On the other hand, L3, L4 and L16 were isolated from different plots and are also closely related. Interestingly, *Pseudomonas* sp. L10 in the *P. koreensis* subgroup is very closely related to *Pseudomonas* sp. Leaf434, which was isolated from *Arabidopsis thaliana* leaves in the effort to construct a representative phyllosphere microbiome, called *At*-LSPHERE [13, 43]. *Pseudomonas* sp. Leaf434 was able to inhibit many *At*-LSPHERE strains and nearly all tested plant pathogenic bacteria [13]. Furthermore, several leaf isolates are closely related to strains with plant growth promoting or disease suppressive abilities, e.g. *Pseudomonas* sp. L2 and *P. capeferrum* WCS358, *Pseudomonas* sp. L8 and *P. defensor* WCS374, as well as *Pseudomonas* sp. L18 and *P. simiae* WCS417 [44]. *Pseudomonas* sp. L14, however, is most closely related to *P. salomonii* LMG22120 that causes spring rot or 'Café au Lait' disease in garlic [45, 46].

Altogether, the chosen approach for isolating fluorescent pseudomonads worked well and a set of interesting leaf strains was identified. The goal to isolate bacteria belonging to the *P. chlororaphis* and *P. protegens* subgroups from leaves was, however, not reached. Either they do not colonise leaves or only in small numbers, or the conditions were not favourable on the sampled radish leaves. Luzia Stalder (manuscript in preparation) sampled leaves from a wheat field naturally infected with *Z. tritici* and discovered that *Pseudomonas* are a dominant bacteria genus in the wheat leaf microbiome and *P. chlororaphis* one of the most abundant subgroups. In the *At*-LSPHERE, however, to

the best of our knowledge no strain belongs to the *P. chlororaphis* subgroup. Potentially, Brassicaceae are not suitable hosts for *P. chlororaphis*. Brassicaceae are known to produce antimicrobial and antifungal substances [47, 48, 49, 50, 51, 52]. However, *P. chlororaphis* PCLRT03 colonised radish roots at high densities in Chapter 2, which was comparable to cucumber and wheat root colonisation [32]. Nevertheless, the environment on the leaves, i.e. the phyllosphere, might still be less suitable in Brassicaceae for *P. chlororaphis* colonisation compared to wheat.

Table 2. **Phylogenetic classification of selected fluorescent *Pseudomonas* bacteria isolates based on sequences of three housekeeping genes.**

Isolate	*rpoD*	*rpoB*	*gyrB*	closest relative(s)
L1	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. lactis* SS101
L2	group *putida*	group *putida*	NA	*P. capeferum* WCS358
L3	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. rhodesiae* LMG17764 T
L4	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. rhodesiae* LMG17764 T
L5	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. lactis* SS101
L6	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. lactis* SS101
L7	NA	SG *fluorescens*	SG *fluorescens*	*P. lactis* SS101
L8	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. defensor* WCS374
L9	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. trivialis* / *P. poae*
L10	SG *koreensis*	SG *koreensis*	SG *koreensis*	*P. koreensis* D26
L11	SG *koreensis*	SG *koreensis*	SG *koreensis*	*P. granadensis* LMG27940 T
L12	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*Pseudomonas* sp. MIACH
L13	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*Pseudomonas* sp. MIACH
L14	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. salomonii* LMG22120 T
L15	SG *fluorescens*	NA	SG *fluorescens*	*P. simiae* CCUG50988 T
L16	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. rhodesiae* LMG17764 T
L17	SG *fluorescens*	SG *fluorescens*	SG *fluorescens*	*P. trivialis* / *P. poae*
L18	SG *fluorescens*	NA	SG *fluorescens*	*P. simiae* CCUG50988 T

Separate phylogenetic trees were constructed by Jordan Vacheron using *rpoD*, *rpoB* and *gyrB* housekeeping gene sequences from a representative selection of fluorescent *Pseudomonas* strains including the 18 new isolates. According to this information, the group or, if possible, the subgroup (SG) was determined; *fluorescens* and *koreensis* are both subgroups of the *fluorescens* group. NA = sequence data not available at time of tree construction. Closest relative was determined using the phylogenetic tree constructed using concatenated *rpoD* and *gyrB* sequences as displayed in Fig. 1; T = type strain.

Two leaf isolates show potent insecticidal activity

Upon injection into *G. mellonella* larvae, several strains exhibited potent insecticidal activity (Fig. S4). The new isolates *Pseudomonas* sp. L6, L7, L8, L14, L15 and L18 could kill 100% of the larvae within 36 or 48 h. L2 and L16 reached nearly 70% mortality within 48 and 36 h, respectively. However, results need to be treated with caution because the positive and negative controls failed at times. In the first repetition (Fig. S4A), *P. bras-*

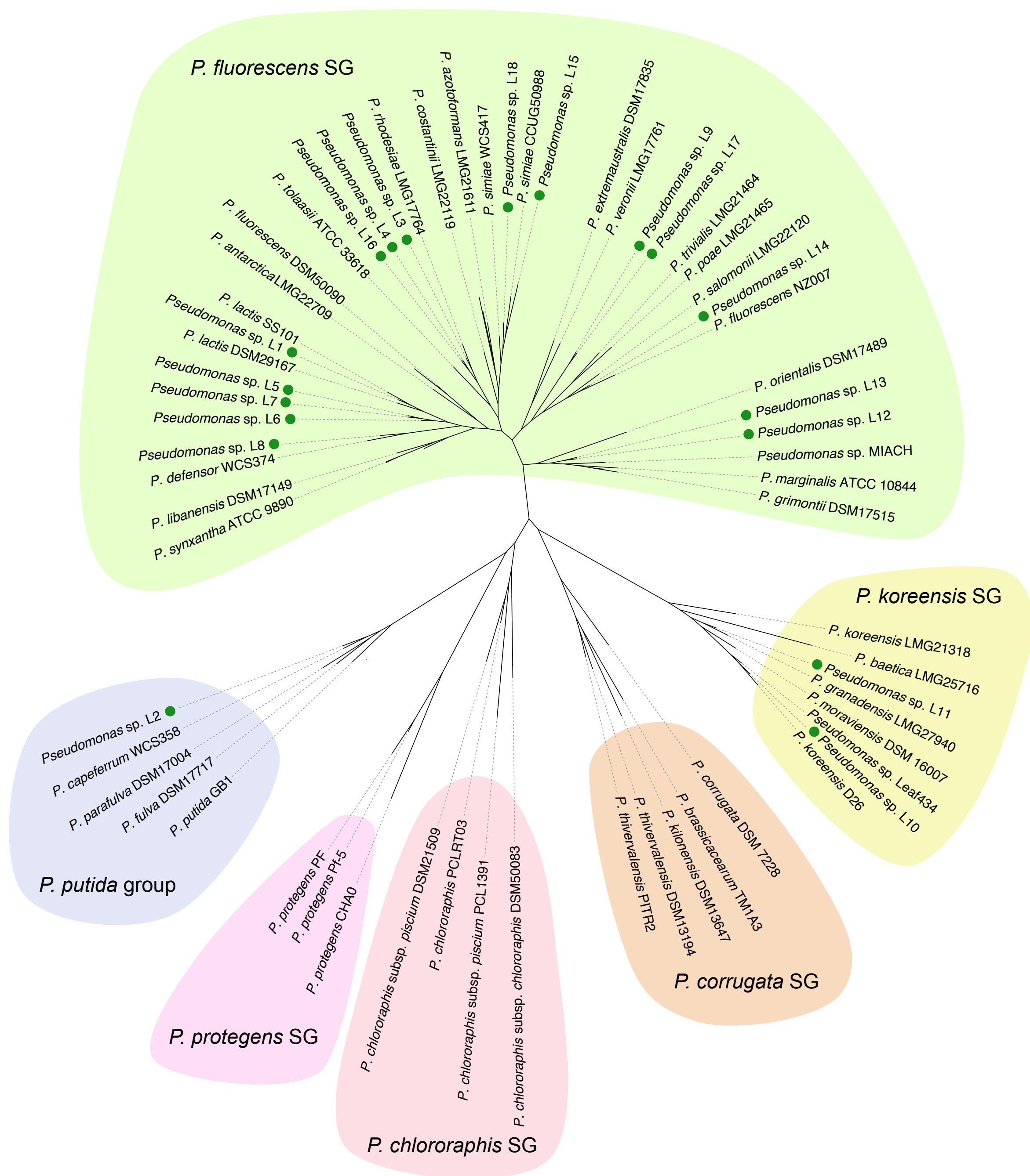

Fig. 1. **Phylogenetic tree including the new fluorescent *Pseudomonas* leaf isolates.**

The phylogenetic tree is based on concatenated *rpoD* and *gyrB* sequences of the new leaf isolates and a large selection of *Pseudomonas* strains covering the *P. fluorescens* and closely related groups. The tree was constructed with Seaview using the maximum likelihood method (PhyML) with a GTR model and a bootstrap approach with 100 replicates. The tree was visualised using iTol and Affinity Designer. For visualisation, strains closely related to the new isolates and relevant reference strains were chosen to allow for a useful classification and comparison. The new leaf isolates – *Pseudomonas* sp. L1-L18 – are depicted with a green circle beside their name. The coloured clouds represent the different *Pseudomonas* groups and subgroups (SG).

sicacearum TM1A3, which does not possess insecticidal activity according to Flury et

al. [29], killed all larvae within 36 h. The larvae melanised shortly after injection and it is possible that the wrong stain was grown by accident. In the third repetition (Fig. S4C), the 0.9% NaCl control had around 30% mortality, yet insecticidal strain *P. protegens* CHA0 reached only 50% mortality. However, the injection assays still give a first insight which strains could possibly be able to exhibit insecticidal activity and were used to select the strains applied in *P. xylostella* feeding assays.

In feeding assays with *P. xylostella* larvae, several isolates exhibited oral insecticidal activities (Fig. 2). *Pseudomonas* sp. L14 was the most promising isolate, causing 100% mortality after 24 h in three repetitions and after 40 h in repetition 3. L7 treatment also resulted in 100% mortality in all experiments and, except for repetition 4, within 48 h. L8 and L15 also significantly enhanced mortality compared to the control in the respective experiments in which they were applied. L18 was used in repetition 1 and 2, yet the survival curve was only significantly different to the control in repetition 2, with L18 causing around 80% mortality (Figs. 2A, B). For L2, the mortality rate was around 50% in the first and 80% in the second repetition. It was both times significantly different to the control, yet it only caused a higher mortality than the control in the second trial. In the first trial, the mortality in the control was nearly 100% at the end of the experiment (Fig. 2A). The food pellets had become yellowish similarly to the bacteria-inoculated pellets, suggesting a contamination of the food pellets with an insecticidal bacteria. Pellets were subsequently plated and high cfu counts also pointed towards a contamination. When comparing the new isolates to the reference strains *P. protegens* CHA0 and *P. chlororaphis* PCLRT03, *Pseudomonas* sp. L14 and, except for the last trial, L7 caused similar mortality levels. L14 was as efficient in killing larvae as CHA0 and significantly more efficient than PCLRT03 in two out of three trials. L7 was significantly different to PCLRT03 in each trial, once slower and twice faster in killing *P. xylostella* larvae. L8, L15 and L18 did not reach the killing speed of the reference strains.

In feeding assays with *P. brassicae* larvae, no leaf isolate was able to significantly reduce larval survival compared to the control except for L8 in the third repetition (Fig. 3). In the first repetition, L7 insignificantly increased mortality compared to the control, and was significantly different to L18 with a low mortality rate and to CHA0 with a high mortality rate (Fig. 3A). Unfortunately, the larvae obtained from the *P. brassicae* rearing in Spring 2022 were less susceptible to EPP infection than observed previously for unknown reasons. In the first repetition conducted in November 2021, the reference strains CHA0 and PCLRT03 caused more than 50% mortality within 50 hours (Fig. 3A), which was not observed in Spring 2022 when the following repetitions were conducted (Figs. 3B-D).

For the third repetition of the *P. xylostella* experiment (Fig. 4A) and the first (Fig. 4B),

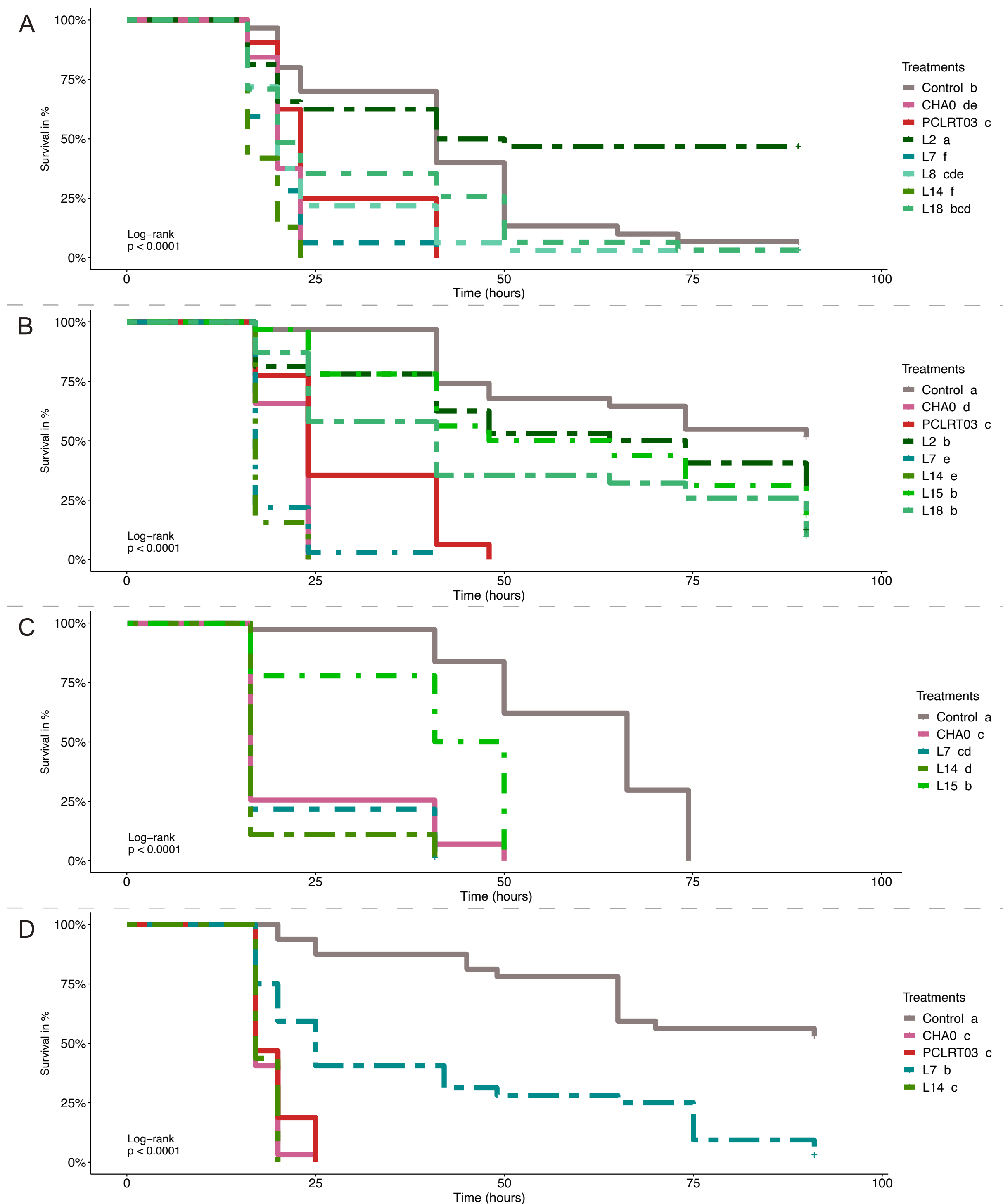

Fig. 2. **Survival of *P. xylostella* after infection with fluorescent pseudomonads.**

Survival of *P. xylostella* larvae after oral infection with a selection of the *Pseudomonas* strains *P. chlororaphis* PCLRT03, *P. protegens* CHA0, *Pseudomonas* sp. L2, L7, L8, L14, L15 and L18 or 0.9% NaCl as control. Four independent repetitions with n = 32 larvae per treatment were performed (A-D). Different lowercase letters beside strain names in the legend refer to significant differences ($P < 0.05$) between treatments according to a pairwise comparison of survival curves.

