Assessing the potential of combining entomopathogenic nematodes and entomopathogenic pseudomonads for the control of the cabbage root fly *Delia radicum*

Master‘s thesis

Michael Brunner, BSc.

Matr. Nr.: 01340880

UH 066 422 Masterstudium Phytomedizin

Supervisors:

Ao. Univ. Prof. Dr. Elisabeth Helene Koschier

Prof. Dr. Monika Maurhofer Bringolf

Anna Spescha, PhD student

Department of Crop Sciences - Institute of Plant Protection, University of life science Vienna

Department of Environmental System Science – Institute of Integrative Biology, ETH Zurich

October/2019

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Acknowledgement

Writing this thesis would not have been possible without the support and help of various people. First of all, and most important I would like to thank my direct supervisor Anna Spescha for helping me with the experimental design, my work in the laboratory and writing. While working with her, she was always very patient and supportive in any situation and working with her was always fun. Additionally to our work, I enjoyed the activities we did together, like climbing and kayaking, a lot which made my stay in Zurich very special. I'm very thankful that she was such a great supervisor and friend and I wish her all the best. I would also like to thank Prof. Monika Maurhofer for giving me the opportunity to do my master project at the Plant Pathology group in Zurich. Her advice on my experiments and writing was very helpful and supportive. Working with her was always fun and very interesting, no matter whether it was on the field or in the lab, I learned a lot from her.

I also would like to thank Prof. Elisabeth Koschier for giving me the chance to do my Master's thesis abroad as well as for correcting and examining the thesis. While I was abroad, she was very helpful with all the administrational tasks in Vienna.

I would not have been able to write this thesis without the help from my family and friends. I want to thank my parents and siblings Christina and Johannes for all their support, patience and help. Especially my mum was always there for me when I needed some help or motivation.

Last, I want to thank Anna for her support as well as Armin, Florence, my brother Johannes, Birgit and Jörg for hosting me while I was writing my thesis.

Abstract

Soilborne pest insects are a major threat to many crops. The damage they cause, leads to quality losses and secondary infections. The cabbage root fly *Delia radicum* is a soilborne pest insect which feeds on roots and bulbs of Brassicaceaen vegetables and arable crops. Current control measurements are either not efficient enough, pollute the environment or cause major concerns about their effect on human health. The use of biological control agents (BCAs) has the potential for control without having negative effects on the environment or human health. BCAs are already in use against many insect pests in the greenhouse or in the field and have shown sufficient control. Entomopathogenic nematodes (EPN) and entomopathogenic pseudomonads (EPP) are most promising for the control of soilborne pests like *D. radicum,* as their natural habitat is the soil. Different EPN species are already in use against soilborne pests (e.g. *Diabrotica virgifera*) and several products are available. Pseudomonads have been known long for their plant beneficial properties and the ability to ward off root pathogens. They are ubiquitous and versatile bacteria and perfectly adopted to colonize the roots and the rhizosphere of plants. Toxicity towards insects has been discovered just recently and they have shown to be toxic when taken up orally by insects. Toxicity seems to be even higher when injected directly into the hemolymph. These findings make them promising for the use as BCAs against soilborne pests.

One major problem of BCAs is their inconsistent performance and susceptibility to environmental influences. As they are living organisms their virulence depends strongly on optimal conditions. One strategy to overcome this problem is to combine two or more BCAs for the control of one pathogen or pest. This way the BCAs might deal with suboptimal environmental conditions successfully while their virulence stays high. Before combining BCAs mechanisms that might lead to antagonistic effects among them need to be uncovered and they must be evaluated for their compatibility. In this study, the compatibility of EPNs and EPPs as well as their potential for the control of the cabbage root fly *D. radicum* has been evaluated. Under greenhouse conditions, single EPP soil treatments of PCL1391 on radish plants, showed a tendency in reducing pupation rate and fly emergence. At the same time, PCL1391 and CHA0 were able to colonize bulbs of radish plants very well at concentrations about $1x10^5$cfu/g bulb. Treatments with five EPN strains under controlled conditions in the climate chamber showed inconsistent effects on pupation rate and fly emergence of *D. radicum*. Experiments with combinations of the EPN strain Sf enema and the EPP strain PCL1391 showed a strong tendency in reducing pupation rate and fly emergence more consistent than single BCA treatments. However, in both repetitions, efficiency of one single treatment was higher. *In vivo* experiments for the compatibility of EPN and EPP showed no antagonistic effects in mortality of *G. mellonella* larva. At the same time survival of EPN seems to be not affected by the presence of EPP when both are mixed *in vitro*.

Zusammenfassung

Bodenbürtige Schaderreger stellen eine große Bedrohung für viele Kulturpflanzen dar, Qualitätseinbußen und Sekundärinfektionen sind oftmals die Folge. Die kleine Kohlfliege *Delia radicum* ist ein bodenbürtiger Schädling, deren Larven an Wurzeln und Knollen von Kreuzblütlern frisst. Aktuelle Pflanzenschutzmaßnahmen sind entweder nicht effizient genug, belasten die Umwelt oder sind bedenklich für die menschliche Gesundheit. Biologische Schädlingsbekämpfungsmittel haben das Potenzial Schädlinge zu bekämpfen, ohne die Umwelt oder die menschliche Gesundheit zu belasten. Sie werden bereits gegen verschiedene Schädlinge im Gewächshaus und im Freiland eingesetzt und erzielen dabei gute Ergebnisse. Entomopathogene Nematoden und entomopathogene Pseudomenaden sind vielversprechend für die Bekämpfung bodenbürtiger Schädlinge wie *D. radicum*, da der Boden Ihr natürliches Habitat darstellt. Einige entomopathogene Nematoden werden bereits kommerziell gegen verschiedene Schädlinge eingesetzt (z.B. *Diabrotica virgifera*) und sind als Pflanzenschutzmittel verfügbar. Pseudomonaden sind schon lange für Ihre Wachstumsfördernden und Pflanzen schützenden Eigenschaften bekannt. Sie sind in der Umwelt allgegenwärtig vorkommende vielseitige Bakterien und damit sehr gut an viele Lebensräume, u.a. an die Rhizosphäre von Pflanzenwurzeln angepasst. Die entomopathogene Wirkung einiger Pseudomonaden wurde erst vor kurzem entdeckt, wodurch sie als vielversprechender Organismus zur Bekämpfung bodenbürtiger Schädlingen gelten.

Eine der größten Herausforderungen für biologische Schädlingsbekämpfungsmittel ist deren unbeständige Wirkung und Anfälligkeit für Umwelteinflüsse. Eine Möglichkeit diese verminderte Effektivität zu vermindern ist die Kombination von zwei oder mehreren Organismen zur Bekämpfung eines Schädlings. Um Organismen erfolgreich zu kombinieren ist es notwendig, ihre Kompatibilität im Detail zu erforschen. In der vorliegenden Studie wurde die Kompatibilität verschiedener Stämme von entomopathogenen Nematoden und Pseudomonaden, sowie deren Potenzial als Schädlingsbekämpfungsmittel gegen die kleine Kohlfliege untersucht. Der entomopathogene Stamm *P. chlororaphis* PCL1391 zeigte im Gewächshaus an Radieschen eine gute Wirkung. Gleichzeitig waren *P. chlororaphis* PCL1391 und *P. protegens* CHA0 fähig, Knollen von Radieschen in Konzentrationen von 10^5cfu/g zu kolonisieren. Experimente mit *Heterorhabditis bacteriophora* und *Steinernema feltiae* zeigten, dass Nematoden die Fähigkeit besitzen, Larven der kleinen Kohlfliege zu befallen. Ihre Wirkung auf die Entwicklung von *D. radicum* Larven wurde in einem Klimakammerexperiment evaluiert, wobei die Entwicklung inhibiert wurde. Experimente mit Kombinationen von *S. feltiae* Sf enema and *P. chlororaphis* PCL1391 inhibierten die Entwicklung von *D. radicum* Larven konsistenter als einzelne Nematoden oder *Pseudomonas* Applikationen. *In vivo* Experimente zeigten keinen antagonistischen Effekt zwischen entomopathogenen Nematoden und Pseudomonaden. In vitro Versuche lassen vermuten, dass das Überleben von entomopathogenen Nematoden nicht durch die Präsenz von Pseudomonaden beeinträchtigt wird

Abbreviations

ANOVA	Analysis of variance
BCA	Biological control agent
cfu	Colony forming unit
ddH_2O	Deionized water
EPF	Entomopathogenic fungi
EPN	Entomopathogenic nematode
EPP	Entomopathogenic *Pseudomonas*
dpi	Days past infection
Fit	P. fluorescens insecticidal toxin
gfp	Green fluorescent protein
H_2O	Water
IJ	Infective juvenile
KB+++	Selective King's B media
KB++G	Selective King's B media containing Gentamicin
LB	Luria-Bertani broth
Log	Logarithm
Mcf	Makes caterpillars floppy
NaCl	Saline
OD_{600}	Optical density at wavelength 600nm
PCR	Polymerase chain reaction
PGPR	Plant growth-promoting rhizobacteria
sp.	Species (singular)
spp.	Species (plural)

1. Introduction

1.1. Soil borne insect pests - why are they so hard to control?

The soil is one of the most valuable resources for any plant. It provides water and nutrients that are essential for plant growth. With their roots, plants grow into the soil to take up nutrients and to stabilize the aboveground parts. Even though the soil seems to be a compact matter, it actually consists of many small particles between which spaces are formed. These spaces and an abundant supply of nutrients and organic matter provide habitats for a wide range of micro- and macro-organisms. Most of these organisms interact with plants. While some organisms are beneficial, others are pathogens or pests, trying to acquire nutrients from the plants. By definition, soilborne pathogens are residents in soil, either for an extended or a short period of their existence. They affect belowground plant parts directly and aboveground parts indirectly (Bruehl, 1987). For insect pests, the soil is a very attractive habitat, especially for the immature stages of many species. The soil protects them from natural enemies and environmental factors. Some larvae like wireworms spend up to two years in the soil to develop sheltered. Adult stages of these insect pests mostly live aboveground to be more mobile for spreading. Immature stages living in the soil, shift within their habitat but do not cover big distances. Finding a suitable host is a challenge for soilborne insect pests and often starts with choices made by oviposition behavior.

Studying the ecology and behavior of soilborne insect pests is crucial to develop improved management strategies. Cultural measures, antifeedants, host plant resistance and biological and chemical control agents all depend upon a knowledge of insect behavior (Villani and Wright, 1990). As insect pests are mobile in the soil, the mobility of control agents plays an important role in their effectiveness. Synthetic soil insecticides (e.g. carbamates, organophosphates, pyrethroids etc.) are only passively mobile and not replicable. Movement of target insects may put them out of the effective zone (Villani and Wright, 1990). Furthermore, the intensive use of pesticides is polluting the environment and supporting the development of resistances among target organisms. Exposure of humans and natural habitants to pesticides is becoming unacceptable. Therefore, new pest control methods need to be found (Goldman *et al.*, 1994).

An alternative control method is the use of biological control agents. Some of them like entomopathogenic fungi (e.g. *Metarhizium anisopliae*) can indeed only move passively but are at least replicable. By replication or growth, they can move towards their host. In contrast, predators and parasitoids (e.g. parasitoid wasps) have the advantage of being mobile and replicable (Villani and Wright, 1990). Antagonistic microorganisms which compete with or directly attack the pathogen are already in use against a wide range of pathogens and pest insects (e.g. *Bacillus thuringiensis*). However, microbial control agents are not widely used in agriculture because many were shown to be ineffective and unreliable under field conditions (Goldman *et al.*, 1994).

1.2. *Delia radicum*: an important pest on Brassicacean crops

In central Europe, the cabbage root fly (*Delia radicum*) is the most important insect pest in Brassicaceaen vegetable plants. At the same time, it has the biggest territorial distribution among Brassicacean pests (Crüger, 2002) and causes severe damage in quality and yield (Schmon *et al.*, 2018). Already very few larvae feeding on tubers can cause significant quality loss and make the product nonmarketable. Damage is often only detected at harvest which intensifies the problem. Plant protection is difficult because larvae live in the soil and feed on belowground parts of the plants like roots and tubers.

1.2.1. Biology of *D. radicum*

The cabbage root fly (*Delia radicum*) is an insect pest belonging to the Anthomyiidae, a subfamily of the Muscidae in the order Diptera. Several *Delia* species are pests on various crops like *D. platura* on cereals, *D. florilega* on onions or *D. floralis* on Brassicacean crops and vegetables. All of them except *D. platura* are polyphagous. Their immature stages damage roots and tubers of the host plants by feeding (Finch, 1989). *D. radicum* overwinters as a pupae and emerges usually in April, when temperatures reach above 14°C. The female flies find their host optically as well as olfactory and lie about one hundred eggs at the bottom of the plant's stem or in the soil close to it. The eggs itself are elongated, white and about 1 millimeter long (Picture 1). Eggs are very susceptible to drought and under field conditions only 10% develop to pupae (Crüger, 2002). After four to eight days, the white to yellow larvae hatch and move towards the roots. Larvae develop through three larval instars while feeding on roots or tubers (Andreassen, 2007). Depending on the temperature, the development takes three to six weeks. Right before they pupate, larvae measure about 9 millimeters in length. For pupation, they form a coarctated pupa for about two weeks. At soil temperatures above 22°C, pupae undergo aestivation, a resting stage that delays fly emergence and sometimes occurs in summer (Crüger, 2002). Adult flies feed on pollen and look alike the common fly but are a little bit smaller in size (Crüger, 2002). In Switzerland the cabbage root fly has three, sometimes four generations (Schmon *et al.*, 2018). Host plants are nearly all Brassicacean vegetables like cabbage, radish, cauliflower but also arable crops like canola. Some wild plants belonging to the cruciferous can also serve as hosts. Damage is highest in an intense Brassicacean crop rotation (Bedlan *et al.*, 1992).

Picture 1: D. radicum *larva feeding on radish bulbs (left) and eggs on radish (right).*

1.2.2. Damage caused by *D. radicum*

Recently hatched larvae start feeding on root hairs and small roots. As they develop, they feed on bigger roots, penetrate them and create furrows (Picture 1). Wounds make roots susceptible for

secondary infections and reduce quality of tubers or bulbs. Often, a few feeding larvae are enough to cause significant damage. Therefore, it is often necessary to control even small populations (Finch, 1989). On seedlings feeding on roots leads to yellowing and wilt of leaves. In the worst case, plants die. The first and second generations are the biggest threat. Up to 90% of seedlings are killed in favorable years by the insect in North America and Europe (Finch, 1989). In regions with an increasing canola cultivation, *D. radicum* has rapidly gained importance as a pest (Andreassen, 2007).

1.2.3. Current measurements against *D. radicum*

Larval stages of the cabbage root fly are very hard to control because they immediately migrate into soil after hatching. In the soil and while feeding in the host plant, larvae are protected from direct control measurements. Preventive, or indirect control measurements before oviposition, like tillage and crop rotation are most important for control. Exploitation of natural enemies can decrease the population of *D. radicum*. Various predators and parasitic wasps, like *Aleochara bilineata, Bembidion sp*. and *Phygadeuon* sp. occur when natural habitats are offered (Crüger, 2002). As direct control measurements, seeds or soil can be treated with insecticides before seeding, e.g. with different organophosphates. However, since only one active ingredient is available, *D. radicum* was already shown to develop resistance (Crüger, 2002). To maintain effectivity, increased doses could be used, but the resulting pollution of soil and environment is ecologically not acceptable (Goldman *et al.*, 1994). Furthermore, the use of insecticides affects natural predators negatively (Andreassen, 2007). For this reason, chemical biocontrol measures are limited. To date, “Spinosad” is the only available plant protection product allowed in organic production systems. In small fields, Brassicacean vegetables can be protected by nets mounted over the plants which prevent oviposition of adult flies (Crüger, 2002; Schmon *et al.*, 2018).