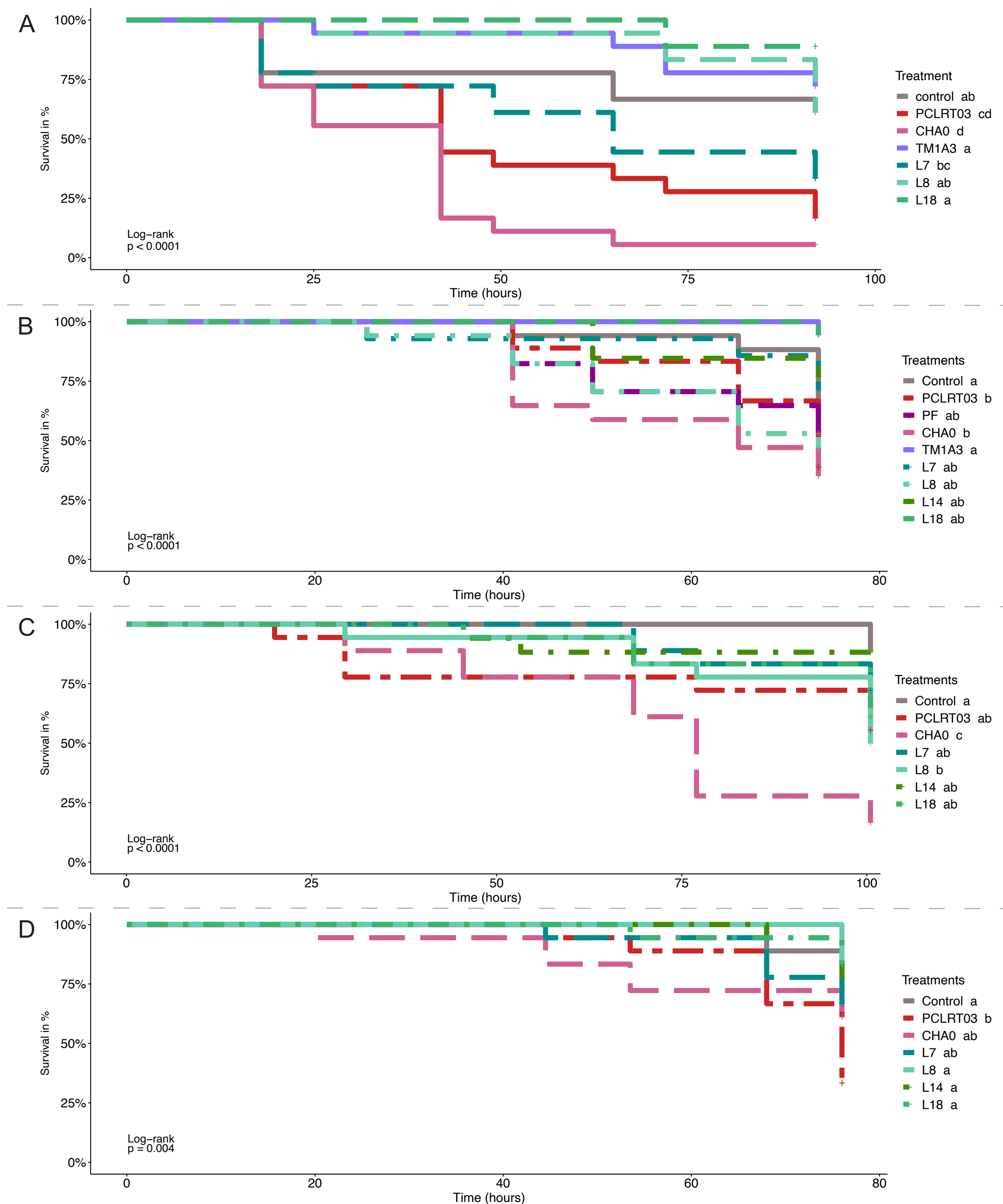

Fig. 3. **Survival of *P. brassicae* after infection with fluorescent pseudomonads.**

Survival of 3rd instar *P. brassicae* larvae after oral infection with a selection of the *Pseudomonas* strains *P. chlororaphis* PCLRT03, *P. protegens* CHA0, PF, *P. brassicacearum* TM1A3, *Pseudomonas* sp. L7, L8, L14 and L18 or 0.9% NaCl as control. Four independent repetitions with n = 18 larvae per treatment were performed (A-D). Different lowercase letters beside strain names in the legend refer to significant differences ($P < 0.05$) between treatments according to a pairwise comparison of survival curves.

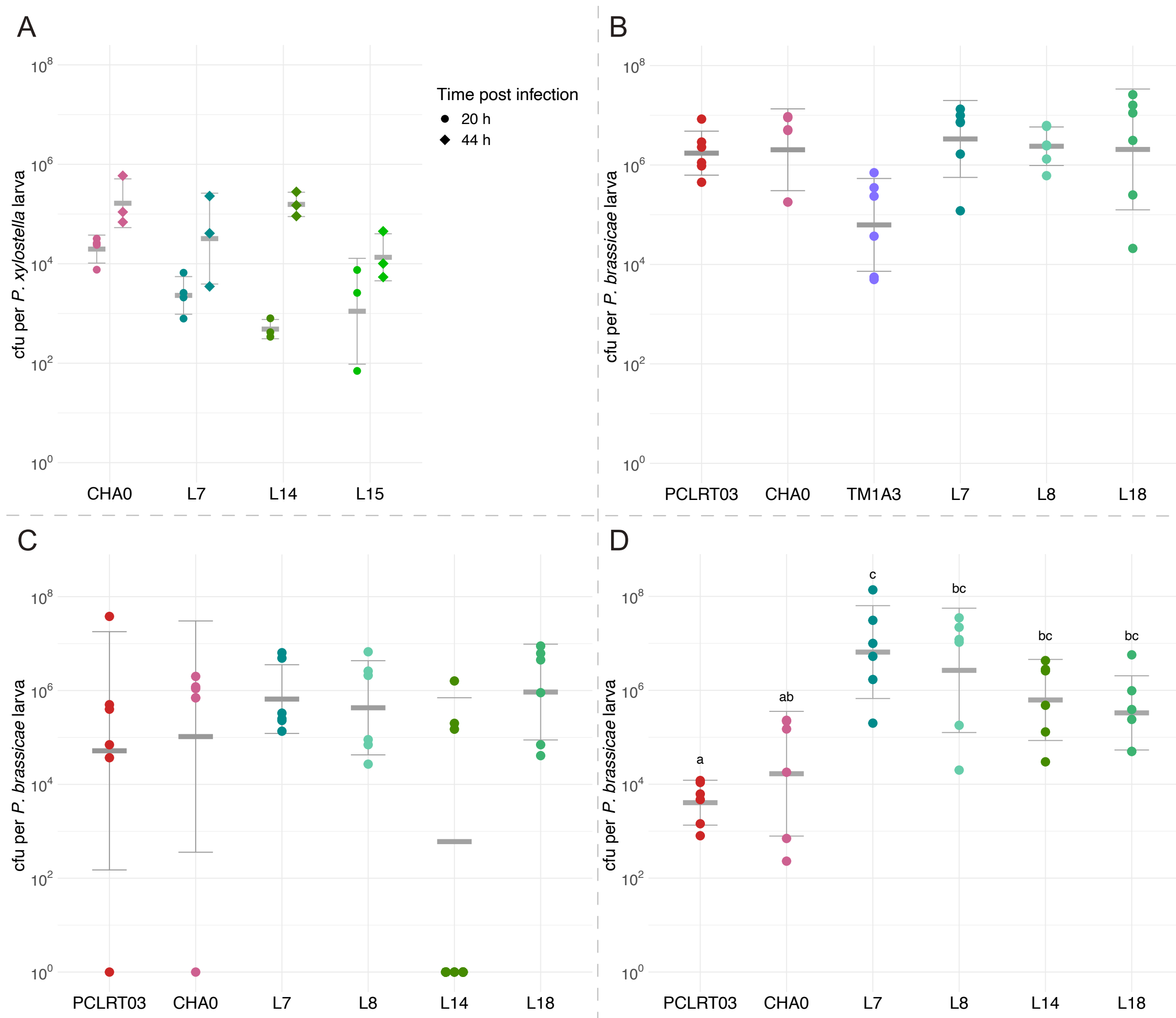

Fig. 4. **Colonisation of *P. xylostella* and *P. brassicae* by fluorescent pseudomonads.**

Colonisation of *P. xylostella* larvae after 20 and 44 hours (A) and of *P. brassicae* larvae after 24 hours (B-D) by *P. chlororaphis* PCLRT03, *P. protegens* CHA0 and *Pseudomonas* sp. L7, L8, L14, L15 and L18. Colonisation was assessed by homogenising six larvae per treatment and plating homogenate dilutions on selective medium in the third repetition of the *P. xylostella* experiment (A) and the first (B), third (C) and fourth (D) repetition of the *P. brassicae* experiment. The crossbars represent the average and standard deviations, and each coloured point represents one data point. Different letters above dots indicate significant differences between treatments ($P < 0.05$) according to a Kruskal-Wallis and post-hoc Pairwise Wilcoxon test (B-D); no letters indicate no significant differences. A was not statistically evaluated due to small sample size per time-point (n = 3).

third (Fig. 4C) and fourth (Fig. 4D) repetition of the *P. brassicae* experiment, colonisation of larvae by pseudomonads was assessed. In *P. xylostella*, CHA0 colonised the larvae to higher levels than the leaf isolates L7, L14 and L15 after 20 h, though L14 reached similar levels after 44 h (Fig. 4A). L14 colonised larvae below 10^3 cfu/larva and CHA0 above 10^4 after 20 h, yet both reached 2 x 10^5 after 44 h. The tested *Pseudomonas* strains were equally able to colonise *P. brassicae* larvae even though the larvae were less susceptible to bacterial infection. In repetition 1, 10^6 - 10^7 cfu/larva were discovered

for CHA0, PCLRT03, L7, L8 and L18, which was insignificantly higher than for TM1A3 at around 10^5 cfu/larva (Fig. 4B). In repetition 3, larvae were colonised by 10^5 to 10^7 cfu/larva if pseudomonads were detected (Fig. 4C). For CHA0 and PCLRT03 one larva and for L14, half of the larvae were not colonised. In repetition 4, the leaf isolates L7, L8, L14 and L18 colonised larvae to significantly higher levels than PCLRT03 (around 5 x 10^3 cfu/larva), and L7 even higher levels than CHA0 (around 10^4 cfu/larva) (Fig. 4D). L7 reached colonisation densities up to 10^8 cfu/larva yet it caused less than 50% mortality after three days. Over all repetitions, the colonisation density at 24 h did not give any indication about the final mortality after three to four days. Higher population densities were observed in *P. brassicae* larvae than in *P. xylostella* larvae despite their lower susceptibility to pseudomonad infection. The higher colonisation densities might be due to the larger size of *P. brassicae* larvae. On the other hand, *P. brassicae* larvae were fed on leaves and could acquire a more natural microbiome than *P. xylostella* larvae that were fed an artificial semi-sterile diet, which would increase competition in the gut. Possibly, pseudomonads could colonise the gut of *P. brassicae* larvae but did not reach sufficient population densities to breach the gut barrier.

Taken together, *Pseudomonas* sp. L14 and L7 showed potent insecticidal activity upon injection into *G. mellonella* larvae and in *P. xylostella* feeding assays and were able to colonise the latter (Figs. S4, 2, 4A). Several other strains showed weak insecticidal activity, namely *Pseudomonas* sp. L8, L15 and L18. Furthermore, L7, L8, L14 and L18 were able to colonise *P. brassicae* larvae at 10^5 - 10^8 cfu/larva after 24 h even though the larvae were not susceptible to bacterial infection (Figs. 3, 4B-D). So far, only medium insecticidal activity was linked to strains belonging to the *P. fluorescens* subgroup. Flury et al. [29] described medium oral insecticidal activity for *P. lactis* SS101 and *Pseudomonas* sp. MIACH, none at all for the *P. fluorescens* type strain DSM50090 and strong activity comparable to *P. protegens* PF upon injection for DSM50090 and SS101, but none for MIACH. Thus the three fluorescens subgroup strains tested by Fluy et al. [29] displayed a lower oral insecticidal activity than any tested *P. chlororaphis* or *P. protegens* strain. Olcott et al. [53] also observed that *P. protegens* Pf-5 was more lethal against *Drosophila melanogaster* larvae and faster in killing them than *P. fluorescens* SBW25, and Pf-5 could colonise larvae to higher levels than SBW25. *Pseudomonas* sp. L18, that expressed a medium oral insecticidal effect in the *P. xylostella* feeding assay, is closely related to *P. simiae* WCS417. *P. simiae* WCS417 has not been evaluated for its oral insecticidal activity, but it was shown to have a systemic effect on plants that can, depending on the plant and insect species, positively or negatively affect leaf feeding insects and their predators [54, 55, 56]. Friman et al. [56], for example, reported decreased *P. xylostella* larval biomass and increased *D. radicum* fly biomass after larvae had been feeding on cabbage plants grown in soil inoculated with WCS417.

They discovered an elevated expression of a defence marker gene in the plants with *P. xylostella* feeding and WCS417 inoculation. Thus, *P. fluorescens* subgroup strains can affect insects in different ways, either upon oral uptake by the insect or by modulating plant defences.

Strains that belong to the *P. fluorescens* subgroup do not possess the *P. fluorescens* insecticidal toxin (fit) complex. They partially harbour the *psl* gene cluster for which a possible contribution to insecticidal activity was suggested [29]. Different cyclic lipopeptides were also found to contribute to insecticidal activity [57]. DSM50090, SS01 and MIACH genomes harbour the cyclic lipopeptides Massetolide and Viscosin, and partially Orfamide A [32, 58]. Orfamide A has been shown to contribute to insecticidal activity in *P. protegens* strains Pf-5 and CHA0 [59, 60]. Toxin complexes (Tc) that were first described for entomopathogenic *Photorhabdus* bacteria were also discovered in these strains, yet it could not be shown that they contribute to insect killing, also because Tc were also found in non-insecticidal fluorescent pseudomonads [29, 58]. Small genes with homology to pathogenicity-linked *reb* genes were discovered in *P. chlororaphis* and *P. protegens* as well as SS01, though they were not further explored so far [29]. Recently, a new insecticidal protein was discovered in a *P. chlororaphis* strain with activity against the western corn rootworm when expressed in transgenic plants [61]. The identified protein shows 82% sequence identity to a protein in a *P. rhodesiae* strain. According to our phylogenetic tree, leaf isolates L3, L4 and L16 are closely related to *P. rhodesiae*. From these strains, only L16 was tested in one *G. mellonella* injection assay, where it killed 70% of the larvae within 36 h. The discovered toxin was specific against rootworms and did not impact several other tested insect species [61]. It can only be speculated which genes could play a role in the insecticidal activity of our new leaf isolates. It is also possible that some strains harbour yet unknown insecticidal traits. This could especially be true for L14 and L7 since they supposedly express a higher oral insecticidal activity than any of the strains from the *P. fluorescens* subgroup tested against insects so far. Therefore, it will be highly interesting to sequence L7 and L14 to gain insights into the mechanisms underlying their insecticidal activity.

Tested leaf strains do not show antifungal activity *in vitro* but protect plants against a root pathogen *in planta*

In the *in vitro* assays against the plant pathogens *F. oxysporum* and *Z. tritici*, the new leaf isolates were not able to inhibit fungal growth (Fig. 5). *Pseudomonas* sp. L7, L8, L14 and L18 could stop mycelial growth of *F. oxysporum* Fol 4287 and Fo 5176 in repetition 1 at the border of their colony, yet all were overgrown in repetition 2. For *Z. tritici* Zt 3D1 and Zt 3D7, they could stop fungal growth at the colony border. The selected reference strains *P. chlororaphis* PCLRT03, *P. brassicacearum* TM1A3, *P. protegens* PF

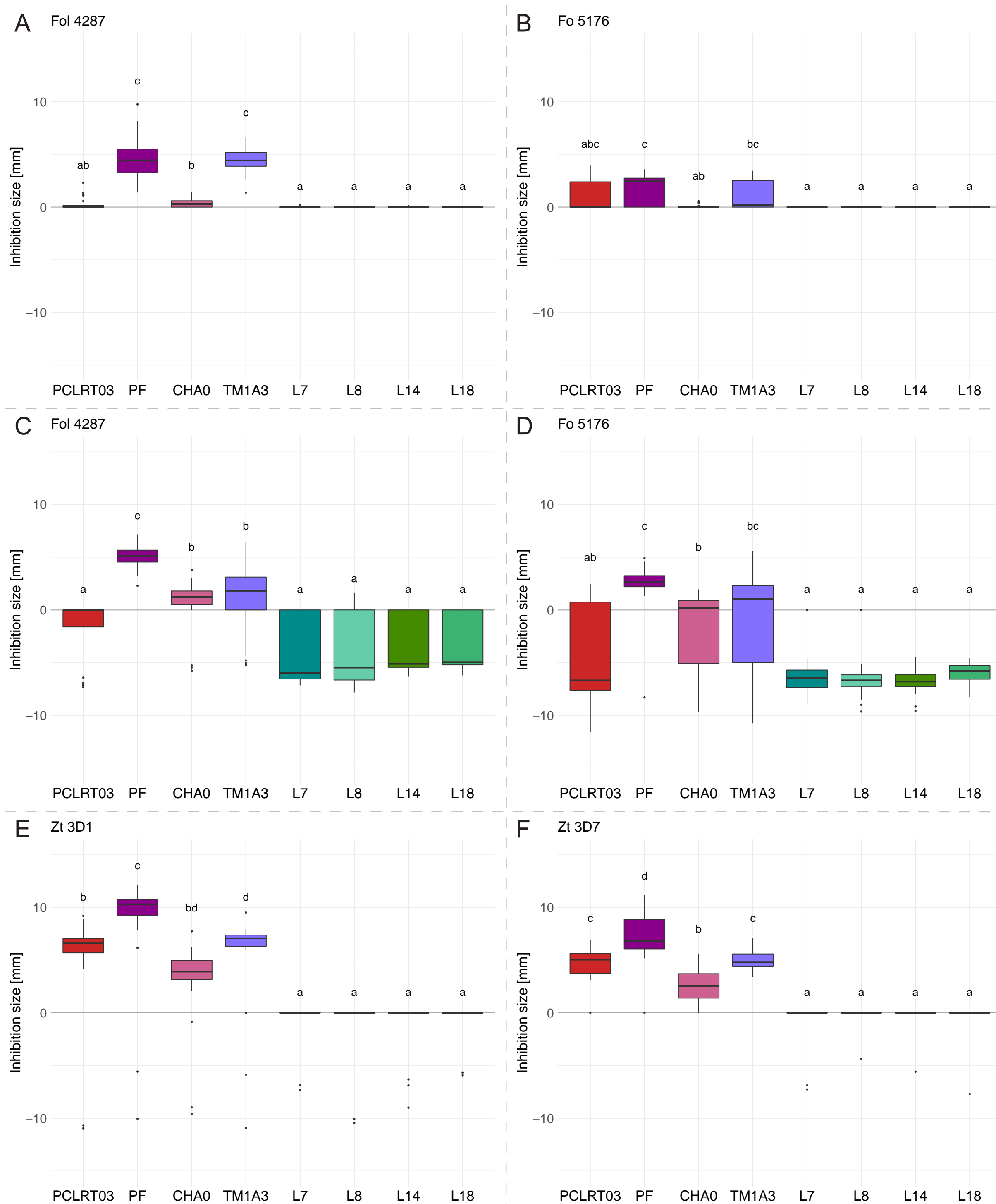

Fig. 5. ***In vitro* inhibition of fungal plant pathogens by fluorescent pseudomonads.**

Inhibition zones exhibited by pseudomonads against *F. oxysporum* Fol 4287 (A and C), *F. oxysporum* Fo 5176 (B and D), *Z. tritici* Zt 3D1 (E) and *Z. tritici* Zt 3D7 (F) *in vitro*. Tested *Pseudomonas* strains: *P. chlororaphis* PCLRT03, *P. protegens* PF and CHA0, *P. brassicacearum* TM1A3 and *Pseudomonas* sp. L7, L8, L14 and L18. For each assay, five square PDA plates with fungal suspensions and two bacteria droplets per strain were prepared; A and B show results from a first, C-F from a second repetition. Negative values in inhibition zone size indicate bacteria colonies overgrown by fungi. Different letters above boxplots indicate significant differences between treatments ($P < 0.05$) according to a Kruskal-Wallis and post-hoc Pairwise Wilcoxon test.

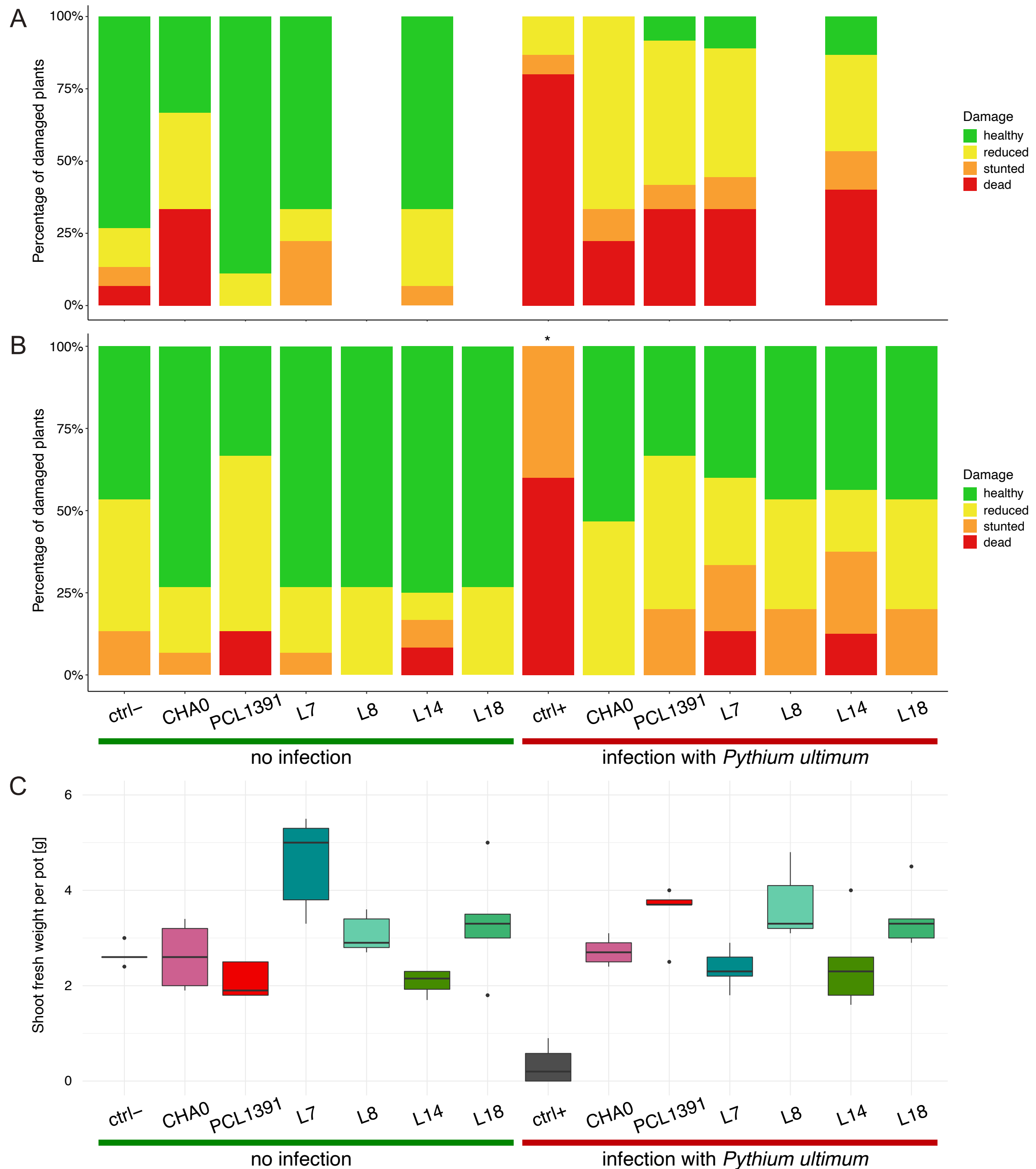

Fig. 6. **Suppression of *P. ultimum* on cucumber roots by fluorescent pseudomonads.**

Percentage of damaged cucumber plants (A, B) and cucumber shoot weight (C) upon infection with the oomycete pathogen *P. ultimum* Pu-11 and inoculation with the *Pseudomonas* strains *P. protegens* CHA0, *P. chlororaphis* PCL1391, *Pseudomonas* sp. L7, L8, L14 and L18 and ddH$_2$O as control (ctrl- and ctrl+). Damage was rated for each plant (A, B) and shoot weight recorded per pot (C) with three plants per pot and three pots per treatment in repetition 1 (A) and five pots per treatment in repetition 2 (B, C). Damage classification: healthy plant; reduced plant growth and/or leaf deformations; stunted plant i.e. heavily reduced growth; dead plant. Due to small sample size, only damage rating in repetition 2 was statistically evaluated and the asterisk indicates that the treatment is significantly different ($P < 0.05$) to all other treatments according to an ordinal regression analysis.

and CHA0 exhibited different abilities in suppressing fungal growth. All four reference strains were able to inhibit the growth of both tested *Z. tritici* strains causing larger inhibition zones against Zt 3D1 than against Zt 3D7. The growth of Fol 4287 was inhibited more strongly than that of Fo 5176 by all reference strains. The most potent reference strain was PF that could cause an inhibition zone of up to 5 mm against Fol 4287 and even 10 mm against Zt 3D1. The closely related CHA0 showed a significantly lower ability to inhibit all fungal pathogens. TM1A3 performed well against most tested fungi except for Fo 5176, which it could barely inhibit and by which it was even overgrown on some plates in the second repetition. PCLRT03 did not show any clear *in vitro* activity against *F. oxysporum*. The only exception was a slight inhibition of Fo 5176 in the first assay but it was overgrown by both *F. oxysporum* strains on most plates in repetition 2. However, PCLRT03 was able to inhibit both *Z. tritici* strains well and performed even significantly better against Zt 3D7 than CHA0.

In vitro inhibition assays with L7 and L14 against *P. ultimum* (Pu) had shown that the isolates could not inhibit pathogen growth (data not shown). However, in a first *in planta* experiment, both isolates were able to limit the amount of dead plants compared to the positive control with Pu infection (Fig. 6A). The assay was repeated with more strains and replicates (Fig. 6B, C). In both repetitions, the positive control with Pu infection led to more than 50% dead plants and 0% healthy plants. In repetition 2, statistical analysis revealed that the plant damage in the positive control (Pu infection) was significantly different to all other treatments, yet no differences were detected between the bacterial treatments with and without Pu infection. *Pseudomonas* sp. L7, L8, L14 and L18 were able to reduce the amount of dead plants, L8 and L18 even to 0% (Fig. 6B). CHA0 was the only treatment with Pu infection without stunted or dead plants, therefore showing the greatest ability to protect cucumber plants from the pathogen. However, plants protected against Pu by PCL1391 had higher plant fresh weights compared to CHA0. Interestingly, shoot weights were even higher in the PCL1391-Pu treatment than in PCL1391 treatment without Pu infection. A reduction in % healthy plants was observed for L7, L8, L14 and L18 when comparing pots with and without Pu infection. However, the shoot weight was only affected by Pu in L7 treatments. In absence of the pathogen L7-treated plants had the highest shoot weights. To conclude, all tested new leaf isolates, namely *Pseudomonas* sp. L7, L8, L14 and L18, could protect cucumber plants from *P. ultimum* infection to a similar extent as *P. chlororaphis* PCL1391 and *P. protegens* CHA0.

The new leaf isolates *Pseudomonas* sp. L7, L8, L14 and L18 were not able to inhibit fungal growth *in vitro*, but could suppress an oomycete in *in planta* assays. Mazzola et al. [62] showed that *P. lactis* SS101, closely related to *Pseudomonas* sp. L7, can suppress *Pythium* spp. *in planta* but not *in vitro*. Several *P. simiae* strains were able to

inhibit fungal pathogens or their toxin production in diverse systems [63, 64, 65, 66]. *P. simiae* WCS417, closely related to *Pseudomonas* sp. L18, and *P. defensor* WCS374, closely related to *Pseudomonas* sp. L8, can elicit induced systemic resistance (ISR) in different plants, but no genes coding for antimicrobial compounds were detected [44]. *P. fluorescens* DSM50090, *P. lactis* SS101 and *Pseudomonas* sp. MIACH cannot produce several of the antimicrobial compounds that the reference strains can produce, but they possess genes to produce the cyclic lipopeptides Massetolide and Viscosin, and partially Orfamide A [29, 32]. In different studies, cyclic lipopeptides including from the Viscosin family were reported to suppress plant pathogens [67, 68, 69]. Although the genetic mechanisms underlying *P. ultimum* inhibition by *Pseudomonas* sp. L7, L8, L14 and L18 cannot be determined without further analysis, it is possible that cyclic lipopeptides might play a role.

P. protegens CHA0 and PF, *P. chlororaphis* PCLRT03 and *P. brassicacearum* TM1A3 all produce different antimicrobial compounds, which can possibly explain most differences in the performance against the plant pathogens. TM1A3 produces DAPG (2,4-diacetylphloroglucinol), CHA0 and PF produce DAPG, pyoluteorin and pyrrolnitrin, PCLRT03 produces phenazines and all strains produce HCN [32]. PF was the strongest inhibitor of the fungal pathogens and is the only strain that produces the toxin rhizoxin [32]. Rhizoxin blocks mitosis by binding to the β-tubulin subunit in eukaryotic cells and a rhizoxin produced by the very closely related *P. protegens* Pf-5 was shown to inhibit growth of *F. oxysporum in vitro* [70]. Potentially, rhizoxin contributes to the strong inhibition of fungal growth *in vitro* (Fig. 5). Furthermore, it was shown that DAPG and dihydroaeruginoic acid contribute to inhibition of *Z. tritici* by PF [71]. The closely related CHA0 could only slightly inhibit growth of *F. oxysporum*, which was similar for TM1A3 except for the first assay, where TM1A3 caused a significantly larger inhibition zone against Fol 4287 compared to CHA0 (Fig. 5A). It was shown that fusaric acid, which is produced by the *F. oxysporum* strains used in this experiments, can prevent DAPG production in CHA0 [72]. Possibly, DAPG production is less affected in TM1A3 than in CHA0. PCLRT03 performed better against *Z. tritici* than *F. oxysporum*, suggesting that phenazines are effective against the first but not the latter.

In conclusion, different fluorescent *Pseudomonas* strains exhibit different antifungal activity mainly due to differences in production of antimicrobial metabolites and toxins. Furthermore, *in vitro* assays do not translate into results obtained in *in planta* assays. The use of detached leaf assays or the development of new, high-throughput *in planta* screening assays should be sought to allow for more accurate determination of biocontrol abilities by new isolates.

New leaf-isolated strains show increased leaf colonisation ability

In a first assay, plant colonisation ability was assessed in two Brassicacean plants, namely for Chinese cabbage leaves one day after inoculation and for radish roots one week after the second inoculation (Fig. 7). On the roots, all tested strains – *P. chlororaphis* PCLRT03, *P. protegens* CHA0, *P. brassicacearum* TM1A3, *Pseudomonas* sp. L7, L8 and L18 – reached colonisation densities between 10^5 and 10^6 cfu/g root. The only exception is L18, which shows a high variability from below detection to 5 x 10^7 cfu/g. However, L18 reached on average the highest leaf colonisation with around 5 x 10^8 cfu/g leaf, which was significantly different to all strains except for TM1A3. L7 and L8 were detected at 5 x 10^6 to 10^7 cfu/g, which was significantly higher than for PCLRT03. Moreover, the leaf isolates showed a lower variability in colonising plant leaves than the root isolates PCLRT03, CHA0 and TM1A3.

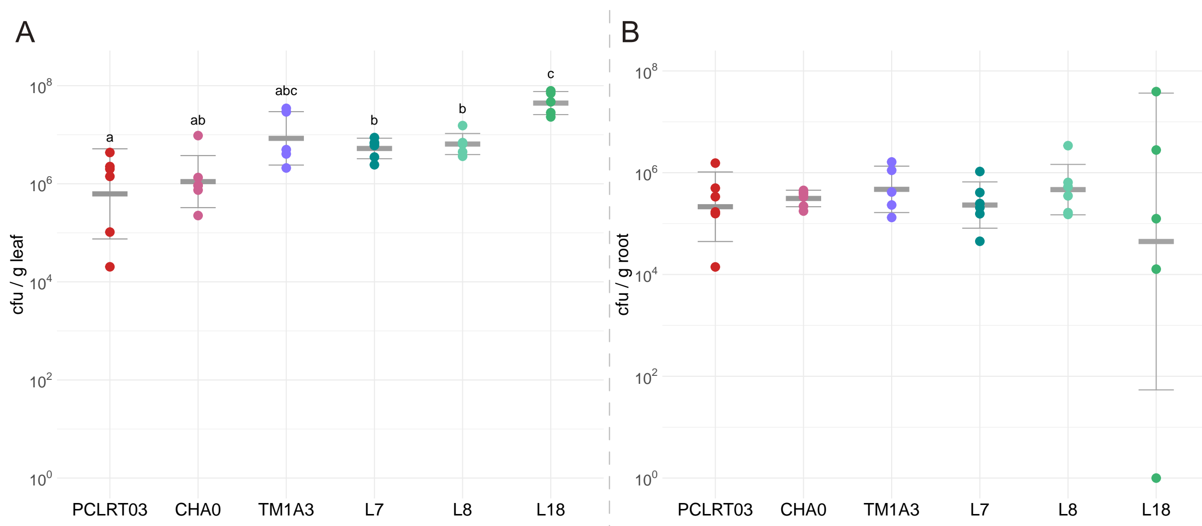

Fig. 7. **Colonisation of Chinese cabbage leaves (A) and radish roots (B) by fluorescent pseudomonads.**

Leaf (A) and root (B) colonisation by *P. chlororaphis* PCLRT03, *P. protegens* CHA0, *P. brassicacearum* TM1A3, *Pseudomonas* sp. L7, L8 and L18. Chinese cabbage leaf samples (n = 6) were taken at 1 day post inoculation (dpi) and radish root samples (n = 6) at 7 dpi. Different letters above dots represent significant differences between treatments ($P < 0.05$) according to Kruskal-Wallis and post-hoc Pairwise Wilcoxon tests; no letters indicate no significant differences.