1.3. Biological control agents as an alternative control method

Populations of all living organism are affected by the actions of predators, parasites, pathogens and antagonist. These processes happen in any balanced ecosystem and have been referred to as “natural control” (Hajek, 2018). When natural enemies are used to suppress the population of a pest organism, it is called biological control or biocontrol (Hajek, 2018; Eilenberg *et al.*, 2001) The aim is not to eradicate pest populations, but to keep the number of pathogens in an acceptable range and avoid an epidemic reproduction. Organisms used in biocontrol are called biological control agents (BCAs) (Börner, 2009). While chemical pesticides can cause serious side effects, leading to major concerns about human health and the preservation of global and local environments, biological control methods are more environmentally friendly. They leave no chemical residues and host specificity is higher in most cases (Hajek, 2018). Strategies for biocontrol include conservation and enhancement of natural enemies, introduction of an exotic BCA for permanent establishment and augmentation without the goal of permanent establishment (Hajek, 2018; Eilenberg *et al.*, 2001).

Various organisms are used as biocontrol agents against pests. Viruses (e.g. *Baculovirus CpGV*) for example are used in apple production against the codling moth. Their biggest advantage is a very high host specificity. Entomopathogenic fungi (e.g. *Metarhizium anisopliae*) are very common in nature and some products are available against *Lepidopteran* and *Coleopteran* species (Copping, 2011). Yet, one problem of applying entomopathogenic fungi is their high susceptibility to environmental factors, resulting in inconsistent performance. Arthropods (e.g. *Phytoseiulus persimilis*), used as BCAs, are either predators or parasitoids. Predators are mostly used in greenhouses while parasitoids are also used in the field (Börner, 2009). Nematodes (e.g. *Steinernema feltiae*) are animals living in the soil and are therefore highly efficient against soil-dwelling pests. Many different products are available and in use in most European countries. (Börner, 2009). *Bacillus thuringiensis* is the most used bacterial biocontrol agent. Their big advantage is a very high effectivity and host specificity. However, many other entomopathogenic bacteria exist (Copping, 2011).
Hence biocontrol agents are living organisms, ecological processes determine their success (Duffy, 1996). Especially in field crops, biocontrol success is strongly influenced by climate, the physical and chemical composition of the rhizosphere, interactions with nontarget pathogens and pests as well as the ability of the biocontrol agent to colonize the rhizosphere (Ownley *et al.*, 1991). So far, most biocontrol success has been achieved in greenhouse cultivation, where ecological parameters are less variable (Paulitz and Bélanger, 2001). Inconsistent performance in the field has proven to be a major obstacle for the development of commercial biocontrol products. A strategy to overcome inconsistent performance is to combine the disease-suppressive activity of two or more biocontrol agents (Meyer and Roberts, 2002).
For the control of *D. radicum,* some biocontrol methods have already been tested. Entomopathogenic fungi have been suggested, but production costs are high (Zimmermann, 1997) and fungi are highly susceptible to environmental factors. In some *in vitro* and greenhouse experiments, isolates of *Trichoderma atroviride*, *Beauveria bassiana*, and *Metharhizium anisopliae* were effective against *D. radicum* (Razinger, 2014). A challenge for sufficient control is the persistence and distribution of the spores in the soil (Finch, 1989). So far, no products for commercial use are available (Copping, 2011). Entomopathogenic nematodes (EPN) have been tested against *D. radicum*, but effectiveness has been inconsistent probably due to suboptimal conditions for the pest. In more recent experiments, a good control of *D. radicum* in the greenhouse was observed (Chen *et al.*, 2003). But under field conditions, the control was not successful. Temperature, soil moisture and soil texture seemed to have the highest impact on effectivity of EPN.

1.3.1. Entomopathogenic nematodes

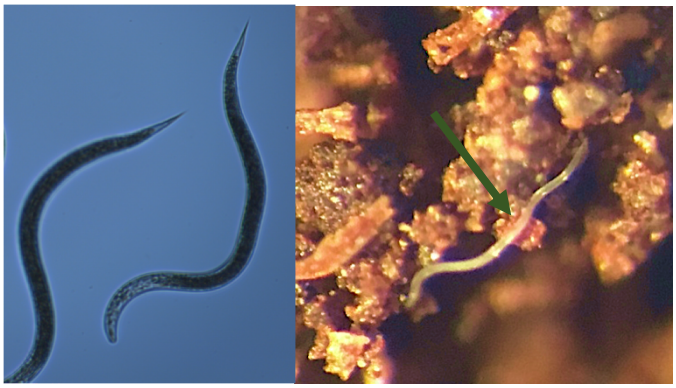
Entomopathogenic nematodes (EPNs) from the genera *Steinernema* and *Heterorhabditis* are already successfully applied against a broad variety of insect pests. Due to a mutualistic association with bacteria in the genera *Photorhabdus* (for *Heterorhabditidae*) and *Xenorhabdus* (for

Steinernematidae), a variety of insects can serve as hosts. These bacteria produce a wide range of toxins and antibiotics (Grewal *et al.*, 2008). Even though more then 30 nematode families are known to parasitize insects, entomopathogenic nematodes from the genera *Steinernema* and *Heterorhabditis* receive the most attention as biocontrol agents against soil insect pests (Lacey *et al.*, 2001; Nickle, 1972).

Table 1: Description of nematode strains applied individually or in combinations in this study.

Strain	Origin or target insect	Species	Reference or comment
Hb enema	nematop®; against black vine weevil	*Heterorhabditis bacteriophora*	e-nema mbH
Sf enema	nemaplus®; against fungus gnats	*Steinernema feltiae*	e-nema mbH
Sf RS5	derived from swiss soil	*Steinernema feltiae*	Jaffuel et al., 2016; Imperiali et al., 2017
Sf MG608	derived from swiss soil	*Steinernema feltiae*	Jaffuel et al., 2016; Imperiali et al., 2017
Sf MG594	derived from swiss soil	*Steinernema feltiae*	Jaffuel et al., 2016; Imperiali et al., 2017

Both EPN form a single, free living stage, called infective juveniles (Picture 2). These infective juveniles carry bacteria of the genus *Xenorhabdus* and *Photorhabdus* respectively. When encountering a suitable host, they enter through the mouth, anus or spiracles and migrate into the hemolymph. *Heterorhabditidae* are even able to enter the host by penetrating intersegmental membranes of the insect (Bedding and Molyneux, 1982). As soon as the IJ has reached the hemolymph, it releases its associated bacteria. Bacteria proliferate in the nutrient rich hemolymph. The insect normally dies within 24-48h and IJs feed on the proliferated symbiotic bacteria and dead host tissue. Afterwards, the nematodes develop through the fourth to the fifth adult stage. In this stage, they reproduce in the host cadaver. Depending on availability of resources one or more generations may occur (Grewal *et al.*, 2008). In the first generation, eggs are laid into the host medium. Later, eggs hatch in the uterus of older female or hermaphrodites and feed on the parental tissue. This process is known as “endotokia matricida” (Johnigk and Ehlers, 1999). The use of parental tissue results in a more effective conversation of insect biomass into nematode biomass. When adequate food supply is available, the juveniles develop to adults and a new generation of juveniles emerges. As soon as food supply becomes limited, juveniles form the stage of infective juveniles and leave the insect



Picture 2: Infective juveniles in water (left) and in soil (right).

cadaver in search for a new host (Grewal *et al.*, 2008). The IJs are morphologically and physiologically adopted to their role of transmission. They have a pair of sensory organs to detect cues that are potentially associated with hosts. Foraging strategies vary from ambush to cruise foraging and are very diverse within different species (Lewis *et al.*, 1992). Ambushing nematodes await host associated cues and only nictate from side to side before they start moving (Hurley, 2018) Nematodes following this strategy, for example *Steinernema carpocapsae* and *S. scapterisciare*, are normally associated with highly mobile, surface-dwelling hosts. *Heterohabditis spp.* are typical cruisers which spend most of their time moving through the soil actively searching for a new host (Hurley, 2018). *S. feltiae* adopts an intermediate strategy and has therefore been shown to be effective against pests with mobile or sedentary habits (Grewal *et al.*, 2008). IJs discriminate directly among potential hosts and prefer some over others. Current knowledge of natural host ranges is limited to accounts of native populations found infecting a host in the field (Peters, 1996). Host range and pathogenicity strongly depends on the nematode's symbiosis with bacteria (Grewal *et al.*, 2008).

Symbiotic bacteria belong to the *Proteobacteria* in the family of *Enterobacteriacea* (Gaugler, 2002). In general, for each nematode species an association with a species or subspecies of bacteria exists (Fischer 1998). *Photorhabdus sp. and Xenorhabdus sp.* are able to produce different kinds of insect toxins (Duchaud *et al.*, 2003). Toxin complexes (Tc) are large orally active toxins that require three components for full toxicity (Bowen *et al.*, 1998; ffrench-Constant *et al.*, 2007). Toxins called "Makes caterpillars floppy", Mcf1 and Mcf2, are active upon injection (Daborn *et al.*, 2002; Waterfield *et al.*, 2003). "Photorhabdus insect-related" PirAB binary toxins have oral and injectable activities in some insects (ffrench-Constant *et al.*, 2007). Differences in pathogenicity among bacterial species have been recorded. Most species of *Xenorhabdus* are highly pathogenic with LD_{50} of less than 20 cells (Akhurst and Dunphy, 1993). Most *Photorhabdus* strains have been reported to have a pathogenicity with LD <100 cells (Akhurst and Boemare, 1990). Some strains of *Photorhabdus* are pathogenic to insects by ingestion, but development of the bacteria in the insect gut has not been reported (Grewal *et al.*, 2008; Blackburn *et al.*, 1998). Antimicrobial organic compounds and bacteriocins active against other *Photorhabdus* and *Xenorhabdus* species and closely related bacteria are produced by all symbiotic bacteria during *in vivo* multiplication to avoid microbial contamination (Gaugler, 2002; Boemare *et al.*, 1992). The symbiosis seems to be protected well by antimicrobial barriers and microbial competitors are eliminated. However, some bacteria like *Paenibacillus spp*. seem to be resistant to theses antimicrobials (Enright and Griffin, 2004).

1.3.2. Pseudomonads as entomopathogens

Bacteria from the genus *Pseudomonas* have been known long for their ability to cause diseases in plants (e.g. *Pseudomonas syringae*), animals and humans (e.g. *Pseudomonas aeruginosa*). But their interaction with plants is not only negative, many metabolic features are beneficial for plants (Silver *et al.*, 1990). Bacteria that provide some benefit to plants can either form a symbiotic relationship with

them, or live free in the soil but are often found near or even within the roots. Several reports confirm that pseudomonads promote growth and reproductive parameters of plants ranging from cereals, pulses, ornamentals, vegetable crops, plantation crops and even trees (Meena, 2014). Therefore, they belong to the plant growth-promoting rhizobacteria (PGPR). Beneficial effects have been attributed to their ability to produce phytohormones, organic acids and siderophores. Other plant-beneficial mechanisms include solubilization of soil phosphate (Glick, 1995). The genus of Pseudomonas spp. (Proteobacteria γ subclass) comprises more than one hundred species. Characteristic for many of these species is their environmental ubiquity, a consequence of their broad colonization ability and high metabolic versatility. Environmental niches they colonize range from oil-spilled seawater to soil, plant surfaces and insect guts (Kupferschmied *et al.*, 2013).

The most promising group of PGPR for biocontrol are the fluorescent pseudomonads. Among them, *Pseudomonas fluorescens*, *P. putida, P. aeruginosa* and *P. aureofaciens* are associated with plants. Many of them have been isolated from suppressive soils for the management of soilborne and foliar diseases. Pseudomonads are natural inhabitants of the soil and the rhizosphere. There they grow well even when introduced artificially (Wilson *et al.*, 1992). Main modes of action fluorescent *Pseudomonas* strains can deploy against plant pathogens are antibiosis, competition for nutrients or niches as well as induction of plant defense mechanisms (induced systemic resistance). PGPR may stimulate the production of biochemical compounds associated with host defense, massive accumulation of phytoalexins and phenolic compounds, increase in the activity of PR proteins, defense enzymes and transcripts and enhanced lignification (Meena, 2014). At the moment, there are different *Pseudomonas* strains available to protect plants from fungal and bacterial pathogens. For example, one *Pseudomonas chlororaphis* strain is used as a fungicide against cereal diseases like *Tilletia spp.* and *Fusarium spp*. It is stimulated by the plant to counteract the pathogen through competition, predation, parasitism or antibiosis (Copping, 2011).

Recently, pseudomonads were shown to be active against certain insect and nematode pests (Ramamoorthy *et al.*, 2001; Péchy-Tarr *et al.*, 2008). These findings make them a promising alternative for pest control, in particular when fighting the notorious problem of soil-dwelling pests. Protein extracts and metabolites of *P. fluorescens* strains like HCN and the lipopeptides viscosin and orfamide were shown to have insecticidal activity (Devi and Kothamasi, 2009; Hashimoto, 2002; Jang *et al.*, 2013). But molecular basis and regulation of this insecticidal activity remains mostly obscure (Kupferschmied *et al.*, 2013). Some *P. fluorescens* strains possess a gene which codes for a protein similar to the insect toxin Mcf1 of the entomopathogen *P. luminescens* (Péchy-Tarr *et al.*, 2008). This mcf1-related gene is part of an eight-gene cluster which was termed "Fit" for *P. fluorescens* insecticidal toxin. The gene encoding for the actual insect toxin is called FitD (Péchy-Tarr *et al.*, 2008). Expression of the fit toxin seems to be activated in a host-dependent manner. Only during infection of insects, the Fit toxin is expressed but not when growing on plant roots. This indicates, that entomopathogenic pseudomonads detects the insect host and induces the production of Fit. A

deletion of the FitD toxin gene did not make the bacteria non-toxic to insects. Therefore, additional virulence factors most likely play a role in insecticidal activity. Hydrogen cyanide and cyclic lipopeptides might contribute to insecticidal toxicity (Flury *et al.*, 2017). Additionally, genes related to the expression of Toxin complexes (Tcs), large multimeric insecticidal protein complexes, can be found in certain strains of *P. chlororaphis* and *P. protegens* (Loper *et al.*, 2012). So far, the FitD gene has only been detected in the plant-associated pseudomonads *P. protegens* and *P. chlororaphis* (Ruffner *et al.*, 2013). Strains of these two species showed toxicity, especially towards lepidopteran insects upon injection. As few as 30 cells of CHA0 per larva were sufficient to cause 100% mortality within 40 hours after injection into hemolymph (Péchy-Tarr *et al.*, 2008). The strains *P. protegens* CHA0 and *P. chlororaphis* PCL1391 also displayed potent oral insecticidal activity in laboratory feeding assays. Both were able to kill larvae of several lepidopteran pests, including *Spodoptera littoralis, Heliothis virescens* and *Plutella xylostella*. Low concentrations of pseudomonads on plant leaves were sufficient to induce high mortality of insects feeding on them (Ruffner *et al.*, 2013). So far, translocation mechanisms from gut into hemolymph are not yet fully understood. Replication in the hemolymph has been shown with a gfp-tagged *P. protegens* CHA0 in larvae of *Pieris brassicae.* The invasion of the insect hemolymph within less than one day after oral uptake suggests that these bacteria should be considered as true insect pathogens (Kupferschmied *et al.*, 2013).

Table 2: Fluorescent Pseudomonas *strains used in this study.*

Strain	**Genotype or phenotype**	**Species**	**Reference or comment**
CHA0	Wild type, isolated from tobacco roots	*Pseudomonas protegens*	Stutz et al., 1986. Jousset et al.,2014
CHA0-gfp	CHA0-gm-gfp-2 mutant with gfp-tag and gentamycin resistance	*Pseudomonas protegens*	Flury, 2016
PCL1391	Wild type, isolated from tomato roots	*Pseudomonas chlororaphis*	Chin-a-Woeng et al. 1998
PCL1391-gfp	PCl1391-gm-gfp-2 mutant with gfp-tag and gentamycin resistance	*Pseudomonas chlororaphis*	Flury, 2016
77	Strain isolated from potato root in Switzerland in 2017	*Pseudomonas chlororaphis*	Vesga, Schneider et al., unpublished
64	Strain isolated from potato root in Switzerland in 2017	*Pseudomonas chlororaphis*	Vesga, Schneider et al., unpublished

Natural interactions of pseudomonads with insects are probably much more widespread than estimated so far. Microbial communities of various insects contain members of the genus *Pseudomonas*. They were already identified as common inhabitants of the intestinal tract or

associated with field-collected larvae, pupae and adults of representatives of the major insect orders (Kupferschmied *et al.*, 2013). The highly versatile pseudomonads may be naturally very well adopted to live inside or exploit an insect as a shelter, vector or food source (Kupferschmied *et al.*, 2013). Several reasons for insect colonization are possible. Pseudomonads might use insects as food sources for proliferation. On the other hand, they could detect insects feeding on plant roots as concurrent and therefore, proteins with insecticidal activity would be weapons to defend the bacteria and their rhizosphere habitat. Another possibility is that bacteria exploit insects as vectors. Although the role of insect colonization is not yet understood, it seems that the oral toxicity does not account towards beneficial insects (Ruffner *et al.*, 2013).

Because of their already proven plant protection ability against fungal and oomycete pathogens, *Pseudomonas protegens* and *P. chlororaphis* are promising candidates for the development of novel microbial products which protect plant roots simultaneously against phytopathogens and herbivorous insects (Kupferschmied *et al.*, 2013). A big advantage of pseudomonads compared to other microbial biocontrol agents is their capability to colonize plant roots (Kupferschmied *et al.*, 2013).

1.4. Combining Biological Control Agents

The use of mixed cultivars or mixed fungicides has shown to be successful to increase and maintain disease control efficiency. A combination of biological control agents might show the same effects. A range of biocontrol agents have potential for combined application (Xu *et al.*, 2011). Given the fact that BCAs performance is strongly influenced by environmental factors, combinations could contribute to higher consistency. Biocontrol agents could complement one another when environmental factors change or favor one of them (Xu *et al.*, 2011). In suppressive soils for example the phenomenon of combined biocontrol agents takes place naturally. There, disease control is manifested by many different agents and their interaction (Xu *et al.*, 2011). Plants are generally not only threatened by one pathogen. A combined application could act against more than just one pathogen and increase the overall plant protection (Meyer and Roberts, 2002).

However, ecological interactions among biocontrol agents play an important role when combining them and must be studied in detail. Knowledge of the organism's ecology is necessary to determine whether they are complementary (Meyer and Roberts, 2002). As biocontrol agents mostly are living organisms, they adopt to changing circumstances to a certain point. This adaptation leads to a very complex net of interactions when combining two or more biocontrol agents. Additionally to the biocontrol-pathogen-environment interaction, the interaction between biocontrol agents must be studied. Biological agents applied against the same pathogen or pest might become food competitors and release for example metabolites to expel or even kill the competitor. Another fact that should be taken into account is that most biocontrol agents often have more than one mode of action that operates to fight the pathogen (Xu *et al.*, 2011). When these modes of actions differ within combined

biocontrol agents a compatibility is more likely. The colonization of different spatial and temporal niches might also influence compatibility of biological control agents (Imperiali *et al.*, 2017).

1.4.1. Challenges when combining nematodes and pseudomonads

Pseudomonads are known to release a wide range of biologically active exoproducts including several broad-spectrum antimicrobial metabolites like 2,4-dacetylphloroglucinol (DAPG), hydrogen cyanide (HCN) and phenazines (PHZ) (Haas and Keel, 2003). These antimicrobial compounds are effective weapons not just against pathogens and pests. Hydrogen cyanide for example is responsible for killing the nematode *Caenorhabditis elegans* (Gallagher and Manoil, 2001). Because many of the antimicrobials produced by pseudomonads are not very specific, a negative effect on bacterial symbionts of entomopathogenic nematodes is possible. On the other side, nematode associated bacteria are capable to produce antibiotics to suppress competing organisms as well (Grewal *et al.*, 2008). More than 30 bioactive secondary metabolites have been reported from *Xenorhabdus* and *Photorhabdus.* Even though most enterobacteria and *Pseudomonas aeruginosa* have been shown to be resistant against antibiotics produced by *Xenorhabditidae,* plant-associated *Pseudomonas* strains have not yet been tested for resistance (Gaugler, 2002). However, antagonistic effects among BCAs must be taken into account when applying them in combination.

Despite challenges, the potential for a combined application of entomopathogenic nematodes and entomopathogenic pseudomonads is high. Multiple modes of action against the target pathogen or pest, the ability to affect more than one stage of the life circle of the target organism, the activity of agents during different times and an increased consistency over a wider range of environmental conditions are potential advantages of biocontrol agents applied in combination (Meyer and Roberts, 2002). A previous study by Imperiali *et al.* (2017) for example showed, that combined application of *Heterorhabditis bacteriophora* and two fluorescent *Pseudomonas* strains (CHA0, PCL1391) improved the performance and protection of wheat. In this master project, the potential of different *Pseudomonas* and nematode strains were tested under controlled conditions against *D. radicum*.

1.5. Description of the project “BeneComb”

This Master thesis was conducted as part of an ongoing Mercator-World Food System center research project. Principal investigators are Prof. Dr. Monika Maurhofer from the Plant Pathology group at ETH Zurich and Dr. Giselher Grabenweger and Dr. Anouk Guyer from the Plant Protection unit at Agroscope. Anna Spescha is working on this project as a PhD student. The project is financed by the Mercator foundation Switzerland.

The aim of this project is to develop a new approach for the control of soil-dwelling insect pests compatible with organic production. Therefore, the biocontrol potential of plant beneficial fluorescent *Pseudomonas* bacteria with entomopathogenic activity is evaluated against the cabbage root fly *D. radicum*. Additionally, entomopathogenic pseudomonads (EPP) are combined with

entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPN) to test the effect of combined applications. Interactions among biocontrol agents are investigated in the laboratory. Screenings for the best strains of each biocontrol agent are performed under controlled conditions in climate chambers and the greenhouse. Later, promising EPP-EPN-EPF combinations are tested in the climate chamber, greenhouse and field against the cabbage root fly *Delia radicum*. The project will give exciting new insights into complex interactions of agriculturally important members of the soil and rhizosphere. New methods based on the combined application of beneficial soil organisms for the control of an important insect pest in organic and conventional vegetable production will be generated. The new methods might have the potential to be adapted to other problematic soil pests (https://worldfoodsystem.ethz.ch/research/research-programs/MRP/BeneComb.html, 10/1/2019).
In previous research, four single EPP strains were already tested under controlled conditions in the climate chamber for their influence on the development of *D. radicum*. The *P. chlororaphis* strain PCL1391 reduced pupation rate and fly emergence in all repetitions. The *P. chlororaphis* strain 77 significantly reduced pupation rate in four out of six repetitions and fly emergence in three out of five repetitions. The *P. chlororaphis* strain 64 reduced pupation rate significantly compared to the control in four out of five screenings and fly emergence in three out of five repetitions. For CHA0 a significant reduction was observed in three out of four screenings for pupation rate and fly emergence was significantly reduced in one out of three repetitions.

Research questions:

1) Can entomopathogenic *Pseudomonas* (CHA0, PCL1391) colonize bulbs of radish plants when applied to soil under greenhouse conditions?

2) Which entomopathogenic *Pseudomonas* strains (64, 77, CHAO, PCL1391) have the highest potential to control *Delia radicum* in radish under greenhouse conditions?

3) Do entomopathogenic nematodes (Hb enema, Sf enema, Sf MG594, Sf MG608, Sf RS5) have the ability to inhibit the development of *Delia radicum* under controlled conditions?

4) What effect has the combination of EPP (PCL1391) and EPN (Sf enema, Sf MG594) on the pupation rate of *Delia radicum* under controlled conditions?

5) How compatible are EPP and EPN when combining them *in vivo* and *in vitro*?

6) Can pseudomonads use EPN as vectors to be transported into the hemolymph of *Galleria mellonella* larva?

2. Material and Methods

2.1. Handling the organisms used in this study

Experiments for assessing the compatibility of EPN and EPP and their effect on the development of *D. radicum* need a sufficient number of infective juveniles, pseudomonads and *D. radicum* eggs. For generating a sufficient number of vital organisms at the timepoint of experiments, an artificial rearing must be stablished. Procedures for rearing EPN and *D. radicum* and for propagating EPP are described in the following chapter.

2.1.1. *D. radicum* rearing

Pupae for *D. radicum* rearing originate from an artificial rearing at the Julius-Kühn-Institute in Germany, the Université de Rennes in France and an already existing rearing at ETH Zurich. One rearing cycle takes about six weeks and starts with pupae which are placed in an insect cage in the climate chamber (Figure 1). Conditions are set at optimum for *D. radicum* development (Table 1). Five days after placing the pupae in the cage, one petri dish with sand (0.3-0.9mm) and water and one petri dish with dry feed is added. The dry feed contains 10g glucose, 10g milk powder, 1g soy-flour and 1g yeast. When first flies start to emerge, after about seven days, wet feed containing 5g honey, 5g soy-flour, 1g yeast and about 6ml sterile water is added to the cage. Water is refilled every second day, while wet feed is replaced every fourth day. Flies start laying eggs about one week after they have hatched. For egg-laying, pieces of cabbage are placed in the insect cage on the sand. Flies are attracted by the cabbage to lay eggs on them or in the sand next to it. After flies have started oviposition, eggs must be collected every second to third day. For collecting the eggs, sand and pieces of cabbage are first rinsed with dH_2O into a beaker. The beaker is shaken in order to float the eggs in the water and then water is poured through filter paper (LS 14 Ø150mm Schleicher Schuell). Eggs are collected from filter paper with a brush and placed on a wet filter paper in a petri dish for further use in experiments or for rearing.

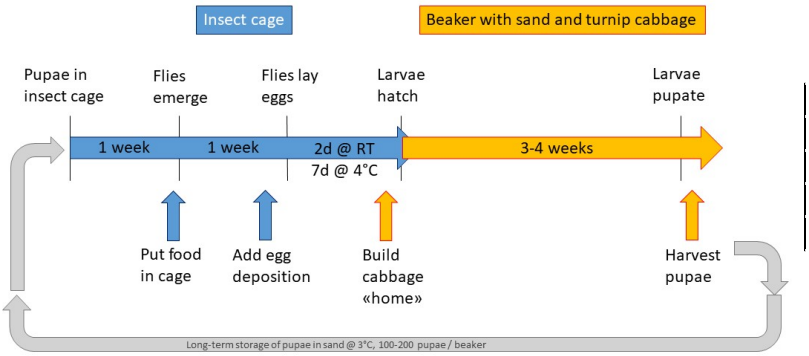


Figure 1: Schematic rearing of Delia radicum.

Table 1: Conditions in the climate chamber for D. radicum rearing and experiments with D. radicum.

	Day	Night
Duration	16 hours	8 hours
Temperature	20°C	18°C
Light intensity	15 kLux	-
Humidity	80%	80%

To generate pupae for rearing, “cabbage homes” are built (Figure 1). Therefore, an 800ml beaker is filled up to 2cm with autoclaved sand (0,3-0,9mm). A filter paper (Ø85mm) with about 70-100 eggs is

placed on top of the sand. Turnip cabbages are washed and sterilized with 70% ethanol. The turnip cabbage is cut into half and placed with the cut face first on top of the filter paper. Each turnip cabbage is covered with autoclaved sand (0,3-0,9mm) and the beaker covered with aluminum foil. In this "cabbage home" larvae will feed on the cabbage and pupate within 21-28 days. The beaker is placed in the climate chamber at controlled conditions (Table 3). After 28 days, pupae can be collected with a sieve (mesh ± 3mm). Therefore, sand and turnip cabbage are washed through the sieve. Pupae are cleaned, dried and stored at 3°C in a beaker filled with sand and covered with aluminum foil.

2.1.2. Entomopathogenic nematode rearing

Nematodes are reared *in vivo* in *Galleria mellonella* larvae. Nematode strains used in this study are from the species *Heterorhabditis bacteriophora* (Hb enema) and *Steinernema feltiae* (Sf enema, Sf RS5, Sf MG594, Sf MG 608). The procedure is the same for all used strains, only the collection of infective juveniles varies slightly between strains.

Four or five *G. mellonella* larva are placed onto a double filter paper in a small petri dish (60mm ø). 400µl nematode suspension is added to each petri dish. The suspension was either dissolved from commercial powder in water or taken from nematode storage in cell culture flasks. Not more than 200 IJs per *G. mellonella* were added. The infected larvae are incubated in the dark at 24°C.

For collecting IJs, white traps are created (Picture 3). Therefore, an ø90mm filter paper is placed on top of a small petri dish (60mm ø). The petri dish is placed in a larger plastic container. Dead larvae are checked for distinct symptoms and then placed on the white trap. After 10-15 days, tap water is added in the container until ground is covered up to ~5mm with water. Normally, nematodes escape from *G. mellonella* and gather in the fresh water. For the strains Hb enema, Sf RS5 and Sf MG594 this worked well. IJs from the strains Sf MG608 and especially Sf enema only left the cadaver after it was cut open. Infective juveniles are collected by pouring the water into an aerated culture flask. Collected IJs can either be used for experiments directly or stored in the aerated culture flasks at 10°C in the dark. For storage, concentration is adjusted to 500-1000 IJ/ml.

Picture 3: White trap for collecting infective juveniles.

2.1.3. *Galleria mellonella*

The greater wax moth *Galleria mellonella* is often used in biomedical studies and studies of new substances or biological control agents that show insecticidal activity. Because of its rapid growth, high fertility, size and short life cycle, *G. mellonella* makes a good model organism for research (Mikulak *et al.*, 2018). For the conducted experiments, larvae from the non-feeding fourth to fifth instar were bought at a fisher shop in Zurich. They were stored at 10°C in the dark and before each experiment sick or dead larva were discarded.

2.1.4. Entomopathogenic pseudomonads propagation

After isolation, aliquots of *Pseudomonas* strains are created to facilitate long-time storage. First each strain is inoculated on KB^{+++} plates (resp. KB^{++G} for gfp tagged strains) (Table 4, appendix). For this, some glycerol stock is spread with a 20µl inoculation loop on a plate under the sterile hood and grown for two days at 24°C (or three days at 18°C). Glycerol stocks must be kept in a cooling box, incubated in the freezer or on ice in order to avoid thawing the culture. 10ml LB liquid culture without antibiotics is inoculated with a 20µl inoculation loop and incubated overnight at 24°C and 180rpm. On the next day, the bacterial culture is mixed in equal parts with 90% glycerol in an 15ml falcon tube. 50µl of suspension is pipetted in small Eppendorf tubes which are incubated for 30 minutes on ice. Afterwards aliquots are stored at -80°C. For experiments, aliquots were spread with a 1µl inoculation loop on KB+++ plates (resp. $KB++^{G}$ for gfp-tagged strains) next to a flame and grown for two days at 24°C (or three days at 18°C). From these, overnight LB cultures were grown and used directly or 200µl overnight culture was spread on KB plates and grown for 24 hours at 24°C.