To monitor the bacteria over time in the phyllosphere, wheat leaf colonisation was monitored during two weeks for the same strains and additionally for *P. protegens* PF and *Pseudomonas* sp. L14 (Fig. 8). One day post inoculation, the leaf isolates *Pseudomonas* sp. L7, L18, L14 and L18 persisted significantly better on wheat leaves than the root isolate PCLRT03 and the wheat leaf isolate PF. L14 was also significantly different to the root isolate TM1A3, and L18 to TM1A3 and the root isolate CHA0. However, values from 1 dpi need to be compared with care since the detection limit of the reference strains was 10x higher than for the new strains at this time-point. At 7 and 14 dpi,

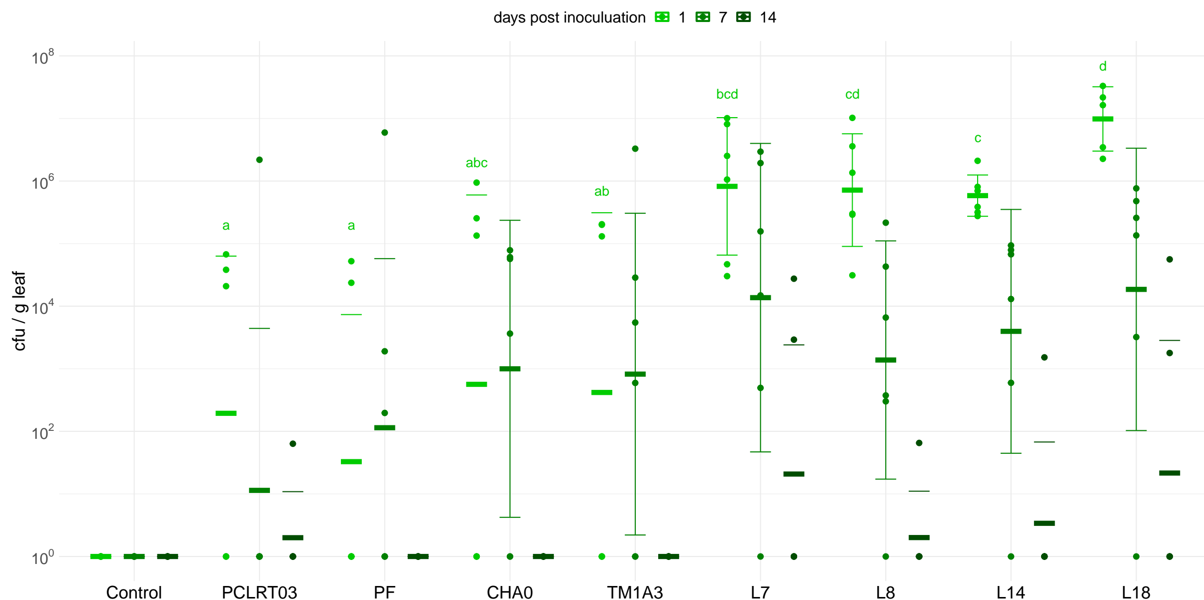

Fig. 8. **Colonisation of wheat leaves by fluorescent pseudomonads.**

Wheat leaf colonisation by *P. chlororaphis* PCLRT03, *P. protegens* PF, CHA0, *P. brassicacearum* TM1A3, *Pseudomonas* sp. L7, L8, L14 and L18. Each six leaves from different plants were sampled at 1, 7 and 14 dpi. Different letters above dots represent significant differences between treatments at the respective time-point ($P < 0.05$) according to Kruskal-Wallis and post-hoc Pairwise Wilcoxon tests; no letters indicate no significant differences.

no significant differences between the strains were detected.

L18 reached the highest wheat leaf colonisation on average with around 10^7 cfu/g at 1 dpi, 2 x 10^4 at 7 dpi and around 10^4 on the two out of six leaves on which it was still detected at 14 dpi. L7 showed similar persistence on plant leaves, and L8 and L14 colonised leaves in around 10 times lower numbers. PF, CHA0 and TM1A3 were found on 3-4 leaves at 7 dpi but not anymore at 14 dpi in contrast to the new leaf isolate for which in some of the samples bacteria could still be detected after 14 days. PCLRT03 was detected on one leaf each at 7 and 14 dpi. The supposed leaf isolate PF did not show a higher ability to colonise plant leaves than the root isolates, and a lower persistence than the new leaf isolates. Possibly, the phylogenetic background might play a more important role for phyllosphere persistence than the origin of isolation. For example, the origin of isolation (arthropods, soil or roots) did not influence the insecticidal activity of *P. protegens* and *P. chlororaphis* strains [32]. However, life in the phyllosphere requires distinct adaptations that are found among different species of phyllosphere strains [10]. Since we did not discover any report describing its isolation in detail we can only speculate that PF was maybe not resident in the phyllosphere but had invaded a wheat leaf at the sampling time-point by chance or that it has lost its phyllosphere adaptations in the three decades since its isolation.

Over all experiments, the root colonisation ability of the new isolates was comparable to the one of reference strains, whereas the new leaf isolates tendentiously showed an enhanced persistence in the phyllosphere. It is unclear which genetic traits underlie the better adaptation of the leaf isolates to the leaf environment. Adaptation to life in the phyllosphere usually includes protection from UV radiation [11]. The root strain *P. protegens* Pf-5, for example, was shown to be very sensitive to UV light in comparison to several phyllosphere isolates from different species [73]. Furthermore, the ability to form biofilms was important for disease suppression by *Bacillus* spp. in roots and on leaves [74, 75]. The cyclic lipopeptide surfactin, for example, contributes to disease suppression as an antimicrobial agent and through its role in biofilm formation [75]. Biofilm formation on leaves was also important for bacteria to protect themselves from dessication [10, 11]. The adaptation to survival in the phyllosphere of the leaf isolates likely includes an increased UV tolerance and enhanced potential to attach to and form biofilms on leaves, possibly including the production of cyclic lipopeptides.

P. protegens PF can inhibit *Z. tritici* reproduction *in planta*

Due to its strong inhibitory effect *in vitro*, *P. protegens* PF was chosen for an *in planta* assay against *Z. tritici* 3D7. Five days after the fungal infection, pictures were taken under a confocal fluorescence microscope. On all leaves with 3D7-mcherry infection, red fluorescing hyphae were growing on the leaf surface and penetrating the stomata as shown in Fig. 9c. On the plants with a second PF-gfp application two days before the imaging, patches of fluorescing bacteria were detected as visible in Fig. 9a. However, it was difficult to detect PF on the leaves where the inoculation took place six days ago. On one occasion, PF were colonising the hyphae and it looked like they were hindering Zt 3D7 from entering the stomata (Fig. 9b). However, the pictures can only confirm that wheat infection with Zt 3D7 was successful and that bacteria could persist for two days and at low numbers for six days on wheat leaves. 14 days post fungal infection, no significant differences in disease symptoms were discovered between treatments with and without PF application (Fig. 10). However, the leaf area covered with lesions was slightly increased when PF was applied twice. At 21 dpi, all leaves were completely covered with lesions and pycnidia had formed. In the treatment with two PF applications, the pycnidia density per leaf and per lesion were significantly reduced compared to 3D7 and 3D7 with one PF application. Two PF applications, one before and one after infection with Zt 3D7 spores could hamper fungal reproduction, whereas one application was not sufficient to exhibit a disease suppressive effect. It is remarkable that PF could inhibit the fungus despite its poor persistence in the wheat phyllosphere observed in a parallel experiment (Fig. 8). The strong inhibitory effect of PF on *Z. tritici in vitro* seemed to translate into a weak inhibitory effect *in planta* when PF was applied twice. Possibly, strains with strong inhibitory effects and low phyllosphere persistence could also be

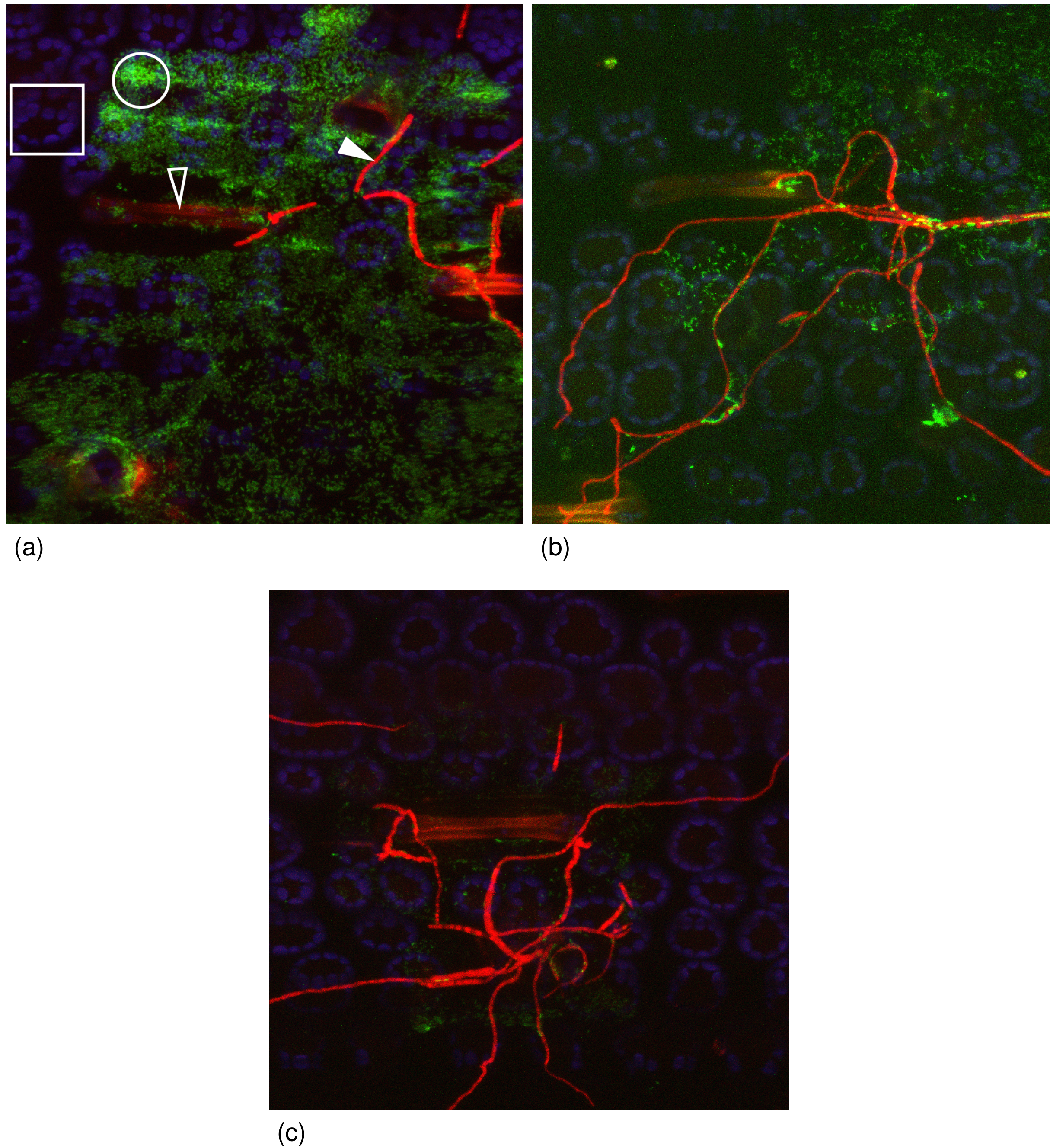

Fig. 9. **Wheat leaves infected with *Z. tritici* 3D7-mcherry and inoculated with *P. protegens* PF-gfp.**

Pictures a, b and c show each a part of a wheat leaf (approx. 0.04 x 0.055 mm) infected with *Z. tritici* 3D7-mcherry (glowing red, filled triangle) and inoculated twice with *P. protegens* PF-gfp (glowing green, unfilled circle). The pictures were taken under a confocal fluorescence microscope 5 days after the fungal infection, and 6 and 2 days after the first and second bacteria application. The blue structures are chloroplasts (unfilled rectangle), and stomata are visible as broad faint red structures (unfilled triangle).

effective for foliar disease control if applied frequently. However, PF might evanesce even faster with exposure to sunlight and its persistence might be insufficient to exhibit a biocontrol effect under field conditions.

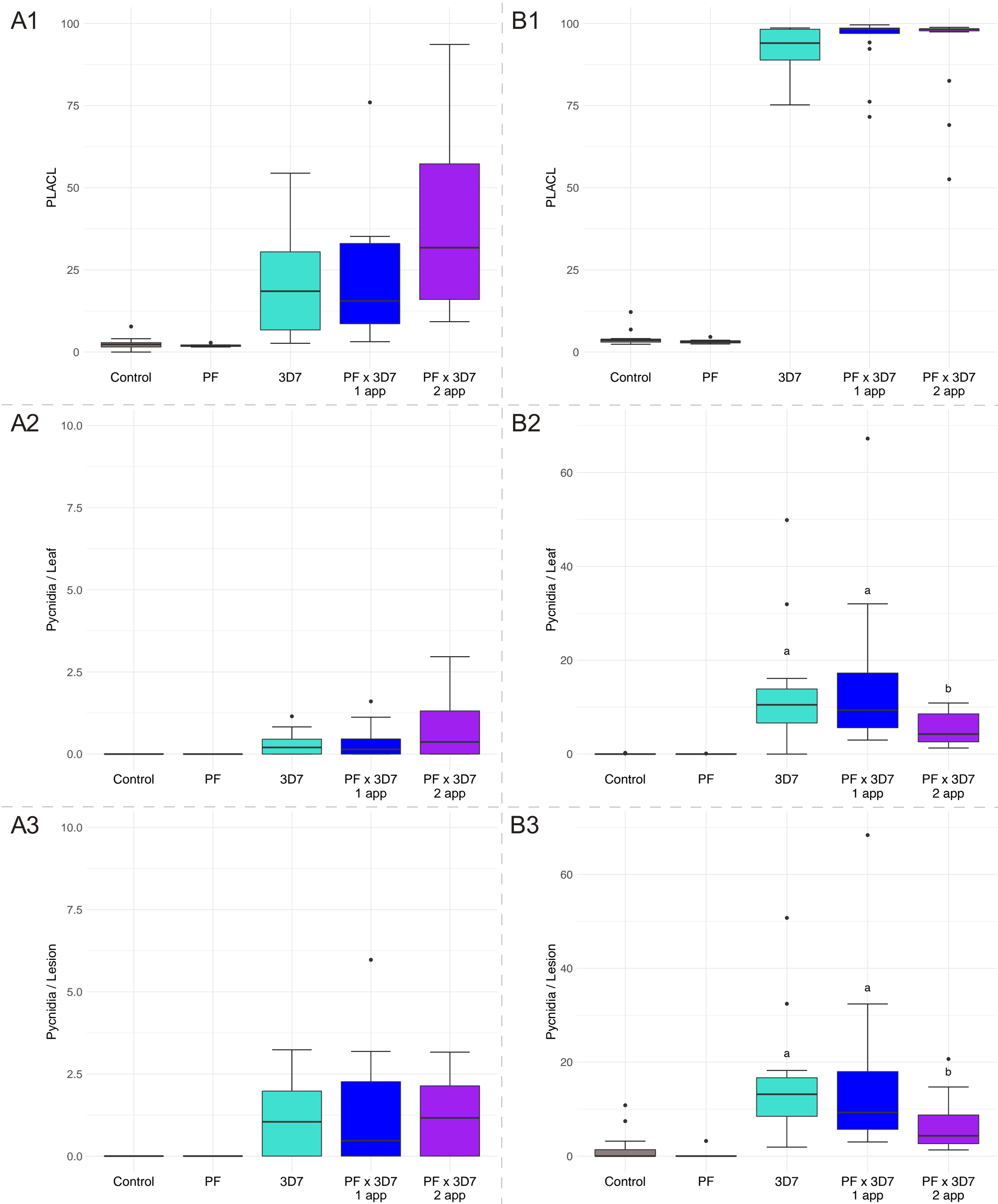

Fig. 10. **Inhibition of *Z. tritici* 3D7 pycnidia formation by *P. protegens* PF *in planta*.**

Z. tritici 3D7 disease progression in percentage of leaf area covered by lesions (PLACL, 1), pycnidia per leaf (2) and pycnidia per lesion (3) at 14 dpi (A; n=12 leaves) and 21 dpi (B; n=16). Treatments: Control = mock inoculation, PF = application of *P. protegens* PF-gfp and mock inoculation, 3D7 = inoculation with *Z. tritici* 3D7-mcherry, PF x 3D7 1 app = one PF application 1 day before infection with 3D7, PF x 3D7 = two PF applications, 1 day before and 3 days after infection with 3D7. Different letters above boxplots represent significant differences between treatments ($P < 0.05$) with 3D7 inoculation according to a Kruskal-Wallis and post-hoc Pairwise Wilcoxon test; no letters represent no significant differences.