Strains with a constitutively expressed GFP tag were generated by means of the Tn7 delivery vectors pBK-miniTn7-gfp1 or pBKminiTn7-gfp2 (Kupferschmied *et al.*, 2014).

2.2. Radish greenhouse experiment to test entomopathogenic *Pseudomonas* for *D. radicum* control

For testing the effect of the *Pseudomonas chlororaphis* strains PCL1391, 64 and 77 and the *P. protegens* strain CHA0 on *D. radicum*, radishes (*Raphanus sativus var. sativus*) were grown in the greenhouse and inoculated with bacteria twice. Within the same setup, the root colonization ability of these strains on radish plants was assessed. Effects of entomopathogenic *Pseudomonas* application on pupation rate, pupal size and fly emergence of *D. radicum* were investigated. Three independent repetitions were performed.

First, radish was sown on sand (3-5mm) in a tray with small holes. This tray was placed in a bigger one and water was added. Both were covered with aluminium foil and incubated for one week in the climate chamber at controlled conditions (Table 1). After seven days, pre-germinated seedlings were planted in soil. About 200g of soil substrate (Jiffy substrate) was filled in each pot and three seedlings were planted per pot. Twelve pots per treatment were prepared. Two pots per strain were prepared for assessing the root colonization ability. Pots were placed in trays in the greenhouse at 22°C during a 16h light period and at 18°C for 8h without artificial light and 70% relative humidity. Trays were watered regularly in order to keep the substrate moist. Once a week, radish plants were fertilized with 25ml per pot of 2ml fertilizer (Wuxal Universaldünger, Maag) dissolved in 1l water.

After transplanting, pots were inoculated with 10^7 cfu/g soil of different *Pseudomonas* strains twice (Picture 4), first at the timepoint of transplanting and second one week before *D. radicum* infestation. Control pots were inoculated with autoclaved water. To obtain the right concentration of vital bacteria, some autoclaved H_2O was added to the incubated KB plates obtained from aliquots (see chapter 2.1.4). Bacteria were scratched off the plates and transferred into a falcon tube. OD_{600} was measured and adjusted to 0.125 (± 4%) which represents a concentration of 10^8 cfu/ml. For measuring OD_{600} ddH_2O was used as blank. 20ml of the adjusted bacteria suspension was added to each pot, resulting in a final bacteria concentration of 10^7 cfu/g soil. Pots for assessing the root colonization ability were inoculated with gfp-tagged strains for CHA0 and PCL1391.

Picture 4: Inoculation of radish plants with different Pseudomonas-strains (left) and infestation with D. radicum eggs (right).

One week after the second inoculation, pots were infested with eggs of *D. radicum* (Picture 3). Eggs were collected from the rearing as explained in chapter 2.1.1. One small black paper slice per pot was bathed in water and placed on a wet filter paper in a petri dish. Ten healthy eggs were placed on each paper slice using a brush. Pots were infested by holding the paper close to the radish plants and flushing eggs with 1ml ddH_2O onto the substrate.

Four weeks after infesting the pots with *D. radicum* eggs, the pupation rate was assessed. Therefore, the stems and leaves of radishes were removed and bulbs were checked for larvae. Soil of each pot was sieved (mesh ± 3mm), pupae cleaned with water, dried with a tissue, counted and placed in a small petri dish. Tools were cleaned and sterilized with 70% ethanol between different strains. Pictures of the pupae were taken and analyzed with the software “ImageJ” to determine their size. Afterwards, pupae were placed in the dark in the climate chamber at controlled conditions (Table 1). After four weeks, fly emergence was determined by counting emerged flies. The fly emergence rate was calculated for each pot by dividing the number of flies by the number of eggs.

To determine the root colonization ability of the pseudomonads, colony forming units (cfu) per g bulb were measured. First, stem and leaf were cut off with a sterile knife. Soil was washed off from bulbs and roots with autoclaved ddH_2O. Bulbs together with roots were placed on a dry tissue and after one minute, placed into a sterile 100ml Erlenmeyer flask and weighed. 10-25ml saline (0.9%NaCl) was added. Erlenmeyer flasks were placed on a shaker for 30 minutes at 400rpm and 3°C. After 30 minutes, 100µl of the suspension in the flask was transferred to a well in a cell culture plate. A dilution series up to 10^{-5} was made and 10µl of each dilution (from undiluted to 10^{-5}) was pipetted on a square KB^{+++}/KB^{++G} plate. The plate was put upright to let each 10µl drop flow downwards. The plates were

incubated for two days at 24°C. After two days, colonies of the countable dilutions were counted under the fluorescent microscope and cfu per gram bulb was calculated for gfp-tagged strains. For the strains 64 and 77 colonies were counted, marked and picked for PCR confirmation with a Fit primer pair specific to *P. chlororaphis* and *P. protegens* (Protocol 1, appendix).

2.2.1 Method optimization

After first experiments, the germination, the amount of *D. radicum* eggs and fertilization were optimized. The substrate was sieved before usage to facilitate sieving for evaluation. The pre-germination step was skipped. Instead, five seeds per pot were sown and redundant seedlings were removed prior to first bacteria inoculation. After second inoculation, some substrate was added to cover developing bulbs. For the last experiment, the number of added *D. radicum* eggs per pot was increased to fourteen due to low pupation rates in previous experiments. Amount of fertilizer per week was increased after the second experiment. Once a week radish plants were fertilized with 1l per tray, containing 2ml fertilizer (Wuxal Universaldünger, Maag).

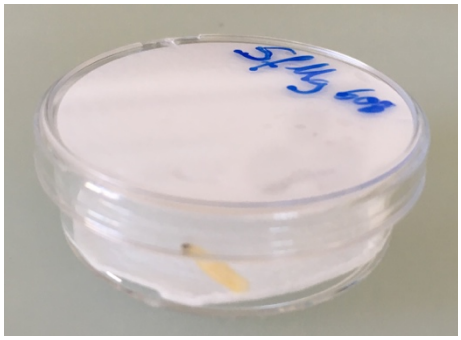
2.3. Climate chamber assay to evaluate entomopathogenic nematode strains for *D. radicum* control

To test the potential of nematodes for the control of *D. radicum*, larvae first were infected directly with five different nematode strains in petri dishes. To assess the effect of nematodes on development of *D. radicum*, screening experiments in the climate chamber were conducted.

2.3.1. Direct infection of *D. radicum* larvae with nematodes

To investigate whether nematode strains are able to infect *D. radicum* larvae, a small number of them was directly infected with nematodes. Survival rate of larvae was determined, and presence of nematodes was investigated under the light microscope. One repetition was performed.

D. radicum larvae were recovered from “cabbage homes” from rearing. Sand of beakers was sieved (mesh ± 3mm) and cabbages opened to recover larvae with tweezers. Four larvae were placed on filter paper in medium sized Petri dishes (ø 60mm). On the lid of the Petri dish, a second filter paper was placed (Picture 5). On each filter paper, 400µl of a 1000IJ/ml nematode suspension was pipetted. Three Petri dishes were prepared per nematode strain. Afterwards, Petri dishes were placed in the dark at 24°C. Survival of *D. radicum* larvae over time was observed. Once a day a sufficient amount of tap water (about 200µl) was added to keep the filter paper moist. Hundred hours past infection, larvae were placed on white traps. Thirteen days after placing larvae on the white traps they were checked for nematodes under the light microscope.



Picture 5: Petri dish in which D. radicum *larvae were directly infected with nematodes.*

2.3.2. Screening nematode strains for the control of *D. radicum*

In order to evaluate the effect of the nematode strains Hb enema, Sf enema, Sf RS5, Sf MG594 and Sf MG608 on development of *D. radicum* eggs, radish experiments in the climate chamber were conducted. Effects on pupation rate, pupal size and fly emergence were investigated. Four independent repetitions were performed.

Radishes were bought at the supermarket on the day when starting the experiment. The stem and leaves were cut off and bulbs were washed with tap water. Afterwards, radishes were bathed in 70% ethanol and dried on a household paper. Bottom holes of small plastic pots (345x276x80mm, Bachmann Plantec AG Switzerland) were closed with packaging tape. Pots were filled up to 2cm with autoclaved sand (0.3-0.9mm). Sterilized radishes were placed, the green facing upwards, on top of sand. At least 20g radishes, usually corresponding to two radishes, were added per pot. Radishes were covered with autoclaved sand (0.3-0.9mm). Eight replicates per treatment were prepared. Nematodes were taken from the storage in the 10°C room. Concentration was adjusted to 1000 living IJ's/ml. Four milliliters of this adjusted suspension was pipetted to each pot. For the control pot four millilitersl of tap water was used. Pots were infested with 10 *D. radicum* eggs (see chapter 2.2). After infestation, pots were covered with aluminum foil and placed in the climate chamber at controlled conditions (Table 1).

Picture 6: Pot at evaluation of pupation rate. Sond is sieved through mesh below pot and pupae collected.

After 24 or 25 days, sand of each pot was sieved (mesh ± 3mm)(Picture 6), pupae cleaned with water, dried with a tissue, counted and placed in a small petri dish. Tools were cleaned and sterilized with 70% ethanol between handling different strains. Pictures of the pupae were taken and analyzed with the software "ImageJ" to determine their size. Afterwards, pupae were placed in the dark in the climate chamber at controlled conditions (Table 1). After four weeks, fly emergence was determined by counting emerged flies. The fly emergence rate was calculated for each pot by dividing the number of flies by the number of eggs.

2.4. Climate chamber assay to evaluate the combination of nematodes and pseudomonads for the control of *D. radicum*

Tests with first combinations of entomopathogenic nematodes and entomopathogenic pseudomonads were tested in the climate chamber. The effect of a mixed application of the nematode strains Sf enema and Sf MG594 with the *Pseudomonas chlororaphis* strain PCL1391 on development of *D. radicum* eggs was assessed. These strains were selected based on their performance in the nematode screening (see chapter 3.2) and the pseudomonads screening which was already conducted in advance to this study (see chapter 1.5). For PCL1391, a gfp-tagged strain was used for

the first repetition. Effects of single, mixed and control applications on pupation rate, pupal size and fly emergence were evaluated. Two independent repetitions were performed.

Radishes and pots were prepared as described in chapter 2.3. After sterilization, radishes were bathed in a bacterial suspension with an OD_{600} of 0.473. To obtain the adequate concentration of vital bacteria, some autoclaved H_2O was added to the incubated plates obtained from aliquots (see chapter 2.1.4). Bacteria were scratched off the plates and transferred into a falcon tube. OD_{600} was measured and adjusted to 0.473 (± 2%), ddH_2O was used as blank. 60ml of bacterial suspension was then filled into autoclaved 100ml beakers. Radishes for both combination treatments and the bacteria control treatment were bathed for ten minutes in this suspension, the green facing downwards (Picture 7). Radishes for the control and single nematode treatments were bathed in autoclaved ddH_2O for ten minutes.

Picture 7: Pots for the combined application of PCL1391-gfp and Sf enema are prepared, and radishes bathed in bacterial suspension.

Radishes bathed in either ddH_2O or bacterial suspension were placed, the green facing upwards, on top of the sand in plastic pots. Two or three radishes with a total weight of at least 20g were added per pot. Radishes were covered with autoclaved sand (0.3-0.9mm). Eight pots were prepared per treatment. Pots for single EPN and combined EPN x EPP treatments were infected with nematodes (see chapter 2.2), and four milliliters of tap water were added to the control and single PCL1391 pots. The addition of eggs and evaluation of experiment was performed as described in chapter 2.3.

2.4.1. Method optimization

For the second repetition, autoclaved tap water was sprayed over pots every third day while placed in the climate chamber to keep the sand moist. Additionally, pots were infected with EPN a second time two weeks before the end of the experiment.

2.5. *In vitro* assay to evaluate the susceptibility of nematodes to pseudomonads

To assess the compatibility of *P. chlororaphis* PCL1391-gfp and different EPN strains, the susceptibility of nematodes to bacteria was assessed. Both were mixed in 96-well plates and survival of nematodes over time was observed. Two independent repetitions were performed. For the first repetition, five nematode strains (Hb enema, Sf enema, Sf MG594, Sf MG608, Sf RS5) were tested. For the second repetition, only Sf enema and Sf MG594 were tested.

Nematodes were taken from the storage in the 10°C room. Concentration was adjusted to 2000 living IJ/ml. To obtain this higher concentration, nematode suspension from storage was filled into a falcon tube. After fifteen minutes nematodes settled on the bottom of the tube and the water on top was pipetted off and discarded. Amount of discarded water depended on nematode concentration at the

beginning. To obtain vital bacteria, they were recovered from aliquots (see chapter 2.1.) Overnight LB cultures were washed, OD_{600} was measured and adjusted to 0.125 (± 4%) which represents a concentration of 10^8 cfu/ml. This suspension was diluted 1:50 with autoclaved ddH_2O to obtain a bacterial suspension of $2x10^6$ cfu/ml.

2000 infective juveniles in autoclaved tap water and $2x10^6$ cfu bacteria per ml autoclaved ddH_2O were mixed in equal amounts. This resulted in a final nematode concentration of 1000 IJ/ml and a bacterial concentration of 10^6 cfu/ml in each well. These concentrations correspond to the concentrations used in the EPN-EPP combination radish assay (see chapter 2.4). Nematode controls were mixed with autoclaved ddH_2O. 100µl of this nematode-bacteria suspension was pipetted into 24 wells for each treatment. Each well contained about 100 living IJ, but accuracy was not determined at the start of the experiment. Afterwards, plates were placed at 200 rpm in the dark at 24°C. The following days, the number of surviving nematodes was counted after 24, 72, 144 and 208 hours in each well. Thereby moving nematodes were considered to be alive. For counting, suspension was first mixed by pipetting up and down three times. Then, 20µl of each well were taken and living IJs were counted using a binocular. For each time point, two wells per treatment were counted and concentration per ml calculated. After 140 hours, a sample of the suspension was taken and checked for PCL1391-gfp bacteria. For this, 50µl were plated onto KB^{++G} plates and incubated for 24 hours at 24°C. Afterwards, plates were checked for bacteria under the fluorescence microscope.

2.5.1. Method optimization

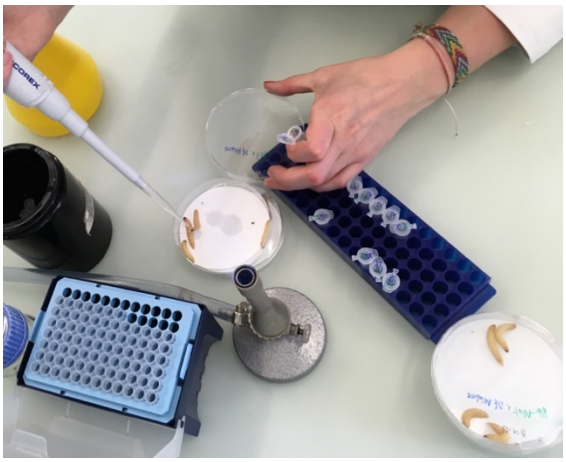
For the first experiment, contamination between the wells, loss of moisture in wells and the length of the experiment caused problems for evaluation. Therefore, second repetition was optimized by using PCR-plates instead of 96-well plates. PCR-plates were sealed with an adhesive film to avoid moisture loss and contamination among wells. Three small holes per well were made with a needle to provide fresh air for EPNs. Moisture loss still occurred after 72 hours and some tap water had to be added prior to counting. In the second repetition additionally to exposing the nematodes to a bacterial concentration of 10^6 cfu/ml, they were also exposed to a concentration of $5x10^7$ cfu/ml. The length of the experiment was shortened in the second repetition to 146 hours. The time point when plating the suspension for survival of bacteria was changed to 48hpi. 100µl were plated and additionally, a dilution serious up to 10^{-5} was done for calculating bacterial concentration.

2.6. Co-infection assay of *G. mellonella* to evaluate the compatibility of nematodes and pseudomonads

Compatibility of entomopathogenic nematodes and pseudomonads was tested by co-infection of *G. mellonella* larvae. Larvae were directly infected with different single EPN and EPP strains as well as with the combination of EPN x EPP. For pseudomonads, the gfp-tagged *Pseudomonas chlororaphis*

strain PCL1391-gfp was used. Survival of larvae over time was observed. After death of *G. mellonella* larvae, cfu per larva was calculated. Two independent repetitions were performed.

Four healthy *G. mellonella* larvae were placed on a filter paper in a petri dish. For the control and all single treatments, one petri dish with four larvae was infected. For the control treatment 1ml autoclaved H_2O was pipetted on the filter paper next to the larva. For single EPN treatments, nematodes from the storage at 10°C were used. Concentration was adjusted to 1000IJ/ml. For the single *Pseudomonas* treatment, PCL1391-gfp overnight cultures were used at a concentration of 10^6 cfu/ml. For combinations, suspensions prepared for single treatments were mixed in equal parts. Afterwards, 1ml of the suspensions were pipetted on the filter paper next to the larvae (Picture 8). Two petri dishes with four larvae were infected for combined infections.



Picture 8: Infection of G. mellonella *larva with PCL1391-gfp suspension.*

Survival of larvae was observed regularly for the next ten days. After 72 hours, two larvae of the control treatment, two of each single treatment as well as four larvae from each EPN x EPP treatment were checked for presence of pseudomonads inside the larvae. Therefore, larvae were washed, and surface sterilized by bathing them for 20 seconds in H_2O with Extravon (soap), 20 seconds in 70% ethanol and 20 seconds in autoclaved H_2O. Larvae were then added to an Eppendorf tube containing 1ml saline (0.9%NaCL). In this tube they were blended with a Polytron PT-MR2100, 500W and put on ice. 100µl of each larval smash was pipetted into one well of a cell culture plate. A dilution series up to 10^{-5} was done and pipetted onto squared KB^{++G} plates (see chapter 2.2). Colonies of the countable dilutions were counted under the fluorescence microscope and cfu per *G. mellonella* larva was calculated. For calculation, initial volume of Galleria mixed with saline in Eppendorf tube was estimated to have a total volume of 2ml.

In the first repetition, emerging nematodes from dead *G. mellonella* larva were checked for PCL-gfp bacteria. Dead larva from first infection were placed on white traps for two weeks (as described in chapter 2.1.2) and emerging nematodes were collected. Nematode suspension was checked for PCL1391-gfp bacteria under the fluorescence microscope.

2.6.1. Method optimization

By mixing the suspension used for the single treatments, concentrations in combined treatments were divided into half. After the first experiment, separate suspensions for the combined treatments were prepared with a concentration of $2x10^6$ cfu/ml for the EPP combinations and 2000IJ/ml for EPN combinations.

2.7. *In vivo* assay to evaluate the potential of nematodes as vectors for pseudomonads

To investigate if nematodes can act as vectors for pseudomonads to enter the hemolymph of insects, the co-infection experiment (see chapter 2.6) was adjusted. *G. mellonella* larvae were infected with combinations of PCL1391 and five different EPN strains as well as with the single entomopathogens. It was assessed if EPN can carry pseudomonads from one larva into another. Therefore, new larvae were infected and the number of cfu per larva was calculated. To test nematodes as vectors for pseudomonads, two different approaches were performed.

For both approaches, four healthy *G. mellonella* larva were placed on a filter paper in a petri dish. For treatments containing single EPN and for the single PCL1391-gfp treatment, three dishes were prepared. For the control treatment, two dishes were infected. Infection of G. mellonella larva was performed similar to infection described in chapter 2.6. After infection, petri dishes were placed in the dark at 24°C and survival was observed every 12 hours for the next three days. After 50 hours hemolymph and whole larvae were checked for presence of PCL1391-gfp bacteria. To check for PCL1391-gfp in hemolymph, larvae were first paralyzed by putting them 10 minutes on ice. Afterwards, they were washed for 20 seconds in H_2O with Extravon (soap) and then surface sterilized by bathing them for 20 seconds in 70% ethanol. After sterilization, larvae were dried on a tissue and hemolymph was collected by cutting off the second last pseudopod on the right (Picture 9). Larvae had to be squeezed to pipette off hemolymph. 10µl were diluted in 90µl saline (0,9%NaCl) and these 100µl plated on round KB^{++G} plates. Remaining hemolymph was plated directly on KB^{++G} plates. Plates were incubated for two days at 24°C. After two days, colonies were counted under the fluorescence microscope and cfu per ml hemolymph was calculated. The squeezed larva from which hemocoel was taken were blended, serially diluted up to 10^{-5} and plated on square KB^{++G} plates (see chapter 2.6). Cfu per *G. mellonella* larva was computed.

Picture 9: Taking hemolymph from infected G. mellonella *larva.*

2.7.1. Transmission of pseudomonads into *G. mellonella* on soil

In a first approach, a dead *G. mellonella* larva from the first infection were placed on autoclaved soil and covered with a petri dish. Four living larvae were added to the soil, unable to touch the dead larva. Nematodes emerging from the dead larva had to make their way through soil to infect living larvae. To investigate if nematodes can act as vectors for pseudomonads into the hemolymph of the living larvae, the presence of PCL1391-gfp inside the added larvae was assessed.

After seven days, one dead larva from previous infection was placed in a plastic container filled half with autoclaved Jiffy substrate. For each EPN x EPP combination and the single PCL1391-gfp (control) treatment, four containers with larvae were prepared. Two containers were prepared for the single nematode treatment. The dead larva was covered with small petri dishes (ø30mm) which reached 1cm deep into the substrate. 25ml autoclaved tap water was added to the containers, which were then placed in the dark at 24°C. After seven days, four living *G. mellonella* larvae were placed on the soil around the dead covered larva (Picture 10). The dead larva and the four living larvae were not able to touch each other. Survival of these four larvae was observed regularly for five days. 100 hours past infection, presence of PCL1391-gfp in surrounding larva was examined (see chapter 2.6).

Picture 10: Four living G. mellonella larva added to the container in which dead larva from first infection were covered with petri dish.

2.7.2. Transmission of pseudomonads by surface sterilized nematodes

For the second approach, nematodes emerging from the first infection (see chapter 2.7) were collected and surface sterilized. Living *G. mellonella* larvae were directly infected with surface sterilized nematodes. The number of cfu per larva and cfu per ml hemolymph from the second infection was assessed. Nematodes from single- as well as from combined infections were used.

Four days after first infection, four larvae per treatment were placed on white traps and incubated in the dark (see chapter 2.1.2). After ten days, nematodes were collected. 200µl of nematode-water suspension was plated onto round KB^{++G} plates to check for PCL1391-gfp bacteria. Plates were incubated for two days at 24°C. After two days, PCL1391-gfp colonies were counted under the fluorescence microscope. Remaining nematodes were surface sterilized by washing them several times in Ringer's solution, 0.01%$HgCl_2$ solution and autoclaved tap water (Protocol 2, appendix). After surface sterilization, nematodes were checked for survival and PCL1391-gfp bacteria. Therefore, 200µl were plated onto round KB^{++G} plates. Plates were incubated for two days at 24°C and PCL1391-gfp colonies were counted under the fluorescence microscope.

For each treatment, two petri dishes containing four *G. mellonella* larvae were infected with surface sterilized nematodes (see chapter 2.6). Concentrations of nematodes were not adjusted. For each treatment, two petri dishes containing four *G. mellonella* larvae were infected. For single nematode treatments, nematodes were taken from the storage at 10°C as a control. Concentration was adjusted to 1000IJ/ml. One milliliter of this suspension was pipetted on the filter paper next to the larvae. Infected larvae were placed in the dark at 24°C and survival was recorded regularly for the next two days. At 39hpi, four larvae of combined treatments were checked for presence of pseudomonads in larvae and hemolymph (see chapter 2.6).

2.8. Statistics

Data were collected with Microsoft Excel and statistical analyses were computed with the program SPSS from IBM. First, data were tested for normality performing a Shapiro-Wilk test. For comparing root colonizing ability and survival of IJ's when exposed to PCL1391-gfp, a Mann-Whitney U test was performed. To determine statistical differences among treatments for pupation rate, pupal size, fly emergence rate and cfu/larva, a Kruskal-Wallis test was performed. As a post hoc test, pairwise comparisons following a one-way ANOVA were performed. Significance values were adjusted by the Bonferroni correction for multiple tests. Based on the pairwise comparison, groups were created manually in the graphs. Survival of *G. mellonella* larvae was analyzed by a Kaplan-Meier test. A Log Rank (Mantel-Cox) test was performed to compare survival between treatments. Statistical significance was for all tests was set to $p=0.05$.

Data are displayed in boxplots created with SPSS. Boxes display the interquartile range from the 25^{th} percentile to the 75^{th} percentile, equal to 50% of all values. The solid line inside the Box is the median of all data values. Whiskers represent the minimum and maximum of the data set up to the 1.5^{th} multiplication of the interquartile range. Outside this range, values are considered as outliers and displayed separate from whiskers as round dots.

3. Results

3.1. Radish greenhouse experiment to test entomopathogenic *Pseudomonas* for the control of *D. radicum*

Root colonization ability of two different pseudomonad strains was evaluated under greenhouse conditions. Therefore, colony forming units (cfu) per g bulb were calculated. In the same setup, effects of selected *Pseudomonas* strains on the pupation rate, pupal size and fly emergence of *D. radicum* were tested. Twelve samples were evaluated for each treatment. Root colonization ability was tested in three independent repetitions. Effects on development of *D. radicum* was assessed in two independent repetitions.

3.1.1. Root colonization ability of different fluorescent *Pseudomonas* strains

To determine the colonization ability of entomopathogenic pseudomonads on the bulbs of radish plants, colonization was measured as colony forming units (cfu) per g bulb fourteen days after inoculation. For measuring cfu, bulbs were shacked in saline. Saline was then plated on a medium which is selective for fluorescent *Pseudomonas* and colonies counted. But medium was not selective enough and other bacteria besides fluorescent *Pseudomonas* grew on the plates. Therefore, it was necessary to count only colonies of distinct pseudomonads. For the strains PCL1391-gfp and CHA0-gfp counting was facilitated as they are gfp-tagged and could be distinguished under the fluorescent microscope. For the strains 64 and 77 which are not gfp-tagged, a validation with PCR was necessary. Three PCRs were conducted, but in all three runs no sample was amplified, not even the positive

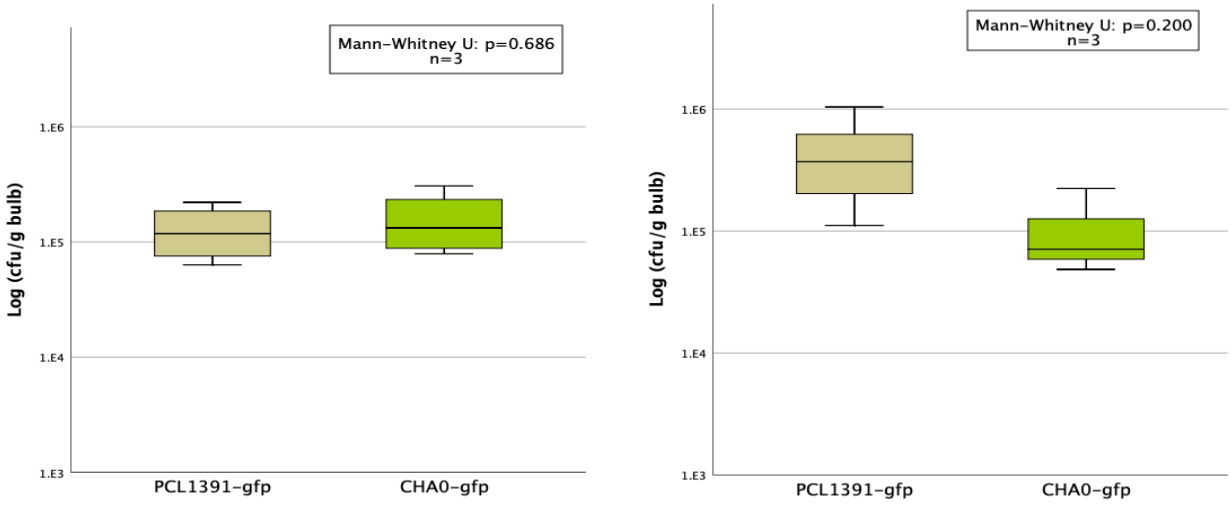


Figure 2: Root colonization ability of P. chlororaphis *PCL1391-gfp and* P. protegens *CHA0-gfp, assessed as cfu per g bulb fourteen days after inoculation of the soil with 1x10^7 cfu/g (first repetition left and second repetition right). Presented data derive from three bulbs per treatment.*

control. PCR was not further optimized due to time restrictions, but overnight LB cultures from all colonies are still stored at -20°C.

In all repetitions, both tested strains were able to colonize the radish bulbs within fourteen days (Figure 2). Bacteria were present in all bulb samples. Colonization was similar for both strains in the first

repetition (p=0.686). On average, bacterial concentration was just above 1x10^{5}cfu/g bulb. In the second repetition of the experiment, colonization was higher for the strain PCL1391-gfp. (Figure 3).

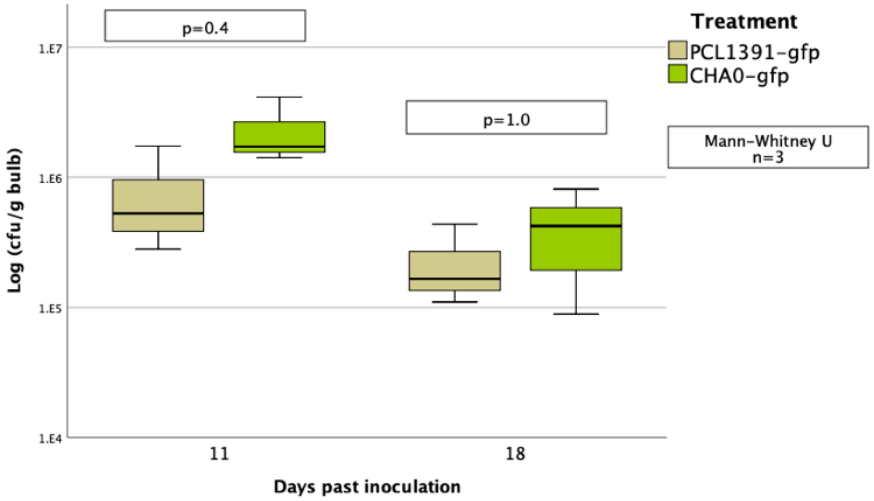


Figure 4: Root-colonizing ability of PCL1391-gfp and CHA0-gfp assessed as cfu per g bulb eleven and eighteen days after an inoculation of the soil with 1x10^{7}cfu/g soil. Presented data derive from three bulbs per treatment at each timepoint.

Root colonization ability was not significantly different between strains for both repetitions. For all samples, the colonization was lower than the initial concentration of 1x10^{7}cfu/g soil.