Potential of pseudomonads to control foliar pathogens and pests

In order to control foliar plant diseases, pseudomonads must possess activity against the targeted pests and pathogens and persist in the phyllosphere long enough to be effective against the target. The reference strains used in this chapter, namely *P. protegens* CHA0 and PF, *P. chlororaphis* PCLRT03 and *P. brassicacearum* TM1A3, did not persist for long in the phyllosphere. However, PF was able to inhibit *Z. tritici* pycnidia production *in planta* after two applications even though the strain probably did not persist on the leaves until pycnidia formation since it was not detected on wheat leaves at 14 dpi in an experiment run in parallel (Figs. 8, 10). Despite the potent oral insecticidal and antifungal activity shown in this thesis and in previous studies [26, 29, 32], the poor persistence in the phyllosphere challenges their utility as biocontrol agents against foliar diseases. The new leaf isolates *Pseudomonas* sp. L7, L8 and L18 are closely related to *Pseudomonas* strains with described plant beneficial properties, yet *Pseudomonas* sp. L14 is closely related to a plant pathogenic strain. L7, L8, L14 and L18 showed insecticidal activity upon injection and feeding, were able to inhibit *P. ultimum in vivo* and could persist on wheat leaves for up to two weeks. However, their disease suppressive abilities were generally lower than for the reference strains. To determine the biocontrol potential of each strain, their performance in the different assays will be discussed in detail.

Among the new leaf isolates, L14 caused the highest mortality and exhibited the fastest killing speed against *P. xylostella*, thereby being almost as lethal as CHA0. However, any plant pathogenic activity needs to be excluded for using L14 in plant protection since it is closely related to plant pathogenic *P. salomonii* [45, 46]. During the wheat leaf colonisation assay, the effect of the bacteria on the plants was not assessed, yet no obvious disease symptoms were recorded for wheat leaves inoculated with L14. In the *P. ultimum* assay in the treatment without infection, a lower shoot fresh weight was recorded after soil inoculation with L14 compared to the other leaf isolates, yet not lower than for CHA0 and PCL1391 treatments. Furthermore, L14 provided, together with L7, the lowest level of protection against *P. ultimum*. L7 also exhibited potent oral insecticidal activity and was with L18 among the isolates that persisted best in the wheat phyllosphere. In the *P. ultimum* assay, L7 had a clear plant growth stimulating effect on cucumber in absence of the pathogen, but was among the weakest strains in term of disease suppression. L8 and L18 showed weak oral insecticidal activity against *P. xylostella* and a similar plant-protective effect than PCL1391 in the *P. ultimum* assay. L18 could colonise Chinese cabbage leaves in significantly higher densities than any other tested strain (PCLRT03, CHA0, TM1A3, L7, L8) after one day, yet its radish root colonisation values showed a much higher variation than the other strains. On wheat leaves, L18 also showed the most consistent and highest colonisation after one day,

and persisted well for two weeks. L8 showed a similar wheat colonisation ability after one day yet numbers decreased more after one and two weeks compared to the other leaf isolates. Overall, L7 and L14 are most promising for biological control of insect pests, while L8 and L18 are more promising against pathogens. L18 is most promising for its persistence in the phyllosphere, though L7, L8 and L14 also persist better in the phyllosphere than the reference strains used in the respective experiments. It would be interesting to test L18 against *Z. tritici*, although the strain did not display any *in vitro* activity. In order to estimate the biocontrol potential of these new leaf isolates, more experiments are needed. Especially *in planta* experiments against foliar fungal pathogens and insect pests should be performed in a next step. Furthermore, several other of the new leaf isolates are closely related to strains with plant beneficial properties and might also be interesting to explore for their biocontrol potential.

In order to efficiently identify strains that can control foliar pathogens or pests, suitable screening approaches need to be chosen. In this chapter, assays that are already established in this lab were performed. The *G. mellonella* and *P. xylostella* assays were effective to screen for insecticidal activity, but not the *P. brassicae* assay mainly due to a recently emerged low susceptibility to *Pseudomonas* infection. In a next step, *in planta P. xylostella* assays could be performed to see whether the strains L7 and L14 exhibit oral insecticidal activity when applied on plant leaves. In such an assay, larval mortality and bacterial persistence on the leaves should be monitored, similar to the *P. brassicae in planta* assay conducted prior to isolation of pseudomonads from radish leaves (Fig. S3). The *in vitro* screenings for antifungal activity did not reveal any effect, yet all screened strains showed a plant protective effect against a root pathogen. This contrast was also observed for other biocontrol agents including fluorescent pseudomonads [62, 76]. Furthermore, the outcomes can vary largely depending on the medium used since pseudomonads do not produce all secondary metabolites on each medium [77]. The *Z. tritici in planta* assay revealed an effect of *P. protegens* PF on pycnidia production, yet the assay is not suitable for screening multiple strains at once. A detached leaf assay might be a suitable approach to screen strains for their antifungal activity, for example by infecting wheat leaves on a water agar plate with *Z. tritici* and subsequently adding bacteria droplets. Such an assay would be much closer to nature than an *in vitro* assay, yet the effort is much lower compared to a whole plant assay. But, compared to plant assays, the plant immune response as well as plant growth promotion or induced systemic resistance potentially triggered by bacterial strains cannot play a role in detached leaf assays. It is yet unclear how much such mechanisms contribute to the plant protective effect against *P. ultimum* exhibited by the new leaf isolates. If they relied largely on systemic mechanisms, a detached leaf assay might not prove more useful for screening for antifungal activity than *in vitro* assays. If isolates with antifungal ac-

tivity are identified in detached leaf assays, the next step would be to perform *in planta* assays. In the *Z. tritici in planta* assay, an effect was only observed when bacteria were applied a second time after fungal infection. Therefore, different application timings and intervals would need to be tested in greenhouse trials to discover an efficient application scheme.

A distinct approach for biocontrol of foliar diseases with pseudomonads might be to apply consortia of *Pseudomonas* strains. Hu et al. [78, 79] chose eight DAPG-producing pseudomonads, namely five *P. fluorescens*, two *P. protegens* (CHA0 and Pf-5) and one *P. brassicacearum* strain, and tested single strains and communities of two, four and eight strains *in vitro* and *in vivo* for plant growth promotion and disease suppression. When growing tomato plants in natural soil, bacterial density in the rhizosphere decreased less over time with increased community richness and colonisation was highest for the eight-strain community [78, 79]. The increased persistence of the eight-strain community in the rhizosphere led to an almost complete suppression of bacterial wilt incidence and *Ralstonia solanacearum* density after 35 days [79]. The eight-strain community also promoted plant growth the most, yet some two- and four-strain communities expressed similar effects [78]. Similarly, consortia of leaf isolates and reference strains could be applied together on plant leaves to control pests and diseases. The higher persistence of the leaf isolates in the phyllosphere might also allow the reference strains to persist better on the leaf, which would enhance their biocontrol activity. In a strain mixture a variety of different antifungal and insecticidal compounds would be produced, which might further increase efficacy. However, pseudomonads also possess weapons for competition with closely related strains, e.g. type VI secretion systems or tailocins as shown for *P. protegens* CHA0 [80, 81]. Therefore, inhibitory effects must be excluded for successful application of consortia. To test the compatibility of a consortium, its members should be monitored after co-application *in planta* using strain or subgroup-specific approaches. This, of course, demands a much higher effort than *in vitro* compatibility assays, but any *in vitro* inhibition that would likely be observed does not necessarily translate into (out)competition under more natural conditions *in planta*. Furthermore, even if a specific strain was not able to persist for long when applied in a consortium, it might still have a beneficial effect on the performance of the consortium. Hu et al. [79] concluded that the diversity rather than the inclusion of a specific strain was important for increased root colonisation and disease suppression. Therefore, we would suggest to test the use of consortia that include phylogenetically diverse strains with good phyllosphere colonisation abilities and with biocontrol activities based on different traits.

Conclusion

The goal of this chapter was to discover pseudomonads that persist well in the phyllosphere and can suppress foliar pathogens and pests. So far, mainly *P. protegens* and *P. chlororaphis* strains were described to control both pathogens and pests, yet the tested *P. protegens* and *P. chlororaphis* strains persisted poorly on leaves. We discovered four leaf isolates from the *P. fluorescens* subgroup with enhanced phyllosphere persistence, similar plant protective abilities and weak to potent oral insecticidal activity when compared to our reference strains. The high lethality of two leaf isolates is a novel finding, since in previous studies, *P. fluorescens* subgroup strains were found to have a significantly lower insecticidal activity compared to *P. protegens* and *P. chlororaphis* strains that contain the Fit insect toxin cluster [29, 53]. However, the application of fluorescent pseudomonads to control foliar pathogens and pests will remain challenging. The leaf isolates, on one hand, were not able to inhibit fungal pathogens *in vitro*, while disease-suppressive root strains, on the other hand, did not persist well in the phyllosphere. To overcome the limitations of both - root strains and leaf isolates - combined application as consortia should be considered. A *Pseudomonas* biocontrol consortium including the most promising leaf isolates as well as *P. protegens*, *P. chlororaphis* and *P. brassicacearum* strains might have an increased persistence in the phyllosphere and the ability to control foliar pathogens and pests. Nevertheless, one main obstacle will be to reach sufficient phyllosphere colonisation to achieve a biocontrol effect under field conditions. However, the observed suppression of a foliar pathogen by a *P. protegens* strain under greenhouse conditions despite its rather poor phyllosphere persistence is promising. Insufficient survival on leaves could be compensated by frequent applications of a *Pseudomonas* biocontrol consortium during critical crop stages. Our findings indicate that it is well worth to continue research on fluorescent pseudomonads for controlling foliar pests and diseases.

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Supplementary Material and Methods

N1	T1	P1	F1	m1
P2	m2	N2	T2	F2
T3	F3	m3	P3	N3
F4	N4	T4	m4	P4
m5	P5	F5	N5	T5

Fig. S1. **Scheme of the leaf sampling from five control plots in a field trial.**

In the field trial described in Chapter 2, EPP strain *P. chlororaphis* PCLRT03 (P), EPN population *S. feltiae* RS5 (N) and EPF strain *M. brunneum* Bip5 (F) were applied as soil drench inoculation alone and in a triple combination (T). In the control (m), no biocontrol agents were applied. The field was split into 5 x 5 plots and treatments distributed according to a Latin square design. Each plot contained 8 rows of radish *Raphanus sativus* L. cultivar 'Andes F1' plants. Biocontrol agents were applied in the inner rectangle representing 4 plant rows of 5 m length (5 x 1.6 m). From the outer rectangle of each control plot (m1-m5), 2 samples consisting of 3 leaves from different plants were taken (s1 & s2).

Media Recipes

King's B medium (KB)

KB medium [82] contains 20 g proteose peptone, 8.4 ml glycerol 87%, 1.5 g $MgSO_4$ x 7 H_2O, 1.5 g K_2HPO_4 x 3 H_2O and 13 g agar bacteriology grade per liter ddH_2O.
KB^{+++} is supplemented with cycloheximide (100 mg/l), chloramphenicol (13 mg/l) and ampicillin (40 mg/l).
KB^{++G} is supplemented with cycloheximide (100 mg/l), chloramphenicol (13 mg/l) and gentamycin (10 mg/l).

Lysogeny Broth (LB)

LB [83] contains 10 g Bacto tryptone, 5 g Bacto yeast extract, 0.25 g $MgSO_4$ x 7 H_2O and 8 g NaCl per liter ddH_2O.

Yeast Sucrose Broth (YSB)

YSB contains 10 g sucrose and 10 g Bacto yeast extract per liter ddH_2O.

Potato Dextrose Agar (PDA)

PDA consists of 39 g BD Difco PDA per liter ddH_2O.

1/2 Potato Dextrose Broth (PDB)

1/2 PDB contains 12 g BD Difco PDB per liter ddH_2O.

Table S1. **Primers used for characterising and sequencing *Pseudomonas* leaf isolates.**

Target	Primer	Sequence (5' to 3')	Length	Anneal.	Ref.
Pseudomonas spp. 16S rRNA	Pse435F	ACT TTA AGT TGG GAG GAA GGG	250	60 °C	[84]
	Pse686R	ACA CAG GAA ATT CCA CCA CCC			
FitD	FitD_66F_DEG	CTA TCG GGT SCA GTT CAT CA	240	60 °C	[57]
	FitD_308R_DEG	TTC TTG TCG GSA AAC CAC T			
P. protegens (Pprot)	DGPf_4F	CGC TGA TCC TCT CGT TGT CTC TGC	1072	64 °C	[85]
	DGPf_4R	ACG CCC TTG TCC ACA TCG			
P. chlororaphis (Pchlor)	DGPf_8F	CCC ACC GAC AGC CAG CAA CG	661	67 °C	[85]
	DGPf_8R	CGG TCT TGT CGC TGA TGC CG			
16S rRNA (sequenc.)	16F27	AGA GTT TGA TCM TGGCTC AG	1465	55 °C	[86]
	16R1492	TAC GGY TAC CTTGTT ACG ACT T			
gyrB	M13R	CAG GAA ACA GCT ATG ACC	966	60 °C	[87]
	M13(-21)	TGT AAA CGA CGG CCA GT			
rpoB	LAPS5	TGG CCG AGA ACC AGT TCC GCG T	1229	68 °C	[88]
	LAPS27	CGG CTT CGT CCA GCT TGT TCA G			
rpoD	PsEG30F	ATY GAA ATC GCC AAR CG	760	55 °C	[89]
	PsEG790R	CGG TTG ATK TCC TTG A			
gyrB	gyrBf	TTC AGC TGG GAC ATC CTG GCC AA	586	65 °C	[90]
	gyrBr2	TCG ATC ATC TTG CCG ACR ACC A			
rpoB	rpoBf1	CAG TTC ATG GAC CAG AAC AAC CCG CT	508	60 °C	[90]
	rpoBr1	CCC ATC AAC GCA CGG TTG GCG TC			
rpoD	rpoDf	ACT TCC CTG GCA CGG TTG ACC A	695	60 °C	[90]
	*rpoD*r	TCG ACA TGC GAC GGT TGA TGT C			

This table displays all primer pairs used in this chapter. Target = species or gene targeted by primer pair. Primer and Sequence = names and sequence of forward and reverse primer. Length = PCR product length in bp. Anneal. = Annealing temperature in PCR reaction. Ref. = reference from which primer pair was obtained from.

Table S2. **PCR reagents.**

Sequencing		Colony PCR	
Component	µl	Component	µl
DNA (5 ng/µl)	2	Template[1]	2.5
dNTP's (2mM)	2.5	dNTP's (2nM)	0.625
Primer F (10 µl)	2	Primer F (10 µl)	0.625
Primer R (10 µl)	2	Primer R (10 µl)	0.625
GC Buffer 5x	10	DreamTaq Buffer 10x	1.25
DMSO 100%	1.5		
Phusion Polymerase	0.4	DreamTaq Polymerase (5 U/µl)	0.175
miliQ water	29.6	miliQ water	6.7
Total volume	50	Total volume	12.5

[1]LB liquid overnight cultures diluted by 1:10 in LB served as template

Table S3. **PCR conditions.**

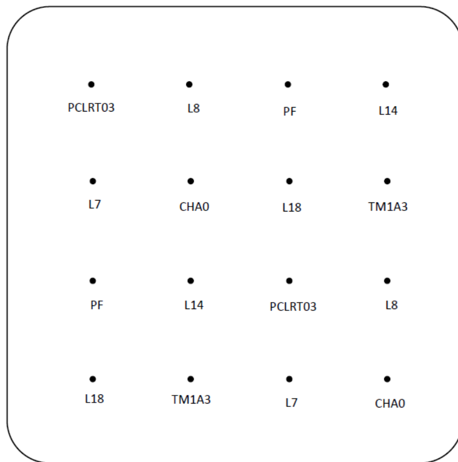
		Sequencing		Colony PCR	
Phase	Step	°C	Time	°C	Time
Pre-Denaturation	1	98	3 mins	94	4 mins
Denaturation	2	98	10 s	94	30 s
Annealing	3	*	15 s	*	1 min
Elongation	4	72	45 s	72	90 s
Final Elongation	5	72	5 mins	72	5 mins
Pause	6	14	∞	14	∞
Cycles	2-4	30x		35x	

* annealing temperature varies according to Table S1.