In the third repetition of the experiment, bulb colonization after eleven and eighteen days was assessed for both strains (Figure 3). The concentration after eleven days was about 1x10^{6}cfu/g bulb for PCL1391-gfp and CHA0-gfp. Over time, concentration decreased for both strains. Eighteen days after inoculation, concentration of bacteria around bulbs had decreased for both strains but was still above 1x10^5 cfu/g bulb. An overall comparison across treatments with a Mann-Whitney-U test showed no significant difference between strains (p=0.337). Also, difference in bacterial concentration over time within the same treatment was not significant (p=0.2 for PCL1391-gfp, p=0.1 for CHA0-gfp).

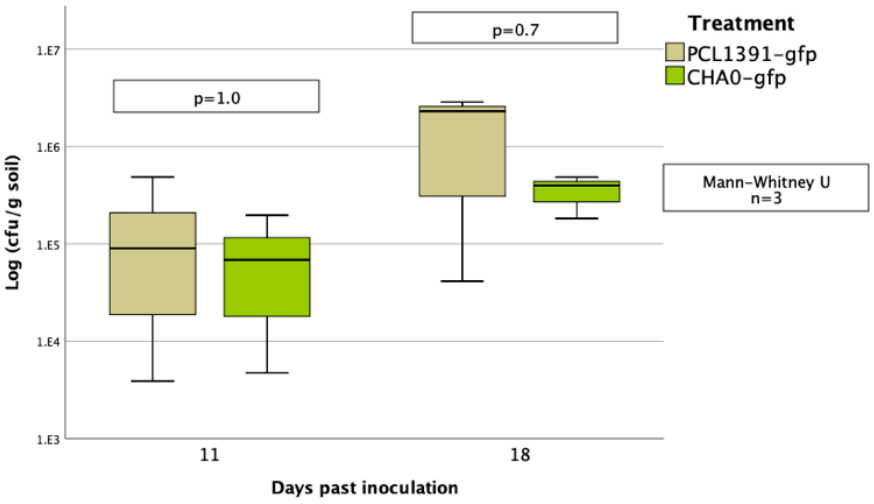


Figure 3: Soil-colonizing ability of PCL1391-gfp and CHA0-gfp assessed as cfu per g soil eleven and eighteen days after inoculation of the soil with 1x107 cfu/g soil. Presented data derive from three soil samples per treatment at each timepoint.

While concentration of pseudomonads on the bulb decreased over time for both strains, concentration in the soil increased for PCL1391-gfp and CHA0-gfp (Figure 4). This increase over time is not significant within treatments (p=0.4 for PCL1391-gfp, p=0.2 for CHA0-gfp). No significant difference in cfu/g soil was observed between PCL1391-gfp and CHA0-gfp.

3.1.2. Effect of entomopathogenic *Pseudomonas* on development of *D.radicum*

The effect of the four EPP strains PCL1391, CHA0, 64 and 77 on the development of *D. radicum* was examined in the greenhouse. Radish plants grown in substrate were inoculated with bacteria and eggs were added. Pupation rate, pupal size and fly emergence rate was assessed. Three independent repetitions were performed. Due to bad egg quality in the first repetition, only the second and third repetition could be evaluated.

Effect on pupation rate. To investigate the effect of different EPP on *D. radicum,* pupation rate was calculated. Counted larva were divided by the number of added eggs per pot. In the second repetition ten eggs were added, while in the third repetition fourteen eggs were added per pot. Average pupation rate of the control was between 31% and 36% for both repetitions and showed a high variance (Figure 5). In general, average pupation rate was lower or equal in pots inoculated with EPP. Variances in

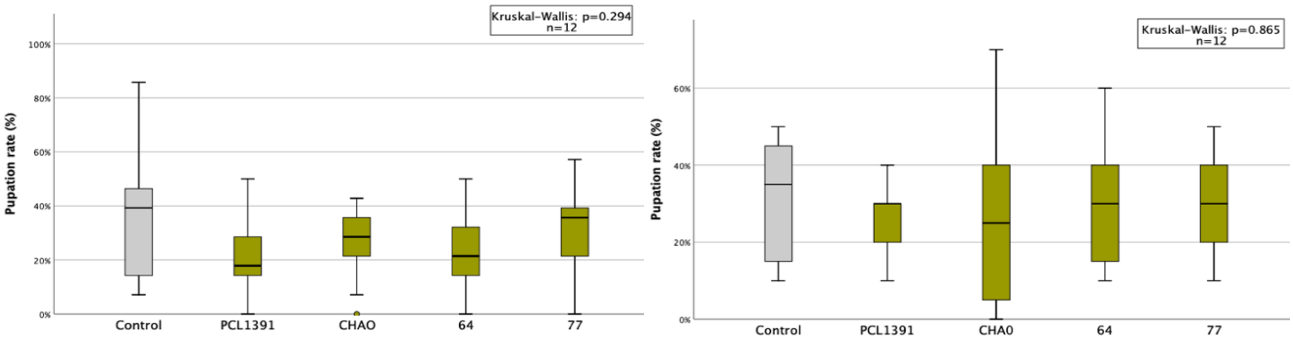


Figure 5: Pupation rates of D. radicum eggs on radish plants in the greenhouse which were inoculated with different Pseudomonas-strains (second repetition left and third repetition right). Presented data derive from twelve replicate pots per treatment. Per pot, ten eggs were added in the first repetition and fourteen eggs in the second repetition.

pupation rate were higher for the second repetition than in the third repetition. PCL1391 reduced the pupation rate in both repetitions most (Figure 5). In the first repetition, application of PCL1391 and CHA0 reduced average pupation rate by 15% (± 2%) compared to the control (Table 6, appendix). Application of the strain 64 reduced average pupation rate by 5%. Application of the strain 77 did not reduce average pupation rate. Overall comparison showed no significant differences (p=0.865). A reduction of 43% on average pupation rate compared to control was observed for PCL1391 in the second repetition. Treatments with CHA0 and 64 reduced average pupation rates by about 30% (± 5%). Application of the strain 77 reduced average pupation rate by 15%. An overall comparison showed no significant differences between treatments (p=0.294).

Effect on pupal size. To investigate effects of EPP on the development of *D. radicum*, the average size of pupae within one sample was calculated by ImageJ. On average, sizes ranged from 9mm^2 to 12mm^2 (Figure 26, appendix). In the first repetition, variances in pupal size in the control group were high. The sizes in this group ranged from 8.8mm^2 to 10.8mm^2. In the second repetition, variances of all treatments were lower. In general, no differences across treatments could be observed (p=0.259 for first repetition, p=0.723 for second repetition). Pupal size varied in the third repetition between

9.5mm^2 and 12mm^2 with low variances in all treatments. In the control and PCL1391-gfp group, two outliers occurred with very low pupal sizes.

Effect on fly emergence. Fly emergence rates were calculated by dividing the number of emerged flies by the number of added eggs per pot at about 60 days after they were added. Fly emergence for twelve pots per treatment were assessed and means compared. In the second repetition, the fly emergence rate of all groups was very low, especially for the control treatment with only 15%. Fly emergence rates of CHA0, PCL1391 and 77 treatments were on average higher than in the control (Table 6, appendix). Only the treatment with strain 64 reduced average fly emergence by 33% compared to the control in this repetition. No significant differences were observed across treatments. Fly emergence for the third repetition was on average 30% for the control and below 20% for all EPP treatments (Figure 6). Variance of the control group was the highest, compared to all the other

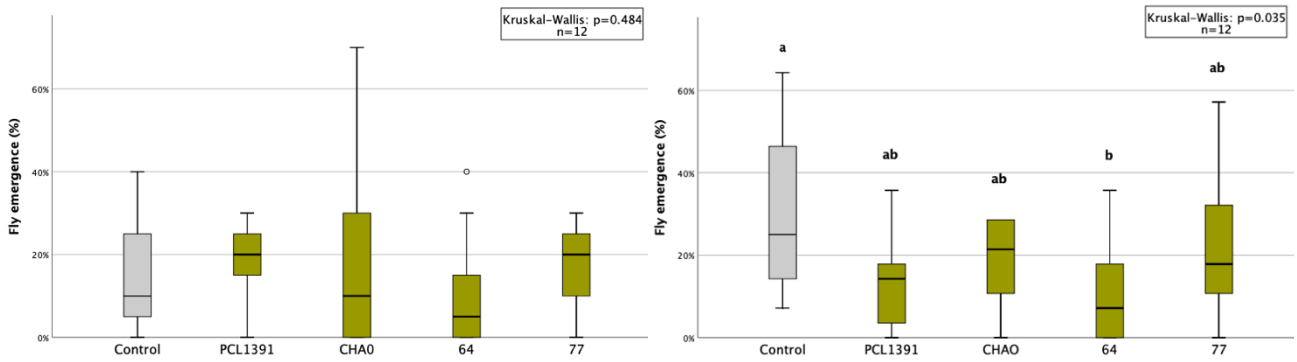


Figure 6: Fly emergence from D. radicum *eggs on radish plants in the greenhouse which were inoculated with different Pseudomonas-strains (second repetition left and third repetition right). Presented data derive from twelve replicate pots per treatment. Per pot, ten eggs were added in the first repetition and fourteen eggs in the second repetition.*

treatments. On average, fly emergence of the control group was the highest and treatment with 64 the lowest. An overall comparison showed a significant difference across groups (p=0.035). Pairwise comparisons showed that fly emergence of the treatment with *P. clororaphis* strain 64 was significantly lower than fly emergence rate in the control (p=0.039). The other treatments also reduced fly emergence compared to the control, but reduction was not significant (p>0.05).

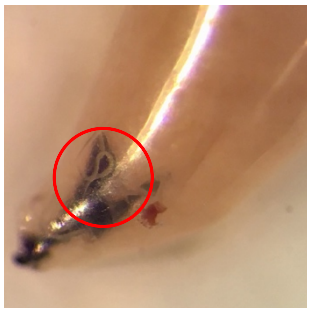
3.2. Evaluation of entomopathogenic nematode strains for *D. radicum* control

The susceptibility of five EPN strains to *D. radicum* larva was tested in two experimental setups. In the first, larvae were directly infected with different EPN and checked for infestation under the microscope. In the second, the effect of five different EPN strains on the development of *D. radicum* eggs on radish bulbs was examined under controlled conditions in the climate chamber. Pupation rate, pupal size and fly emergence were computed for each treatment as described in chapter 3.1.2. Four independent repetitions were performed. In each repetition, eight pots per treatment were evaluated.

3.2.1. Direct infection of *D. radicum* larvae with nematodes

After direct infection, all EPN-strains were able to invade at least one of four *D. radicum* larvae. Living nematodes were detected under the light microscope (Picture 12). To investigate the effect of different EPN-strains on mortality of *D. radicum*, survival is displayed with a Kaplan-Meier plot (Figure 27, appendix). First larva died 18 hours after infection. In the next 80 hours, larvae of all treatments died slowly. The highest mortality was observed for Sf RS5 with 58% dead larvae after 100 hours, followed by Sf enema with 50% (Table 7, appendix). It was lowest for Sf MG608 with 16%, followed by Sf MG594 and Hb enema with 33%. Sf RS5 killed significantly more larva than Sf MG608. EPN could not be detected in all infected *D. radicum* larvae and typical signs of nematode infection, like coloring was observed only in some (Picture 11).

Picture 11: D. radicum *larvae ten days after they were infected with EPN.*



Picture 12: Dead D. radicum *larva infested b Hb enema.*

3.2.2. Effect of different EPN on *D. radicum* development on radish bulbs

In general, average pupal sizes, pupation rates and fly emergence rates varied strongly among treatments and repetitions (Table 3). Pupation rates and fly emergence rates were low in the first two repetitions for nearly all treatments, including the control. Pupal sizes were low in the second and third repetition, especially for the control treatment. At the same time the standard deviation was high for most parameters and not many significant reductions compared to the control were observed.

Effect on pupation rates. On average, pupation rates were between 30% and 50% for the control groups in all repetitions with highest pupation rates in the last two repetitions (Table 3). All treatments with EPN showed lower average pupation rates, except in the first repetition where pupation rate of

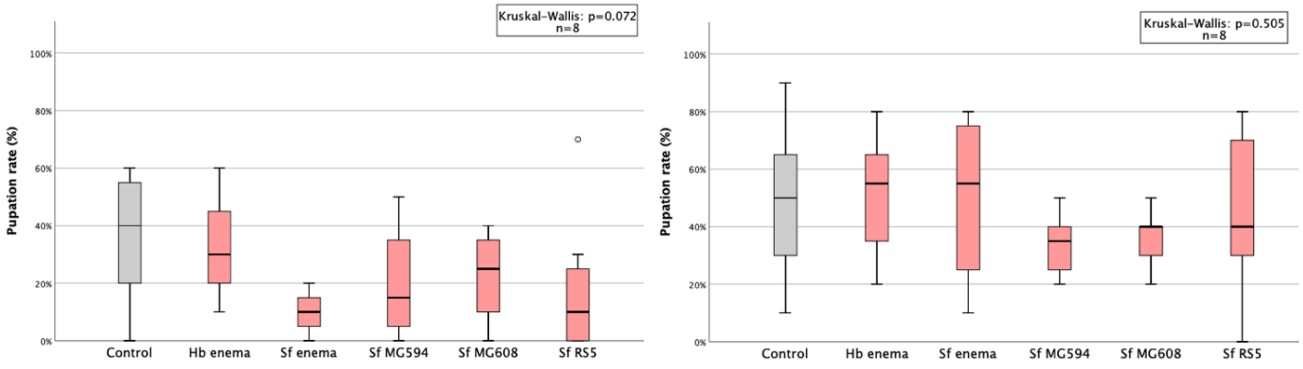


Figure 7: Pupation rate of D. radicum eggs added to pots filled with sand and radish bulbs in the climate chamber which were infested with different EPN-strains (second repetition left and fourth repetition right). Presented data derive from eight replicate pots per treatment with twelve eggs per pot.

the control group was the lowest with 33% (Table 3). Variances were high for most treatments in all repetitions. A few treatments with EPN had small variances in some repetitions. However, the small variances in these few treatments were not consistent over repetitions. When small variances were

observed, average pupation rates were lower than in the control group. Sf enema reduced average pupation rate by 72% compared to the control group in the second repetition (Figure 7), but this reduction was not consistent over repetitions. Sf MG594 and Sf MG608 reduced the average pupation rate by 31% (± 4%) in the second repetition and by 28% (± 3%) in the fourth repetition compared to the control. Overall comparison of treatments showed no significant difference in pupation rate in any repetition. In general, all EPN treatments, except Hb enema, reduced the pupation rate in one or two repetitions. However, reduction by any EPN strain is inconsistent over repetitions and no tendency could be observed.

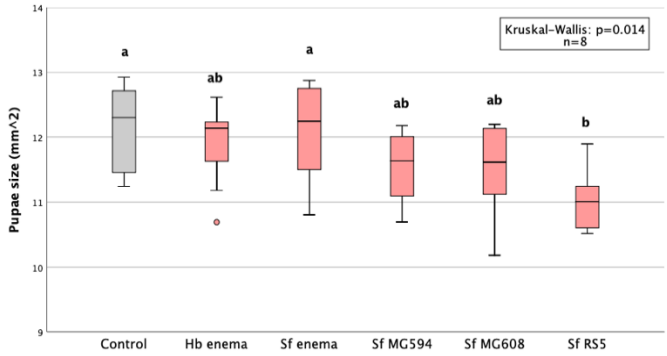


Figure 8: Pupal size of D. radicum pupae added to pots filled with sand and radish bulbs in the climate chamber which were infested with different EPN-strains (first repetition). Presented data derive from eight replicate pots per treatment with twelve eggs per pot.