Table S4. **List of selected fluorescent *Pseudomonas* spp. leaf isolates.**

Isolate	Plot	Sample	Colony	16S	FitD	Pprot	Pchlor
L1	m1	s1	c9	p	n	n	n?
L2	m2	s1	c1	p	p?	n?	n
L3	m2	s2	c1	p	n	n	n
L4	m3	s1	c1	p	n	n	n
L5	m3	s1	c2	p	n?	n?	n
L6	m3	s1	c6	p	p?	p?	n
L7	m3	s1	c7	p	p?	p?	n
L8	m3	s2	c4	p	n	n	p?
L9	m3	s2	c7	p	n	n?	n
L10	m4	s1	c4	p	n	n?	n
L11	m4	s1	c5	p	n	n	n
L12	m4	s1	c6	p	n	n	n
L13	m4	s1	c7	p	n	n	n
L14	m4	s2	c5	p	n	p?	n
L15	m4	s2	c8	p	n	n?	n
L16	m5	s1	c5	p	p?	n	n?
L17	m5	s1	c8	p	n	n	p?
L18	m5	s2	c11	p?	n	n?	n?

This table displays information about the 18 *Pseudomonas* isolates that were selected for DNA extraction, housekeeping gene sequencing and further characterisation. A selection of it was used in the experiments presented in this chapter.
Isolate = lab name; Plot, Sample (consisting of three leaves) & Colony refer to the sampling origin of each new isolate; 16S, FitD, Pprot and Pchlor refer to the different primer pairs with which PCRs were performed (Table S1); Gel electrophoresis results are displayed as following: p = band present at expected height, positive result; n = no band at expected height, negative result; p? = faint band around expected height (unclear result); n? = faint smear around expected height.



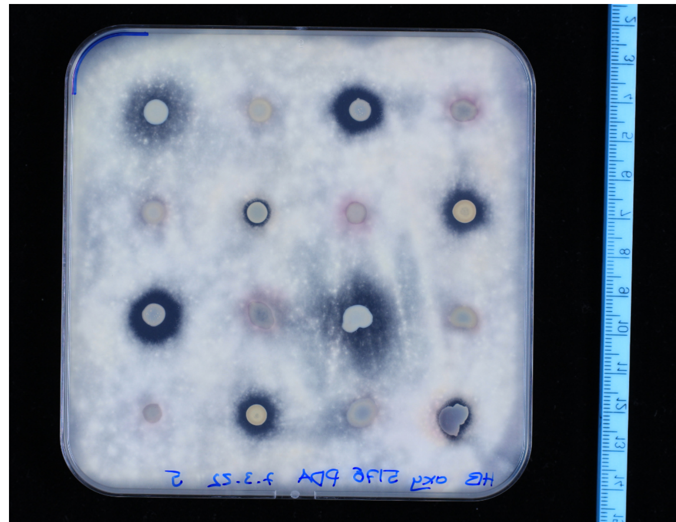


Fig. S2. **Inhibition of *F. oxysporum* by fluorescent *Pseudomonas* bacteria *in vitro*.**

The inhibition of *F. oxysporum* Fol 4287 and Fo 5176 and *Z. tritici* Zt 3D1 and Zt 3D7 by *P. chlororaphis* PCLRT03, *P. protegens* PF, CHA0, *P. brassicacearum* TM1A3, *Pseudomonas* sp. L7, L8, L14 and L18 was assessed *in vitro*. On the left, the scheme shows how bacteria droplets were added on PDA plates. Prior to that, fungal spores were spread evenly on the plate. On the right, an example picture shows the inhibition of Fo 5176 at 3 dpi in the second experiment.

Supplementary Results

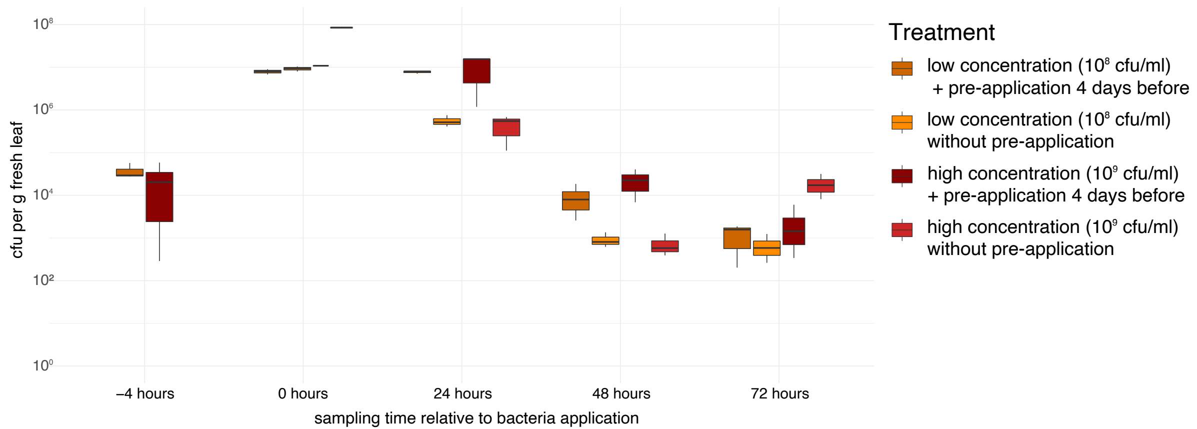


Fig. S3. **Colonisation of Chinese cabbage leaves by *P. chlororaphis* PCLRT03.**

The figure shows Chinese cabbage *Brassica rapa* subsp. *pekinensis* leaf colonisation by *P. chlororaphis* PCLRT03. The horizontal axis shows the time point at which the treated leaves of Chinese cabbage were analysed for PCLRT03 colonisation. For inoculation, leaves were sprayed with 10^8 cfu/ml and 10^9 cfu/ml PCLRT03-gfp until runoff at 0 hours. For treatments with pre-application, 10^8 cfu/ml PCLRT03-mturq were applied 4 days before inoculation with PCLRT03-gfp.

In parallel to the colonisation assessment, *P. brassicae* larvae were placed on Chinese cabbage plants to feed *ad libidum*. Mortality did not exceed 15% for any treatment after 3 days. 3 days after inoculation, 6 larvae per treatment were analysed for bacteria colonisation. Around 2-4 x 10^3 cfu/larva were detected across treatments.

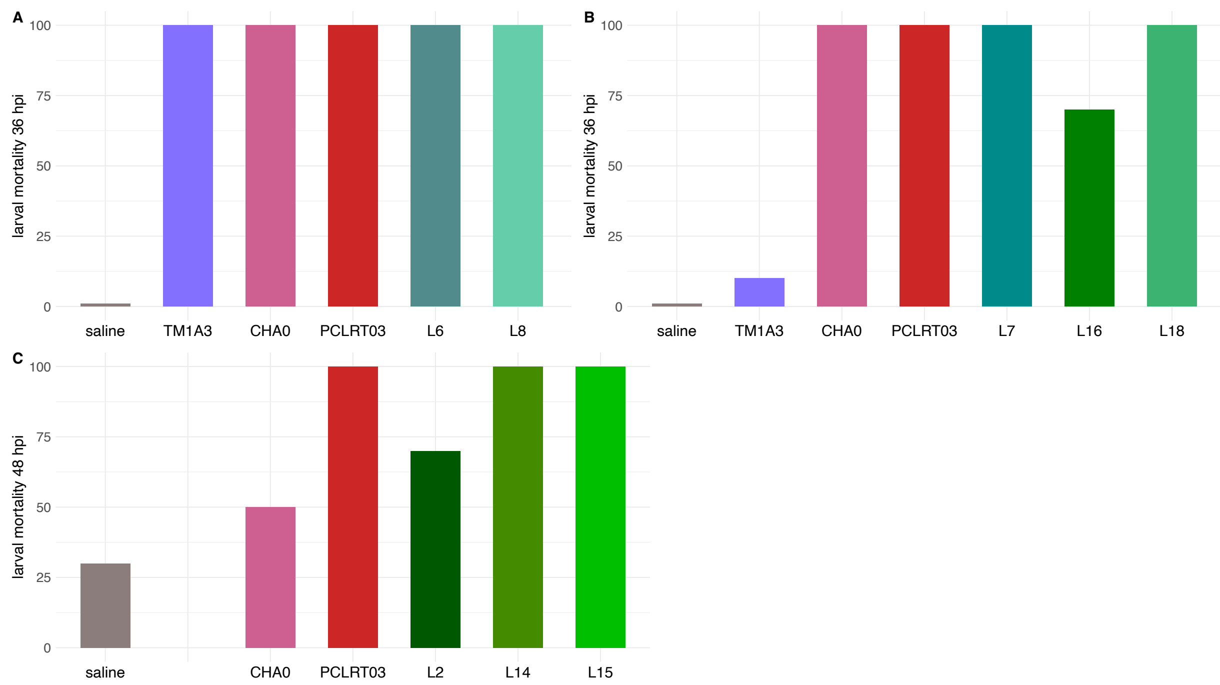


Fig. S4. **Mortality of *G. mellonella* larvae after injection with fluorescent pseudomonads.**

The figure shows mortality of *G. mellonella* larvae after injection with 0.9% NaCl (saline), *P. brassicacearum* TM1A3, *P. protegens* CHA0, *P. chlororaphis* PCLRT03, *Pseudomonas* sp. L2, L6, L7, L8, L14, L15, L16 and L18 at 36 (A, B) or 48 (C) hours past infection (hpi).

Chapter 5

General Discussion

5.1. A holistic *Pseudomonas* biocontrol concept – wishful thinking or practicable?

The entomopathogenic bacterium (EPB) *Bacillus thuringiensis* (Bt) is the most widely applied biocontrol agent (BCA), yet more often transgenic crops that express its Cry toxins are planted [1]. The wide-spread use especially of Bt-corn led to pest populations with resistance to Cry toxins [2]. As an alternative to Bt-corn, DuPont expressed a novel insecticidal protein discovered in a *P. chlororaphis* strain, designated IPD072Aa, in corn plants [3]. This toxin is orally active against the Western corn rootworm *Diabrotica virgifera virgifera* – also against Bt-resistant insects – but does not affect non-target Lepidopteran and hemipteran species. On one hand, this illustrates the potential of pseudomonads for insect control, but it does not solve the problem that resistant pests emerge when one toxin is frequently applied over a large range and over time. Besides that, the *P. protegens/P. chlororaphis* Fit toxin does not display oral toxicity against insects [4]. Moreover, transgenic crop plants are largely banned for cultivation in Europe. Additionally, fluorescent pseudomonads are well known for producing antimicrobial substances and suppressing plant pathogens. This leads us to propose the application of entomopathogenic pseudomonads (EPP) as bacterial non-Bt biocontrol agent for dual biocontrol of pests and pathogens.

One major aim of this thesis was to investigate whether entomopathogenic pseudomonads are suitable to control insect pests. In previous studies, EPP from the subgroups *P. protegens* and *P. chlororaphis* were shown to cause mortality upon ingestion in Lepidoptera (*Plutella xylostella, Pieris brassicae, Galleria mellonella, Spodoptera littoralis, Lymantria dispar dispar, Helicoverpa armigera, Heliothis virescens*), Diptera (*Drosophila melanogaster, Musca domestica, Lucilia caesar*), Hemiptera and Coleoptera (*Diabrotica virgifera virgifera, Diabrotica barberi*) [3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13]. Both EPP species can target a range of insect species, yet there are large differences in lethality.

In a lab study, we observed that *P. protegens* CHA0 can persist in wireworms upon oral uptake for several weeks but it did not induce mortality (unpublished results). Overall, leaf-feeding Lepidopteran pests seem to be more susceptible to *P. protegens* infection than root-feeding Dipteran or Coleopteran pests [14]. This seems to be similar for *P. chlororaphis*: In laboratory assays, *D. balteata* larvae were less susceptible to *P. chlororaphis* infection than *P. brassicae* larvae (chapter 3). In *D. balteata*, *P. chlororaphis* PCLRT03 significantly increased mortality compared to the control by about 20% in only one out of four repetitions, and this was similar to the result by Bruno et al. [15]. Vesga et al. [16] even suggested that EPP are commensal to Coleopteran insects and only become pathogenic if they can access the haemolymph. This poses two challenges for EPP insect control: Can EPP control below-ground pests, or are root-feeders generally tolerant to EPP infection? Could rhizosphere-competent EPP strains be suitable to control above-ground pests, e.g. susceptible Lepidoptera?

In this thesis, we showed that *P. chlororaphis* strain PCLRT03 can effectively control the cabbage maggot *Delia radicum* in laboratory, greenhouse and semi-field assays as well as in a field trial (chapter 2). There were large differences in efficacy between the two EPP species but also between strains. In the screening assays, all tested *P. chlororaphis* strains significantly reduced fly emergence by more than 50%, while *P. protegens* strains reduced fly emergence by around 30%, which was statistically not significant (chapter 2). In the greenhouse, *P. chlororaphis* PCL1391, PCLAR03 and *P. protegens* CHA0 insignificantly reduced fly emergence rate, while only PCLRT03 was significantly different to the control (unpublished results). This suggests that *P. chlororaphis* has the potential to control Dipteran below-ground insect pests but that strain selection is very important. For Coleoptera, strain selection might be even more important. Vesga et al. [16] discovered that two *P. chlororaphis* strains isolated from healthy Coleoptera had delayed insecticidal activity against *P. xylostella*. On the other hand, DuPont discovered a specific toxin against the Coleopteran pest *D. virgifera* in a *P. chlororaphis* strain [3]. Furthermore, in field trials using well-described *P. protegens* and *P. chlororaphis* strains, one each, these EPP reduced *D. virgifera* damage, larval survival or increased yield [17]. Possibly, suitable *P. chlororaphis* candidate strains for controlling Coleopteran pests could be identified when testing different strains against a target pest. One could try to isolate strains from deceased Coleopteran larvae found in the field or from the rhizosphere and the soil in fields with a high pest pressure.

For selecting a suitable biocontrol strain against below-ground pests, insecticidal activity against the target pest seems to be the most important factor. For controlling above-ground Lepidopteran pests, phyllosphere persistence might be a more important screening criterion, since in the study of Vesga et al. [16] all tested *P. protegens* and *P. chlororaphis* strains, with the exception of two Coleoptera isolates, displayed very similar

activity against leaf-feeding *P. xylostella* larvae. However, we have observed that EPP persist poorly in the phyllosphere under greenhouse conditions (chapter 4). We hoped to isolate EPP strains from the *P. protegens* and *P. chlororaphis* subgroups from radish leaves and discovered two *P. fluorescens* subgroup strains with insecticidal activity and elevated persistence on leaves (chapter 4). Yet there is evidence that *P. chlororaphis* are common on wheat leaves (Luzia Stalder, personal communication). Therefore, the chances are high that EPP strains that show an adequate phyllosphere persistence can be isolated from leaves. Even though root isolates likely cannot be used directly to control leaf-feeding pests due to their bad phyllosphere persistence, they might still impact leaf-feeders. For example, *P. lactis* SS101 and *P. simiae* WCS417 induced systemic resistance and increased plant resistance against leaf-feeding insect pests [18, 19]. However, Löser et al. [20, 21] did not find any decrease in herbivory for a selection of fluorescent pseudomonads but one strain repelled a predator. Such effects depend heavily upon host plant, pseudomonad and pathogen or pest [22], which is challenging to exploit in augmentative biocontrol, where the goal is that one product can be applied on different plants and ideally also against different pests. Therefore, for developing *Pseudomonas* products for foliar application, the emphasis should lay on isolating EPP from the phyllosphere and select them based on their phyllosphere competence and their insecticidal activity against leaf-feeding pests.