Effect on pupal size. In the first and third repetition, pupal sizes varied between 10mm^2 and 14mm^2, while in the fourth repetition, sizes varied from 6mm^2 to 14mm^2, showing the highest variances among all repetitions (Table 3). Sf RS5 reduced pupal size in the first and third repetition. In the first repetition, reduction was significant compared to the control (p=0.014) (Figure 8). Other than that, no significant differences were observed, also due to high variations within treatments. Sf enema reduced the pupal size in all repetitions compared to the control, except in the first. Sf MG594 and Sf MG608 reduce pupal size in all repetitions except in for fourth one. However, these reductions were not significant at the 5% level. In general, no clear effect of EPN treatments could be observed.

Effect on fly emergence. Fly emergence rates highly varied within treatments in all repetitions, except for the second repetition. There, fly emergence was in general low with emergence rates on average below 30% (Figure 9). Because of high variances, no significant differences between treatments could be observed in any repetition. A reduction in average fly emergence by all EPN treatments was observed in the second repetition (Figure 9). Hb enema did not reduce fly emergence in the first and fourth repetition, but in the second and third. Sf enema reduced fly emergence in the second and fourth repetition by 73% and by 7% compared to the control (Table 9, appendix). Sf MG594 reduced fly emergence in the second and third repetition most. Reduction varied between 52% and 10%. Sf MG608 reduced fly emergence in all

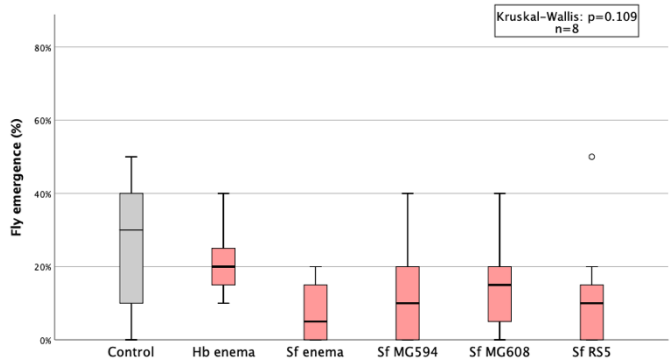


Figure 9: Fly emergence of D. radicum eggs added to pots filled with sand and radish bulbs in the climate chamber which were infested with different EPN-strains (second repetition). Presented data derive from eight replicate pots per treatment with twelve eggs per pot.

repetitions except for the first one by 43% in the second repetition, by 3% in the third repetition and by 18% in the fourth repetition. Sf RS5 also reduced fly emergence in all repetitions except the first one by 52% in the second repetition, by 37% in the third repetition and by 17% in the fourth repetition.

Table 3: Average pupal sizes, pupation rates and fly emergence rates when evaluating the effect of different entomopathogenic nematodes on the development of D. radicum *on radish bulbs in the climate chamber. Presented data derive from eight replicate pots per treatment with twelve eggs per pot.*

	Pupae size (mm^2)		Pupation rate (%)		Fly emergene rate (%)	
	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation
1. Repetition						
Control	12,14	0,69	33%	15%	30%	15%
Hb enema	11,91	0,64	53%	16%	42%	15%
Sf enema	12,08	0,75	47%	22%	41%	24%
Sf MG594	11,54	0,55	39%	19%	33%	9%
Sf MG608	11,5	0,75	42%	26%	31%	22%
Sf RS5	11,02*	0,46	42%	13%	34%	11%
2. Repetition						
Control	8,79	2,08	36%	23%	26%	19%
Hb enema	9,82	1,7	33%	18%	21%	10%
Sf enema	7,51	2,13	10%	8%	8%	9%
Sf MG594	8,26	2,02	20%	19%	13%	14%
Sf MG608	8,47	2	23%	16%	15%	13%
Sf RS5	10,02	1,82	18%	24%	13%	17%
3. Repetiton						
Control	12,09	1,25	50%	27%	39%	24%
Hb enema	11,55	0,65	51%	16%	35%	18%
Sf enema	11,6	0,56	59%	12%	43%	12%
Sf MG594	11,75	0,83	53%	18%	35%	18%
Sf MG608	11,79	0,27	49%	12%	38%	16%
Sf RS5	11,73	0,9	36%	17%	24%	13%
4. Repetiton						
Control	9,08	1,26	49%	26%	34%	19%
Hb enema	10,35	1,33	51%	20%	30%	14%
Sf enema	8,48	1,36	50%	28%	31%	27%
Sf MG594	10,08	1,14	34%	11%	24%	11%
Sf MG608	10,7	0,67	36%	9%	28%	14%
Sf RS5	10,23*	0,58	45%	27%	28%	26%

* indicates a significant difference compared to the control

3.3. Climate chamber assay to evaluate the combination of nematodes and pseudomonads for the control of *D. radicum*

To investigate the potential of a combined application of EPN and EPP, effects of single and combined applications on pupation rate, pupal size and fly emergence of *D. radicum* developing on radish bulbs were examined. Two independent repetitions with eight replicates per treatment were performed. The setup of the second repetition differed slightly from the first repetition. In the second repetition pots were moistened with sterilized tap water during the experiment. While PCL1391-gfp was used in the first repetition, wildtype PCL1391 was used in the second experiment.

3.3.1. Effect of a combined entomopathogenic nematode - *Pseudomonas* application on the development *of D. radicum*

Effect on pupation rate. Pupation rate was on average between 55% and 60% for control groups of both repetitions, with a variation from 0% to 110%. In the second repetition, one control pot had a pupation rate of 110%. In this case, either too many eggs were added to one pot, or larva migrated from another pot. In general, variance of pupation rate in both control groups was high. All treatments

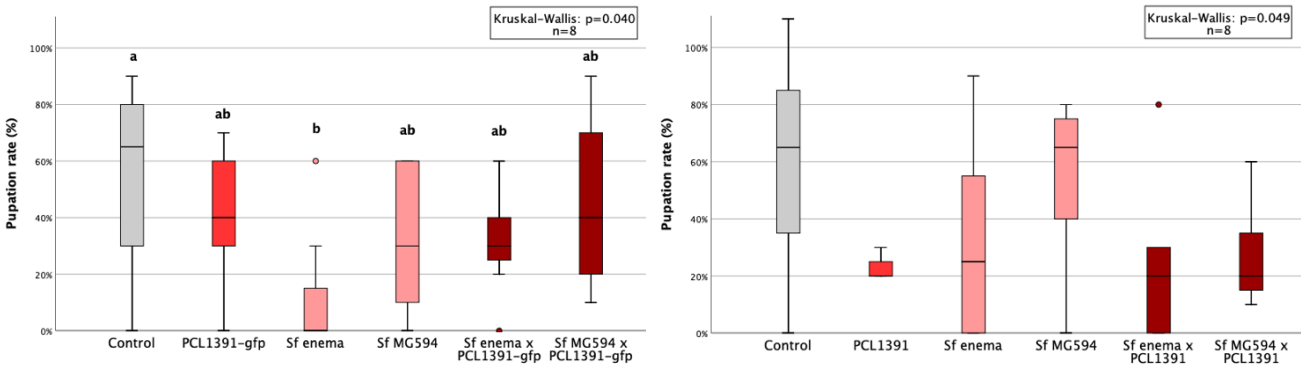


Figure 10: Pupation rates of the EPN – EPP combination assay. D. radicum *eggs were added to pots filled with sand and radish bulbs which were infested with two single EPN-strains, one single EPP strain and combinations of these EPP and EPN (first repetition left and second repetition right). Presented data derive from eight replicate pots per treatment with ten eggs added to each pot.*

reduced the average pupation rate compared to the control group in both repetitions (Figure 10). In the first repetition, average pupation rate was significantly reduced by the single application of Sf enema compared to the control. Single PCL1391-gfp treatment and combined Sf MG594 x PCL1391-gfp treatment reduced average pupation rate by 22% (± 3%) compared to the control (Table 8, appendix). The combination of Sf enema x PCL1391-gfp reduced average pupation rate by 43% compared to the control. Single application of Sf MG594 reduced average pupation rate by 41%. In the second repetition, the control treatment had a high average pupation rate of 60%. The combination of Sf enema x PCL1391 reduced average pupation rate by 58% compared to the control (Figure 11). Single PCL1391 treatment reduced the average pupation rate most by 62% with a very low variance. Both single EPN applications reduced average pupation rate by 48% for Sf enema and by 23% for Sf MG 594 compared to the control. The overall comparison of all treatments showed a slightly significant difference (p=0.049), but the pairwise comparison did not detect any significant differences among treatments after the Bonferroni correction for multiple tests.

Effect on pupal size. In the first repetition, average pupal sizes varied between 6mm^2 and 10mm^2. Variances were low for all treatments except for Sf enema. Pupal sizes from both combined applications were on average highest. The two single EPN treatments had slightly smaller pupal sizes compared to the control. The pupae from the PCL1391-gfp treatment showed a small reduction in size compared to the control. An overall comparison across treatments showed a significant difference in pupal size between the treatment Sf enema and Sf enema x PCL1391-gfp (Figure 11).

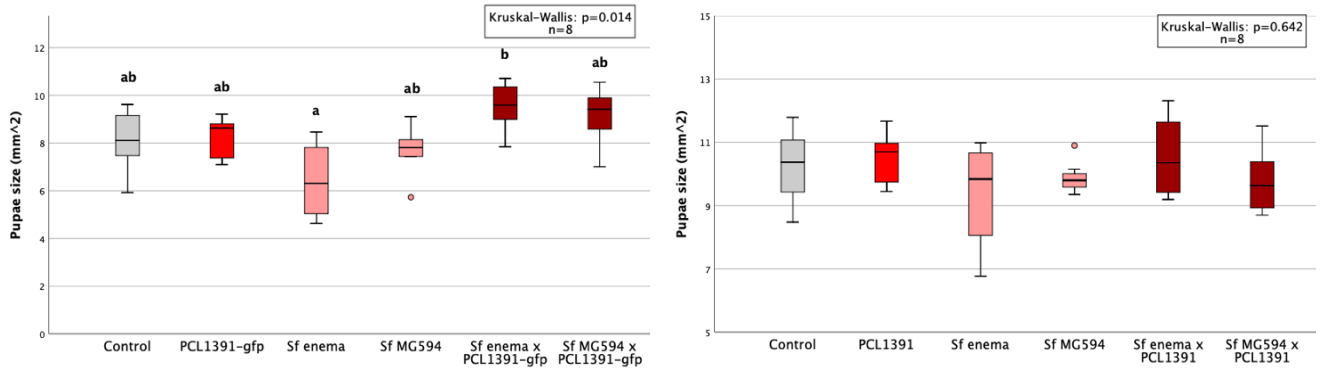


Figure 11: Pupal size from the EPN – EPP combination assay. D. radicum *eggs were added to pots filled with sand and radish bulbs which were infested with two single EPN strains, one single EPP strain and combinations of these EPP and EPN (first repetition left and second repetition right). Presented data derive from eight replicate pots per treatment with ten eggs added to each pot.*

In the second repetition average pupal size was between 9mm^2 and 11mm^2. In general pupae were bigger than in the first repetition. Variances were bigger in the second repetition except for Sf MG594. An overall comparison did not detect any significant differences in pupal size across treatments.

Effect on fly emergence. Fly emergence rate was very low for all treatments in the first repetition. The overall comparison showed a significant difference (p=0.014), but after Bonferroni correction, the pairwise comparisons were not significantly different (Table 9, appendix). Most flies emerged from the single PCL1391-gfp and combined Sf MG594 x PCL1391-gfp treatment, with an average

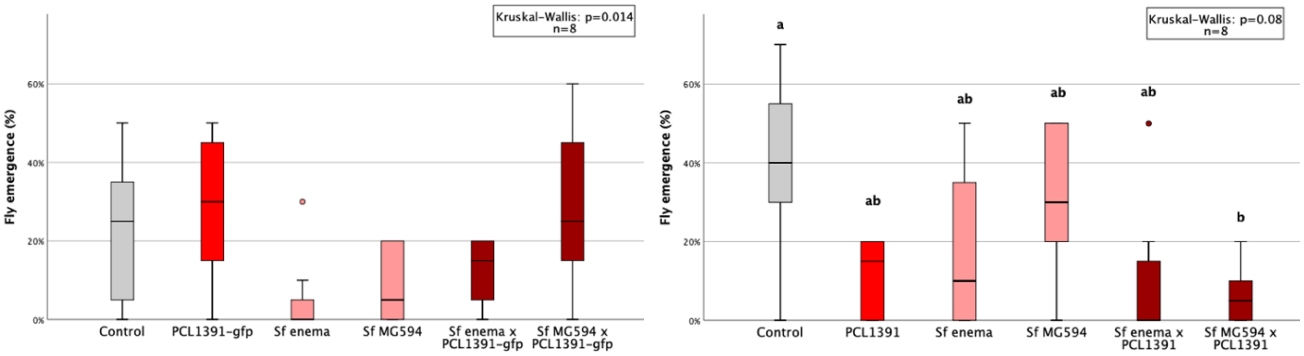


Figure 12: Fly emergence from the EPN – EPP combination assay. D. radicum *eggs were added to pots filled with sand and radish bulbs which were infested with two single EPN-strains, one single EPP strain and combinations of these EPP and EPN (first repetition left and second repetition right). Presented data derive from eight replicate pots per treatment with ten eggs added to each pot.*

emergence rate of 29% (Figure 13). Both were higher than the control which had an average fly emergence rate of 23%. The single EPN treatments could reduce average fly emergence by 88% for Sf enema and by 61% for Sf MG594.

In the second repetition, the control had an average emergence rate of 40% (Figure 13). Single EPP and both EPP x EPN combinations reduced average emergence rate to below 9% (± 2%). A significant difference in fly emergence was observed between the combination of Sf MG594 x PCL1391 and the control treatment. Sf enema and the combination of Sf enema x PCL1391 were the only treatments that reduced fly emergence in both repetitions. Reductions by all other treatments were less consistent and occurred only in one of the two repetitions.

3.3.2. Fluorescent *Pseudomonas* presence

After evaluating the first repetition of the experiment, sand, radishes and one dead larva were checked for gfp-tagged PCL1391 pseudomonads. Two sand and two radish samples were tested from the combined treatments of EPN and EPP and the single EPP treatment. PCL1391-gfp was present in all samples (Figure 13). Concentrations ranged from a few hundred cfu/g up to $1x10^7$ cfu/ g. In the sand and radish samples of Sf MG594 x PCL1391-gfp, concentrations were lower than in the Sf enema x PCL1391-gfp treatment. Bacterial concentration in the PCL1391-gfp treatment was lower than in the combined treatment of Sf enema x PCL1391-gfp. In the one larva tested from the Sf MG594 x PCL1391-gfp treatment, the concentration was $4x10^5$ cfu/g.

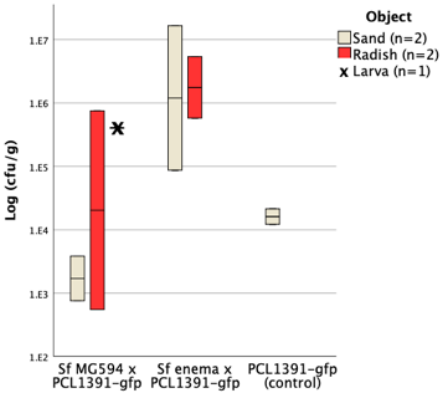


Figure 13: Concentrations of PCL1391-gfp bacteria in sand, radish and one dead larva after the end of experiment. Presented data derive from two replicates per treatment.