For successful disease control, EPP must establish at sufficient densities on the plant organ under attack. Haas and Défago [22] established a threshold of 10^5 cfu/g for biocontrol activity against soil-borne pathogens. In multiple greenhouse, three semi-field and one field trial, PCLRT03 colonised radish roots at 10^6-10^7 cfu/g upon two inoculations, which was sufficient for reducing the pest and its damage (chapter 2, unpublished results). *P. xylostella* larvae were fed on pellets inoculated with 10^6 cfu and this led to 90-100% mortality within two (*P. protegens*) to three (*P. chlororaphis*) days [8, 16]. Force-feeding *G. mellonella* larvae with 10^6 cfu of *P. protegens* CHA0 resulted in approx. 50% mortality within one week (Maria Zwyssig, personal communication). *P. brassicae* larvae were fed with approx. 10^7 cfu on a Chinese cabbage leaf (chapter 3) or 5 x 10^6 cfu on an artificial food pellet [11], which resulted in 50-80% mortality within four days. It can only be speculated that the threshold for pest control is higher than for disease control. For disease control, the whole bacteria population is effective, while only a small part is taken up by plant-feeding pests. Furthermore, the pseudomonads encounter a hostile environment in the insect gut and only a small amount reach the hemolymph, where they express the Fit insect toxin [23]. Therefore, the higher the root colonisation and the more insects eat, the higher the chances that pseudomonads can overcome the gut barrier and the insect's immune system to kill it. Depending on the feeding behaviour of the target pest, colonisation densities of 10^6 cfu/g could be sufficient to have

a potent oral insecticidal effect. The *P. chlororaphis* products on the European market are applied as seed-dressing [24]. For commercialisation, it needs to be tested how well PCLRT03 can establish in the rhizosphere upon seed treatment. Likely, a second application 2-3 weeks after planting is necessary to reach sufficiently high colonisation densities. For bacterial blight disease control on apple flowers, *P. fluorescens* needs to be sprayed weekly during blossom [25]. For controlling foliar pests, insecticidal pseudomonads must likely also be applied frequently. The leaf isolates described in this thesis were found at 10^6-10^7 cfu/g on wheat leaves after one day, and colonisation decreased to 10^3-10^4 cfu/g after one week (chapter 4). The strain that persisted best was found at 10^5-10^6 cfu/g on four out of six leaves after one week. However, the persistence in the field and in a formulation with UV protectants will be different than in the greenhouse upon aqueous spray applications.

The strain *P. chlororaphis* PCLRT03 was isolated from potato roots in 2017 and showed insecticidal activity against *P. xylostella, P. brassicae, D. radicum*, could colonise insects to high densities, established well on roots and suppressed the soil-borne pathogen *P. ultimum* and the foliar pathogen *Z. tritici* (chapter 2, 3, 4, [16]). These results and especially its efficiency in controlling *D. radicum* under field conditions imply that it might be a suitable candidate for commercialisation. The major challenge - besides the additional field trials to prove its efficacy against target pests and pathogens - is the arduous registration process mentioned in the introduction. The European and Swiss regulatory instances demand an increasing amount of tests that are not useful to establish the safety of a species and a specific strain. To render PCLRT03 safe for use, it is important to establish that it does not have significant impacts on non-target species, that no large amounts of secondary metabolites remain on the produce and that it cannot infect humans. Non-target effects are very unlikely to occur since EPP are part of the natural soil and root microbiome [26]. Furthermore, it was shown that EPP do not harm neither bumblebees nor a parasitoid [27, 28]. To determine the amount of toxic substances that humans would potentially consume, the produce, e.g. the radish, could be harvested and analysed for the toxins and antimicrobial substances PCLRT03 is known to produce. Measuring the amount of toxins produced after growing in nutrient-rich liquid medium for 16 h might be easier, yet it does not allow for any estimation how much might reach a consumer. The insecticidal Fit toxin, however, is only expressed inside the insect and not on plant roots [29, 23]. A simple test to exclude any pathogenic behaviour in humans is to assess whether a strain can grow at human body temperature, i.e. at 37 °C [30]. In a simple KB agar plate growth assay, PCLRT03 was heavily reduced in growth at 33 °C and could not grow at 37 °C (+/- 1 °C).

Taking together more recent findings on insecticidal activity with decades of research on pathogen suppression, entomopathogenic pseudomonads are promising biocontrol

agents for dual pest and disease control. Their versatile life-style and their ability to adapt to different environments renders the discovery of EPP strains with good persistence in the phyllosphere or with activity against Coleopteran pests likely. Yet, as for most BCA, pseudomonads are susceptible to the varying environmental conditions which might render their field performance variable. One way to overcome this problem is to build consortia either of different *Pseudomonas* strains or to combine EPP with other insecticidal BCA. In this project, we combined EPP with entomopathogenic nematodes and fungi to increase efficacy and stability.

5.2. Combining pseudomonads with entomopathogenic fungi and nematodes for increased efficacy and reliability

A second major aim of this thesis was to investigate the biocontrol potential of a consortium of EPP, EPF and EPN strains. We had two hypotheses: first, the consortium increases the efficacy i.e. the consortium increases insect mortality or decreases plant damage better than the single BCA; second, the consortium increases the stability, i.e. the consortium reduces the pest or the damage consistently over repetitions, while single BCA show variable effects. In *D. radicum* screening assays, combinations of EPP with EPN or EPF resulted in synergistic effects and were more stable over repetitions (chapter 2, unpublished results). In the greenhouse experiments, the double combinations also seemed to be slightly more stable over repetitions, but no increase in efficacy was observed (chapter 2). In one semi-field trial, the EPP-EPN-combination increased efficacy, while the triple combination in the field trial was as efficient as the EPP alone, and the stability cannot be assessed due to a lack of repetitions (chapter 2). In the laboratory assays with *P. brassicae* and *D. balteata*, the triple combination was both more stable and more efficient in killing larvae (chapter 3). Taken together, consortia seem to increase efficacy and stability under laboratory conditions. Under more natural conditions, consortia can possibly increase efficacy if the environmental conditions are not favourable for all applied BCA as it was observed in the semi-field trial. We have indications that the stability of a consortium could be increased under field conditions, but we do not have any data to support that conclusion. One additional major benefit of the triple combination could be its increased target range. Different single BCA and double combinations were the most effective in controlling either *D. radicum*, *P. brassicae* or *D. balteata* (chapters 2, 3). However, the triple combination had a great effect on *D. radicum* survival and damage (chapter 2), and killed most *P. brassicae* and *D. balteata* larvae the fastest (chapter 3). The triple consortium might be applied against a wide range of below-ground pests and, due to the EPP, could possibly also suppress soil-borne pathogens. However, more research and especially more field trials are needed to show the efficacy, stability and host range of our BCA triple consortium.

To allow for uptake of a consortia-based strategy by farmers, an easy application scheme and cheap product(s) would need to be developed [31]. Ideally, all three BCA would be formulated in one product that is applied once and gives protection throughout the whole cropping season. However, looking at current application techniques of EPP, EPF and EPN as well as results from *in vitro* inhibition assays, this does not seem feasible. EPF are commonly applied by mixing fungus-colonised barley kernels (FCBK) into the field during tillage [32]. Pseudomonads, e.g. Cerall or Proradix, are often applied as seed dressing or soil drench inoculation and can be bought as liquid suspension or as powder [24]. EPN are usually formulated as powder that is dissolved in water for spray application or through the irrigation system [33]. From the results of our field trial (chapter 2), the BCA could be applied as follows: EPF as fungus-colonised barley kernels at tillage, EPP as seed dressing at sowing, and 2-3 weeks after sowing a combined EPN-EPP application. Even though the different application techniques are more effort than one combined application, they are well compatible with standard farming practices. From our observations in the lab, a combined EPN-EPP application in a tank mixture should be fine or even the development of a combined product might be possible. In trials in our group, both EPN and EPP survived well when formulated together in alginate beads (Pascale Flury, Daniela Schönholzer, personal communication). For EPF, barley kernel formulations are unsuitable for combined application, while liquid (e.g. blastospores) or bead formulations could be combined with EPP and/or EPN. However, any inhibitory effects would need to be excluded for combined applications and combined products. The development and registration of a combined product might be very challenging, so pursuing combined applications might be easier. The frequency and timing of application depend on the pest pressure and environmental conditions [30]. For EPP, it is very important to apply them at least twice to enable them to establish well on the roots which is essential for controlling insect pests. In our field trial, EPN and EPF were applied once and significantly reduced *D. radicum* damage compared to the control, yet were significantly less effective than the EPP that were applied twice (chapter 2). Therefore, it might also enhance efficacy of EPF and EPN to apply them twice or more, especially for crops with longer culture duration. Given that all three BCA are available as products and recommendations for application schemes have been developed, the costs and the benefits of applying a consortium are critical for its uptake. Field vegetables are often high value specialty crops with limited availability of pesticides, while for arable crops, a range of pesticides is available for crop protection throughout the season [31]. Biocontrol solutions are therefore more attractive for vegetable production, but might also gain importance for arable crops the less pesticides are available. The costs for buying three or four BCA products are likely to be higher than for pesticide sprays, depending on the pest and the necessary applications. If the consortium provides reliable control of important below-ground pests and soil-borne pathogens of

different crops, farmers might be willing to apply it.

In this thesis, we gained important knowledge on the interaction between the three studied BCA. First, we showed the compatibility between BCA in combined applications against an important below-ground pest (chapter 2) and second, we studied their interaction during co-infections in two insect pests (chapter 3). We can only speculate how often co-infections occur when all three BCA are applied in the same field. For co-infections to occur, an insect must get in touch with EPF spores, feed on roots colonised by EPP, and be detected and hunted by EPN infective juveniles. If all three BCA are abundant, chances are high that co-infections occur frequently. However, EPN have been shown to avoid cadavers that are already infected by EPF [34]. In this case, EPP-EPF and EPP-EPN co-infections probably happen more often in the field than EPF-EPN or triple co-infections. It is unclear how this influences the overall efficacy of a combined application: we do not know whether the synergistic effects in the *D. radicum* screening are due to co-infections or because two BCA would infect more larvae (chapter 2) and we do not know how much the increased killing speed and mortality in the *P. brassicae* and *D. balteata* would boost biocontrol efficacy (chapter 3). We hypothesise that chances are higher that a larvae is infected by at least one BCA in combined applications where a higher abundance of BCA is present, and that the insect's defence system surrenders faster and in more cases if attacked by two or three BCA. Furthermore, an insect cadaver in the field attracts saprophytes and scavengers, and it is likely that two or three BCA can defend a cadaver better than a single BCA since a larger array of antimicrobials is produced. In co-infections, either EPF or EPN win the cadaver for reproduction and, in rare cases, both reproduce on the same cadaver [35]. It is unclear how the presence of EPP influences the reproductive success of EPF and EPN. We have hints but no hard proof that EPF can sporulate with EPP present (chapter 3). We have seen EPN reproduction in the presence of EPP, yet did not assess the fitness of the offspring or whether EPP were associated with the IJ (chapter 3). The recent studies on the natural association of EPP with EPN open up further research questions [12, 36]. Are EPP involved in EPN pathogenesis, as Ogier et al. [36] suggest? Can EPN carry EPP to new hosts after a co-infection? If yes, where are the EPP located? How do EPP and NB interact if they were both inside the IJ? In our group, we are also addressing these questions and have first indications that infective juveniles can carry EPP to a new host after co-infections (Maria Zwyssig & Tabea Patt, personal communication). From previous studies, we know that EPP use insects for dispersal [10], so we could speculate that they use EPN to reach new hosts.

For identifying a consortium of three BCA that are compatible and effective in controlling a target pest, it was important to study the interaction of the BCA under different conditions. The screening in the radish bulb sand system allowed to identify strains with high

D. radicum killing potential and also to study first interactions of EPP with EPN and EPF (chapter 2). The greenhouse was a very important step in the upscaling to the field trial even though it was challenging to achieve a decent and stable fly emergence rate in the negative control treatment. The interactions between BCA turned out to be different at the different test levels. The synergistic effects observed under screening and semi-field conditions were neither observed in the greenhouse nor in the field trial. Overall, this step-wise approach led to the identification of a consortium that is compatible and can decrease *D. radicum* survival and damage. Furthermore, the same consortium was also highly effective in killing *P. brassicae* and *D. balteata* larvae and there, faster and deadlier than the single BCA (chapter 3). This leads us to speculate that the same consortium might be effective on several crops against several pests. Yet it is possible that a consortium might be more effective against other pests when replacing one member with another species or strain, or when building up an entirely new consortium of EPP, EPF and EPN. Possibly, BCA consortia comprising EPP, EPF and EPN could be built using different species and strains according to the target pest. However, other species or strains might interact differently in a consortium. In the screening assay, the tested *B. bassiana* strain was compatible with *P. chlororaphis* PCL1391 (chapter 2) and fluorescent pseudomonads have successfully been combined with *B. bassiana* [37]. Similarly, co-infections of *B. bassiana* and *S. feltiae* were shown to result in additive and synergistic effects [38]. Furthermore, results from our group suggest that *P. protegens* are compatible with *S. feltiae* in *G. mellonella* co-infections (Maria Zwyssig, personal communication). Even though these BCA seem to be generally compatible, for each consortium the compatibility and the efficacy of the respective strains needs to be assessed. Studying the interactions in such depth as done in this thesis is not feasible for frequently building new consortia of EPF, EPN and EPP against different pests. From my experience gathered during this thesis, I suggest the following procedure: 1) identifying the most potent strain against a target pest using literature and in a screening approach, 2) testing the compatibility of the most potent strains in laboratory co-infection assays, 3) performing greenhouse assays against the target pest while monitoring BCA survival in the rhizosphere and the soil, 4) performing field trials, best by using the same inoculation methods as for commercially available products. Hopefully, this approach can be used to select BCA consortia against different insect pests.

5.3. Contribution of biocontrol to a sustainable world food system

In this section, I want to place this thesis – the BeneComb project (short for beneficial combinations) – and biocontrol into a bigger context. The World Food System needs to undergo many changes to feed the world and reduce damage to the environment via

e.g. greenhouse gas emissions, biodiversity loss and soil degradation. It is frequently stated that global food production needs to double by 2050 to feed the increasing world population [39]. Such statements are often used to legitimate the promotion of unsustainable practices like the use of synthetic fertilisers and pesticides and to devalue organic agriculture, mainly with the argumentation that in comparison with conventional farming there is an approximately 20% yield gap [40, 41, 42]. However, the impact of agriculture on the environment and the climate also needs to be taken into account. On one hand, intense high-input systems must become more ecological while keeping stable yields or with little yield decreases. On the other hand, low-output systems need to sustainably increase yields. For some time now, the concept of 'ecological intensification' or 'sustainable intensification' is used. It refers to increasing agricultural outputs while reducing external inputs by using natural functionalities that ecosystems offer [43, 44]. Another concept promoting transitions to more sustainable food systems, which is applicable to all agricultural systems, is agroecology. The FAO also promotes agroecology as a holistic approach that uses ecological and social concepts to develop more sustainable farming and food systems [45]. The transition towards sustainable food production is a huge challenge for farmers and society, which requires a holistic transition within a relatively short period [46]. One challenge among the many are adopting crop protection measures apart from synthetic pesticides. In this respect, the eight principles of integrated pest management (IPM) should be considered because they match well with sustainable food production [47]. Biological control is one of the main non-chemical disease control methods in IPM [47].

The implementation of biological control varies largely among crops, countries and farming systems. At the moment, Europe and North America are the largest markets for commercial biocontrol products, while the most growing markets are South America and Asia [48]. However, synthetic pesticides cannot simply be exchanged by biopesticides, and the interrelation between plant beneficials and crops, farming practices, environmental conditions and other products also needs to be considered [46]. Therefore, biological control, together with ecological farming practices, should be implemented as part of IPM and as part of sustainable intensification or of agroecological developments. Conservation biocontrol matches well with measures to increase biodiversity on farmland, e.g. wildflower strips. These measures increase the abundance of natural antagonists of plant pests and diseases, but it is challenging to selectively increase the abundance of beneficial soil microbes by cultural measures. In a long-term Swiss field trial with the aim to monitor differences between conventional, organic, and biodynamic farming systems (DOK trial), the microbial biomass, richness and activity was increased in organic farming compared to conventional plots with exclusively mineral fertilisation [49]. However, Jaffuel et al. [50] found that EPN abundance was overall very low and

was not influenced by the farming system in the DOK trial. Similarly, Schneider et al. [51] observed that EPF community structure was not significantly influenced by the farming system in the same trial. Dennert et al. [52] observed in a Swiss farming system and tillage experiment that a specific group of pseudomonads with antifungal activity was enriched in conventionally farmed plots with tillage compared to organically farmed plots with reduced tillage. Interestingly, the opposite was found for soil disease suppressiveness, which was higher in the organic compared to the conventional plots. Even though the farming system had an impact on the *Pseudomonas* community, there was no correlation between the abundance of *Pseudomonas* antimicrobial-producing strains and disease suppressiveness. With increasing research and knowledge on soil and root microbiomes it was suggested to engineer the microbiome by applying synthetic communities (SynComs) of keystone taxa or by breeding plants that select for a beneficial microbiome [53, 54, 55]. These approaches are very interesting, but much further research on the interaction between different soil types, plants and their microbiomes is needed in order to exploit root microbiome functions target-oriented or to define selection criteria for plant breeding. The application of well-described biocontrol agents, i.e. augmentative biocontrol, and of plant-growth promoting microorganisms as biofertilisers are at the moment the most straight-forward and feasible approaches to promoting plant health and growth based on beneficial soil microbes.

Plant beneficials have a range of activities that can be exploited. The biocontrol abilities especially of entomopathogenic pseudomonads, nematodes and fungi were already extensively discussed in this thesis. Fluorescent pseudomonads are also used as biofertilisers for their plant-growth promoting abilities and frequently, new beneficial properties are discovered. Several fluorescent pseudomonads including certain *P. fluorescens* and *P. putida* strains were shown to alleviate drought stress [56, 57, 58, 59]. *P. putida* as well as mycorrhizal fungi were observed to augment salt stress tolerance [60, 61]. A consortium of *Pseudomonas* sp. and *Curtobacterium* sp. could alleviate cold stress in plants [62]. O'Callaghan et al. [63] describe that soil microbial inoculants can be used for biofertilisation (N fixation, phosphate solubilisation), plant growth promotion, protection against soil-borne pathogens and below-ground pests, improving abiotic stress tolerance of crops, bioremediation, greenhouse gas mitigation and the enhancement of soil characteristics (e.g. soil structure, water retention). However, the efficacy of plant beneficials depends on the environmental conditions and soil factors they encounter in the field [63]. Moreover, the choice of beneficials should be adapted to the prevailing conditions in the field. For example, pseudomonads which promote plant-growth via phosphate solubilisation do not enhance plant growth when plants encounter sufficient amounts of available phosphate and do not depend on microorganisms to solubilise phosphate [64, 65]. Therefore, it is important to know under which conditions

beneficials are effective and useful. O'Callaghan et al. [63] propose the use of microbial consortia to enhance establishment and efficacy of beneficials yet identify the selection of compatible consortium members as major challenge. According to the findings of this thesis, the compatibility of consortia can be assessed in standard laboratory assays. However, the efficacy of the beneficials and, later, the behaviour of consortia in the field still needs thorough investigation because lab and greenhouse observations do not necessarily translate into field efficacy [63]. Ideally, a toolbox should be established containing well-described beneficials that are compatible with each other and that can be used under different conditions i.e. in different soils, climatic conditions and on different crops and for different purposes, e.g. biofertilisation, protection against different diseases and pests, and improvement of stress tolerance. From this toolbox, consortia could be built and individually adapted to farmer's needs. The use of well designed consortia could greatly contribute to ecological intensification and agroecological approaches.

5.4. Conclusion and Outlook

This thesis represents one of the first attempts to apply insecticidal pseudomonads for biocontrol of insect pests. We could show that a *P. chlororaphis* strain can suppress the Dipteran root pest *D. radicum* under four different conditions, including a field trial. This strain was compatible with entomopathogenic nematodes and fungi for pest control and the consortium was more efficient in killing a Lepidopteran and a Coleopteran pest insect in laboratory assays than the single agents. Furthermore, *P. fluorescens* subgroup strains isolated from leaves showed potent insecticidal activity and persisted well on leaves. In summary, these findings suggest that insecticidal pseudomonads can be applied to fight below- and above-ground pests and diseases, alone and in combination with entomopathogenic nematodes and fungi (Fig. 1). However, as any research project, it opened up at least as many research question as it answered. In the last decade, an amazing amount of factors that contribute to the insecticidal activity were described, yet little is known about the ecology of *Pseudomonas*-insect-interactions. An ongoing project in our group uses an experimental evolution to study the adaptation of *P. protegens* to an insect host (Maria Zwyssig, personal communication). Understanding the role insects play for pseudomonads e.g. as means of dispersal, or as alternative hosts and nutrient source and the conditions determining pathogenic or commensal relationships is important for developing successful biocontrol strategies. Understanding the interactions between pseudomonads, nematodes and fungi in the soil and in insect hosts is also important for combined applications in biocontrol. This thesis has outlined their basic compatibility for joint application, but interesting new research questions have emerged as discussed above. Regarding biocontrol, one main question is:

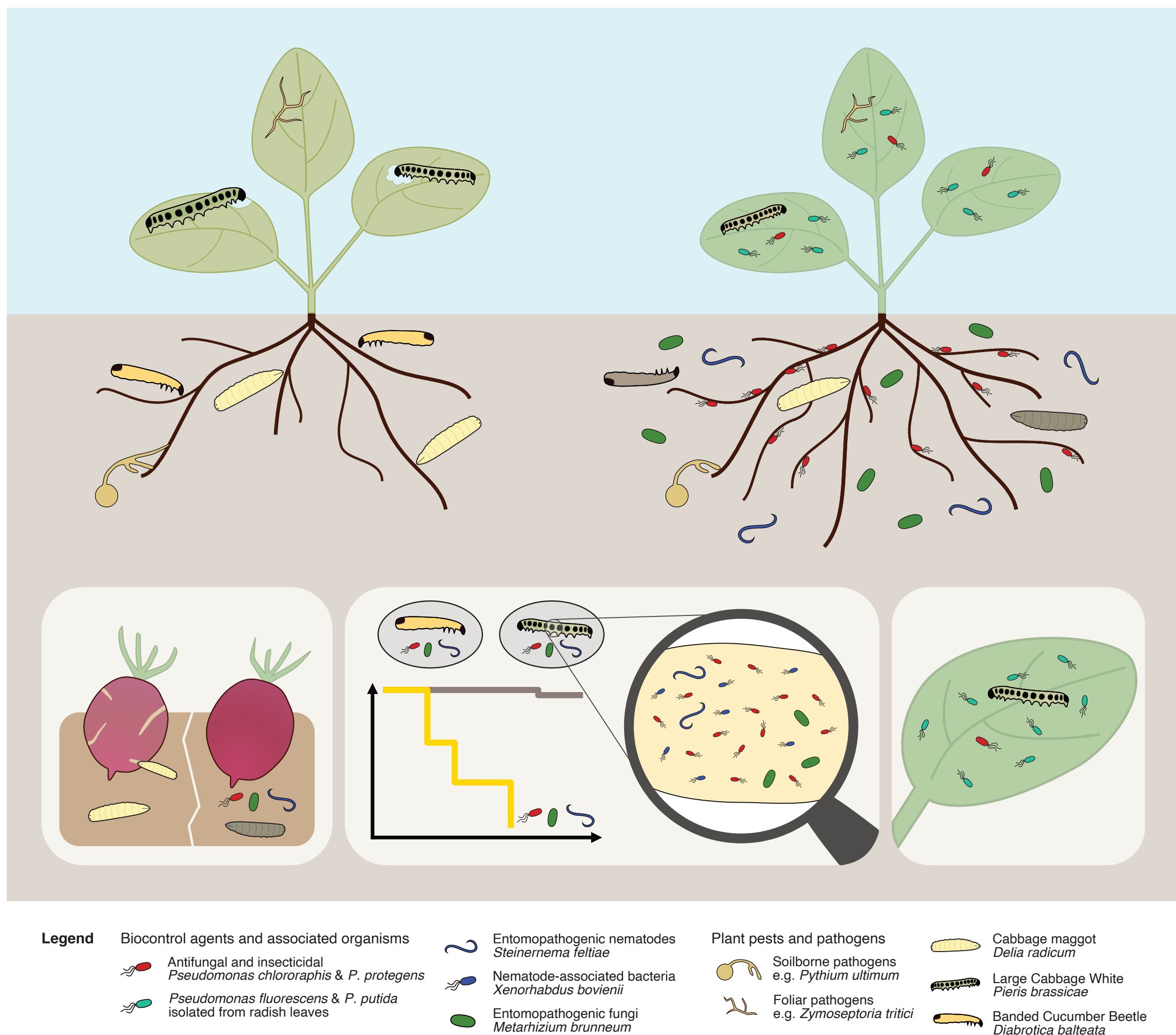


Fig. 1. **Beneficial Combinations protect plants from pests and pathogens.**

Plants are attacked by insect pests and microbial pathogens as shown on the upper left. Pests and pathogens can damage leaves, roots and/or the produce and thereby reduce food production. Under ideal conditions, the different biocontrol agents studied in this thesis protect plants from below-ground pests, soil-borne pathogens, above-ground pests and foliar pathogens as depicted at the right side. In Chapter 2 (lower left), a combination of *Pseudomonas chlororaphis*, *Metarhizium brunneum* and *Steinernema feltiae* protected radish plants from *Delia radicum* damage. In Chapter 3 (lower middle), this consortium was highly efficient in killing *Pieris brassicae* and *Diabrotica balteata* larvae and all three biocontrol agents together with the nematode-associated *Xenorhabdus bovienii* were detected inside single larvae. In chapter 4 (lower right), leaf isolates from the *P. fluorescens* subgroup showed potent oral insecticidal activity and plant protective abilities. Possibly, consortia of insecticidal fluorescent pseudomonads, entomopathogenic nematodes and fungi can protect plants from a wide range of pests and diseases.

How often do co-infections happen when the three agents are applied together in the field, and what are the implications for the efficacy of the consortium?

The results of this thesis strongly indicate that entomopathogenic pseudomonads could

successfully be used to control insect pests. Therefore, more applied research should gather further evidence to promote product development and registration. Unfortunately, there is a gap between university research and biocontrol companies, which adds to the little availability of *Pseudomonas* biocontrol products while a vast amount of research focuses on these versatile bacteria. Collaborations between university and applied research institutes are important to bring together knowledge about infection mechanisms with field performance. A further link towards commercialisation are joint projects by applied research institutes and companies funded by a third party (e.g. state offices for agriculture or innovation funds like Innosuisse). At the moment, several European Horizon 2020 research projects focus on biocontrol or integrated pest management, e.g. IPM Popillia (www.popillia.eu) and Excalibur (www.excaliburproject.eu). Such collaborations comprising of research institutes and companies hold great potential to promote biocontrol as whole as well as new biocontrol solutions. Alternatively, applied research institutes could develop products themselves and use the profit for further research. For example, the French and Swiss agricultural research institutes, INRAE and Agroscope, launched a grapevine breeding program to develop disease resistant varieties in order to produce wines without pesticide use and the returns from the soon-to-be-registered varieties will flow back into grapevine breeding [66, 67]. Based on all these considerations, a collaborative research project with different research institutes and industry partners should be sought to further study the consortium of three biocontrol agents developed in this thesis. In a first step, field trials at different field sites and over years need to be performed to further test its efficacy against the cabbage maggot. In these trials, different application schemes should be tested to optimise plant protection by the consortium. If the consortium can reliably and efficiently protect plants from cabbage maggot damage, product development and registration need to be tackled. Further research should then test the consortium against other below-ground pests in different crops. Hopefully, the consortium could finally be applied to protect a variety of crops from several below-ground pests. As van Lenteren et al. [48] stated on the development of biological control, there are 'plenty of new opportunities'. The challenge is to bring together basic and applied research, companies, legislation and farmers for optimal uptake of biocontrol solutions that are promising in lab and field trials.

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Scientific Contributions

Published Work

Daniel Ariza-Suarez, Beat Keller, **Anna Spescha**, Johan Steven Aparicio, Victor Mayor, Ana Elizabeth Portilla-Benavides, Hector Fabio Buendia, Juan Miguel Bueno, Bruno Studer, Bodo Raatz, 2022. Genetic analysis of resistance to bean leaf crumple virus identifies a candidate LRR-RLK gene. *The Plant Journal*. doi:10.1111/tpj.15810

Pamela Bruno, Carla Arce, Ricardo Machado, Gaia Besomi, **Anna Spescha**, Gaétan Glauser, Charlyne Jaccard, Betty Benrey, Ted Turlings, 2022. Sequestration of cucurbitacins from cucumber plants by *Diabrotica balteata* larvae provides little protection against biological control agents. *Journal of Pest Science*. doi:10.1007/s10340-022-01568-3

Anna Spescha, Joana Weibel, Lara Wyser, Michael Brunner, Mathias Hess Hermida, Aurélie Moix, Franziska Scheibler, Anouk Guyer, Raquel Campos-Herrera, Giselher Grabenweger, Monika Maurhofer, 2023. Combining entomopathogenic *Pseudomonas* bacteria, nematodes and fungi for biological control of a below-ground insect pest. *Agriculture, Ecosystems and Environment*. doi:10.1016/j.agee.2023.108414

Anna Spescha†, Maria Zwyssig†, Mathias Hess Hermida, Aurélie Moix, Pamela Bruno, Jürg Enkerli, Raquel Campos-Herrera, Giselher Grabenweger, Monika Maurhofer, 2023. When competitors join forces: Consortia of entomopathogenic microorganisms increase killing speed and mortality in leaf- and root-feeding insect hosts. *Microbial Ecology*. doi:10.1007/s00248-023-02191-0
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Conferences and Presentations

2022

1st International and 10th National Conference on Biocontrol in Agriculture and Natural Resources (05-06.2.2022, hybrid meeting, Ahvaz, Iran)

Oral presentation (Keynote Speaker): Biological Control: Fighting below ground insect pests with entomopathogenic *Pseudomonas* bacteria, nematodes and fungi.

Plant BioProTech (28-30.06.2022, Reims, France)

Oral presentation: Biological Control: Fighting below ground insect pests with entomopathogenic *Pseudomonas* bacteria, nematodes and fungi.
(accepted, not held due to Sars-CoViD-2 infection)

International Congress on Invertebrate Pathology and Microbial Control & 54th Annual Meeting of the Society of Invertebrate Pathology (01-04.08.2022, online, Nelson Mandela Bay, South Africa)

Oral presentation: Biological Control: Fighting below ground insect pests with entomopathogenic *Pseudomonas* bacteria, nematodes and fungi.

18th International Symposium on Microbial Ecology (ISME18, 14-19.08.2022, Lausanne, Switzerland)

Poster and Poster Pitch: Biological Control: Fighting below ground insect pests with entomopathogenic *Pseudomonas* bacteria, nematodes and fungi.

2021

Second International Congress of Biological Control (ICBC2) (26-30.04.2021, online, Davos, Switzerland)

Poster: Biological Control: Fighting below ground insect pests with entomopathogenic *Pseudomonas* bacteria, nematodes and fungi.

World Food System Center (WFSC) summer school (16.08.2021, Eschikon, Switzerland)

Workshop: Plant Pests vs. Biological Control Agents – insights into the BeneComb research project.

FOOD DAY @ ETH (WFSC Research Symposium, 05.11.2021, Zurich, Switzerland)

Poster: Biological Control: Fighting below ground insect pests with entomopathogenic *Pseudomonas* bacteria, nematodes and fungi.

Patterns in Nature and Plant Sciences (Plant Science Center (PSC) Symposium, 08.12.2021, online, Zurich, Switzerland)

Poster: Biological Control: Fighting below ground insect pests with entomopathogenic *Pseudomonas* bacteria, nematodes and fungi.

2020

WFSC Research Symposium (11.12.2020, online, Zürich, Switzerland)

Poster: Biological Control: Fighting below ground insect pests with entomopathogenic *Pseudomonas* bacteria, nematodes and fungi.

2019

International Congress on Invertebrate Pathology and Microbial Control & 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group 'Microbial and Nematode Control of Invertebrate Pests' (28.07-01.08.2019, Valencia, Spain)

Poster: Biological Control: Fighting below ground insect pests with *Pseudomonas* bacteria.

Herbsttagung der Schweizerischen Gesellschaft für Phytomedizin (12.09.2019, Fribourg, Switzerland)

Oral presentation: Biologische Schädlingsbekämpfung der kleinen Kohlfliege *Delia radicum* mit entomopathogenen Pseudomonaden, Pilzen und Nematoden

WFSC Research Symposium (31.10.2019, Zurich, Switzerland)

Poster: Biological Control: Fighting below ground insect pests with *Pseudomonas* bacteria.

Plant Response to Environment across Scales (PSC Symposium, 11.12.2019, Zurich, Switzerland)

Poster: Biological Control: Fighting below ground insect pests with *Pseudomonas* bacteria.

2018

WFSC Research Symposium (08.11.2018, Zurich, Switzerland)

Poster: Biological Control: Fighting below ground insect pests with *Pseudomonas* bacteria.

Breakthroughs in Plant Sciences (PSC Symposium, 05.12.2019, Zurich, Switzerland)

Poster: Biological Control: Fighting below ground insect pests with *Pseudomonas* bacteria.

Curriculum Vitae

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Born 6th January 1995
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2018 - 2022 Doctor of Science (PhD), ETH Zurich
Doctoral Program: Plant Sciences
Project: BeneComb - Biological control of soilborne insect pests using combinations of pseudomonads, nematodes, and fungi
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2016 - 2018 Master of Science in Agricultural Sciences, ETH Zurich
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Master Thesis: Generating herbicide tolerant cassava plants using CRISPR-Cas9
Supervisors: Christelle Chanez, Dr. Emily McCallum

2017 Master Internship (4 months)
International Center for Tropical Agriculture (CIAT), Cali, Colombia
Project: Virus resistance in common bean *Phaseolus vulgaris* L.
Supervisor: Dr. Bodo Raatz

2013 - 2016 Bachelor of Science in Agricultural Sciences, ETH Zurich
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Supervisor: Dr. Andrea Sánchez-Vallet

2013 Maturitätsabschluss (High School Diploma), Kantonsschule Zug
Matura Thesis: Graffiti - Kunst oder Vandalismus?

Political Engagement
2018 - 2021: Member of Cantonal Council of Zug (Kantonsrat Zug)
2017 - 2018: Member of City Council of Zug (Grosser Gemeinderat Zug)