3.4. *In vitro* assay to evaluate the susceptibility of nematodes to pseudomonads

Susceptibility of EPN-strains to the entomopathogenic *Pseudomonas chlororaphis* strain PCL1391-gfp was tested to evaluate their compatibility as biological control agents. EPN and EPP were mixed in microtiterplates and incubated at temperatures favorable for EPP. Single EPN treatments with about the same amount of IJs were incubated at the same conditions. After 24, 72 and 144 hours, IJs were counted and the concentration of living infective juveniles per ml was calculated for mixed and single EPN suspensions. The survival of IJs in combined mixtures was compared to the survival of IJs in single EPN suspensions of the same strain. The experiment was first performed using two replicates per EPN strain, while all five nematode strains were tested. For this repetition, results were not reliable because some samples were cross contaminated with bacteria. For the second repetition, only the two strains Sf enema and Sf MG594 were tested as these strains were chosen for the combined application in chapter 3.3.

3.4.1. Survival of nematodes when exposed to *P. chlororaphis* PCL1391

Even though concentrations for single EPN and mixed EPN x EPP were adjusted to 1000IJ/ml, concentrations varied for EPN strains in the first repetition. For single and mixed Hb enema treatments and the single Sf RS5 treatment, concentrations of living IJs were higher than 1000IJ/ml. In the first repetition, two replicates per treatment were counted at each timepoint.

After 24 hours, no big difference in concentration was observed between single EPN treatments and respective EPN x EPP mixtures in the first repetition. After 144 hours, nearly all wells were empty and no living IJ could be counted neither in the wells with EPN alone nor in the combined treatments. In the first repetition, differences between single and mixed treatments could be observed best after 72 hours (Figure 14). For the combination of Sf MG608 x PCL1391-gfp as well as for the single Sf MG608 treatment, all IJs were dead after 24 hours. Therefore, this strain was excluded from analyses. Differences between single EPN and respective EPN x EPP mixtures were compared using a Mann-Whitney U test. No significant difference in concentration of living IJs between single EPN treatments and mixtures could be detected. But the number of replicates (n=2) was too low in this repetition to make a statistically correct statement.

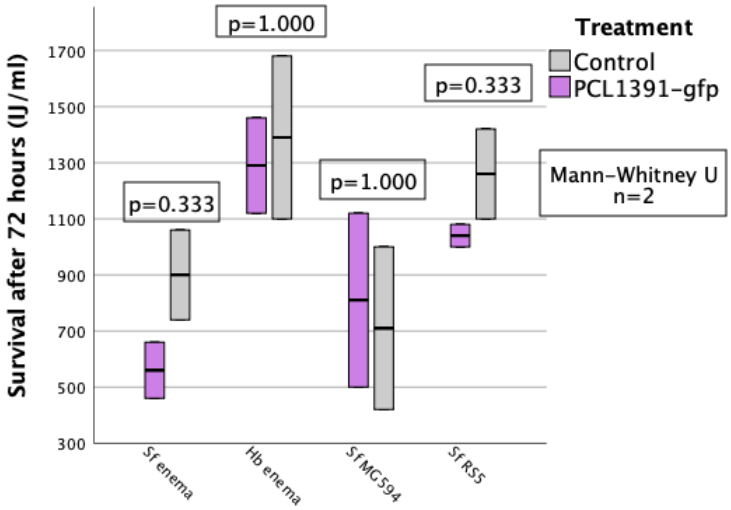


Figure 14: Survival of four different EPN-strains after 72 hours of exposure to 10^6 cfu/ml of PCL1391-gfp. Presented data derive from two replicates of each treatment.

In the second repetition, survival of IJs after 72 hours was measured for two concentrations of PCL1391-gfp with. EPN were exposed to concentrations of 10^6cfu/ml and $5x10^7$cfu/ml. For the single Sf MG594 treatment and its combination with PCL1391-gfp, all infective juveniles died already after 24 hours. Therefore, this strain was excluded from analyses. For the strain Sf enema, differences in survival of IJs between single and mixed treatments can be observed with both concentrations of PCL1391-gfp (Figure 15). The exposure to a concentration of $5x10^7$cfu/ml of PCL1391-gfp bacteria results in a lower survival of EPN after 72 hours. However, difference in survival is not significant (p=0.222) compared to the control treatment. Surprisingly, the survival of EPN after exposure to $1x10^6$cfu/ml was higher compared to the control treatment, but difference is not significant (p=0.056). Comparing the survival of nematodes exposed to a concentration of $5x10^7$cfu/ml to an exposure of 10^6cfu/ml shows a significant difference (Mann-Whitney U, p=0.016). Survival of

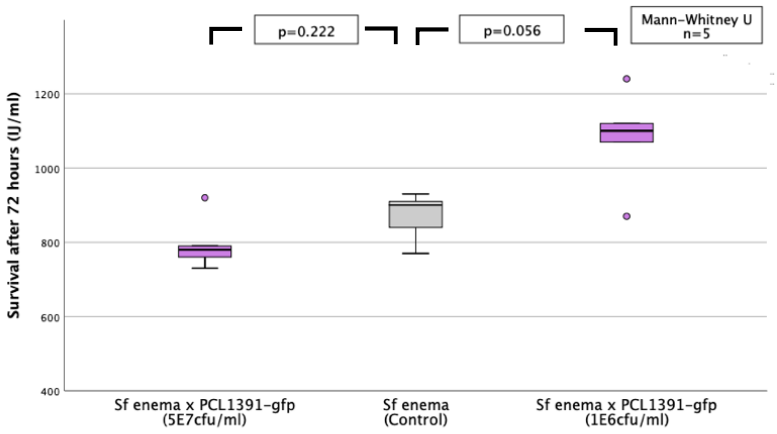


Figure 15: Survival of Sf enema after 72 hours of exposure to 10^6 cfu/ml and $5x10^7$ cfu/ml of PCL1391-gfp. Presented data derive from five replicates for each concentration.

nematodes was significantly higher when they were exposed to a concentration of 10^6cfu/ml compared to an exposition to a concentration of $5x10^7$cfu/ml.

3.4.2. Survival of pseudomonads when mixed with nematodes

Within the second repetition of the experiment, survival of bacteria after 48 hours was checked. For each concentration of PCL1391-gfp, two samples were taken for each treatment and bacterial concentration assessed. For all replicates treated with concentrations of 10^6 cfu/ml, no PCL1391-gfp pseudomonads were alive after 48 hours (Figure 16). At an initial concentration of $5x10^7$ cfu/ml, concentration decreased strongly after 48 hours. When PCL1391-gfp bacteria were exposed to Sf enema, concentrations decreased to about 10^4cfu/ml in both replicates. When exposed to Sf MG594, concentrations decreased slightly less to about 10^6cfu/ml in both replicates. For assessing the susceptibility of EPN to EPP the fact that bacteria were not present any more after 48 hours must be taken into account for the evaluation. The persistence of single PCL1391-gfp bacteria as a control was not assessed in this experiment.

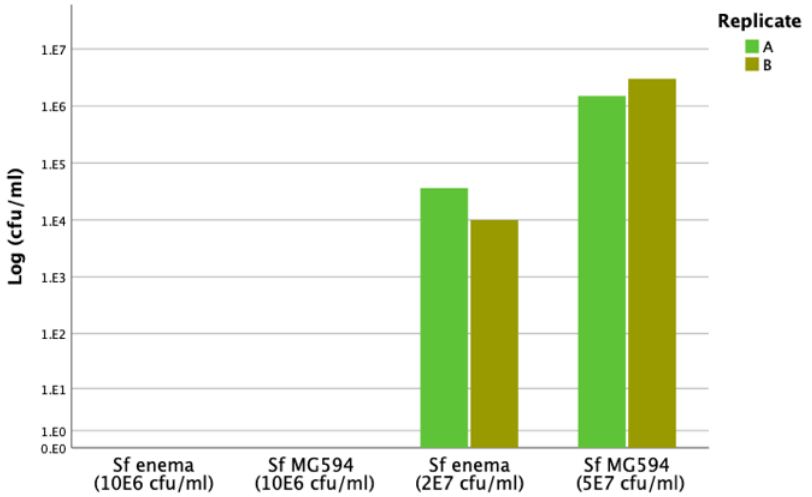


Figure 16: Cfu per ml of mixture in the two tested combinations after 48 hours. For each treatment, two replicates were checked for survival of pseudomonads.

3.5. Co-infection assay of *G. mellonella* to evaluate the compatibility of nematodes and pseudomonads

To investigate the compatibility of EPN and EPP the effect of single and combined applications on survival of *G. mellonella* larvae was evaluated. Effects of EPP on killing efficiency of EPN were evaluated by survival of larvae over time. The possibility of antagonistic or synergistic effects between EPN and EPP combinations in terms of pathogeny was evaluated.

3.5.1. Effect on survival of *G. mellonella* larvae

Survival of *G. mellonella* larva is assessed the first time at 72 hours after infection. From there on, survival is monitored every 24 hours. For the single applications of EPN and EPP, four replicates were performed, while eight were tested for the combined treatments. Larvae infected with EPN or a combination of EPN and EPP did not survive longer than 180 hours in both repetitions.

In the first repetition, survival was significantly higher for the control and for PCL1391-gfp compared to all other treatments (Figure 17). Log Rank test comparing the survival between single EPN and combined treatments for each strain was not significant for any EPN strain. Within the control

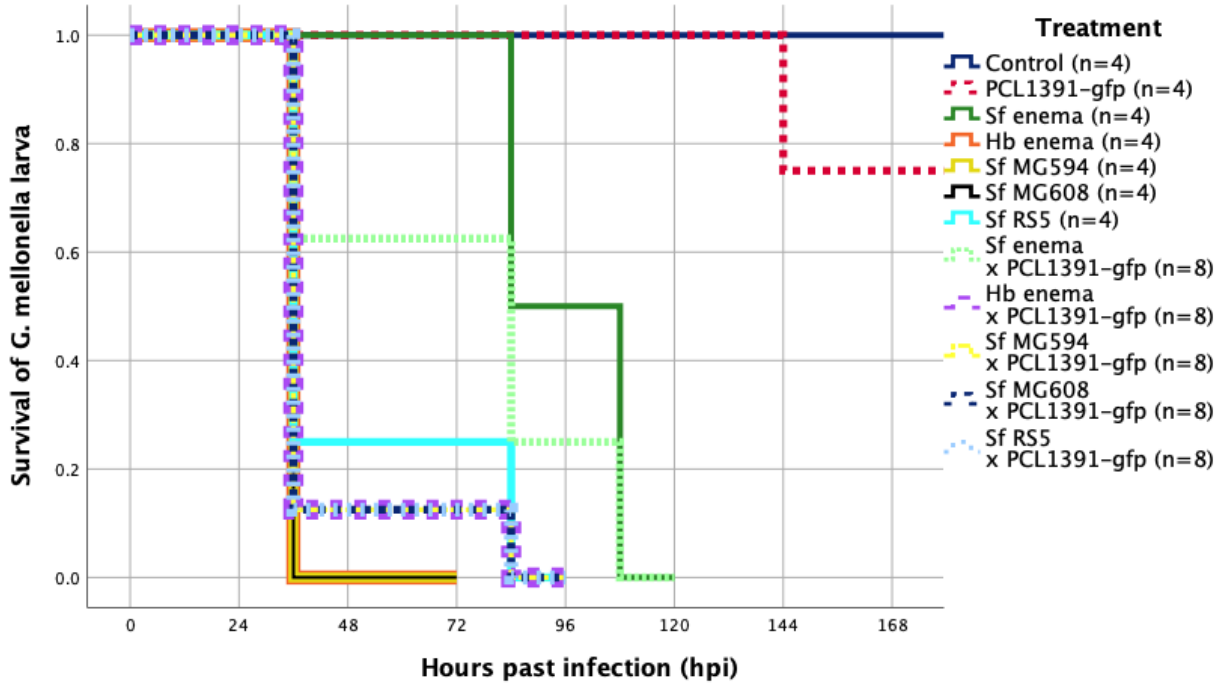


Figure 17: Survival of G. mellonella *larva over time after infection with different EPN-strains and their combination with PCL1391-gfp. Presented data derive from larvae infected with single EPN (1000IJ), single PCL1391-gfp (10^7cfu) or the combination of both (1000IJ plus 10^7cfu). For single treatments as well as for the control, survival of four replicates per treatment was observed. For combined treatments, survival of eight replicates per treatment was observed (first repetition).*

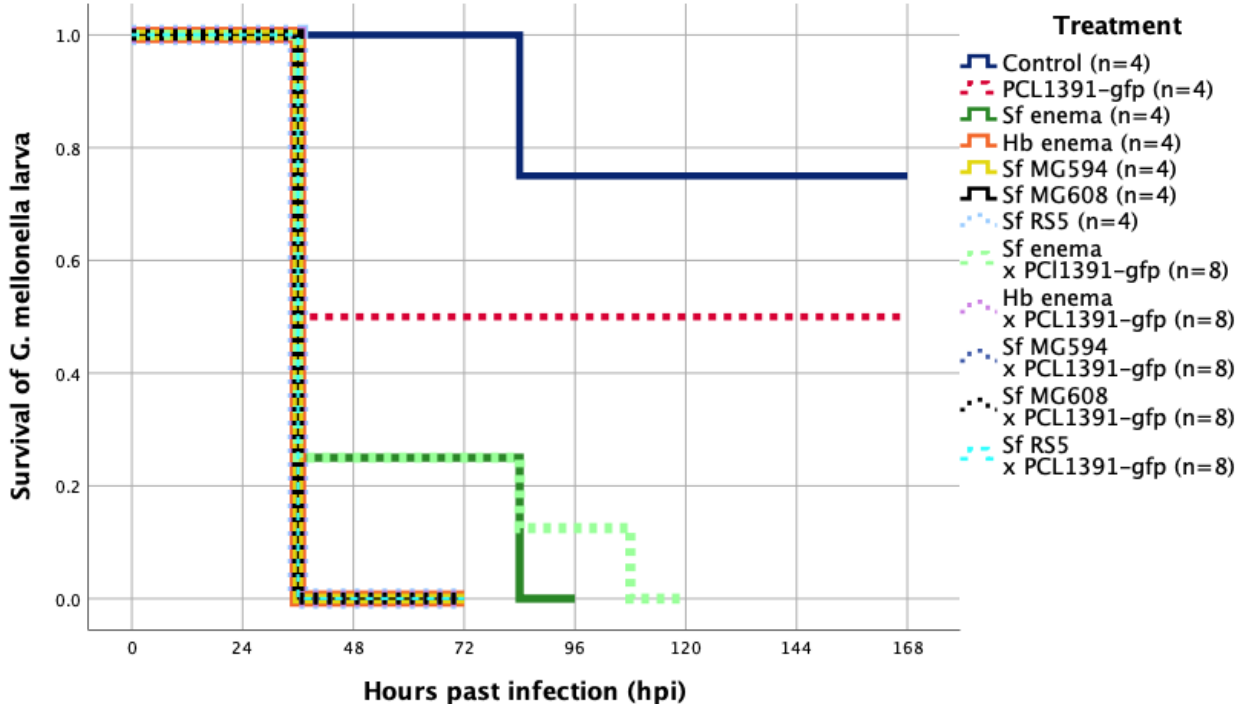


Figure 18: Survival of G. mellonella *larva over time after infection with different EPN-strains and their combination with PCL1391-gfp. Presented data derive from larvae infected with single EPN (1000IJ), single PCL1391-gfp (10^7cfu) or the combination of both (1000IJ plus 10^7cfu). For single treatments as well as for the control, survival of four replicates per treatment was observed. For combined treatments, survival of eight replicates per treatment was observed (second repetition).*

treatment, all larvae survived until the end of experiment at 250 hours past infection. From the PCL1391-gfp treatment, 75% of *G. mellonella* larvae survived until the end of the experiment. Larvae

treated with Sf enema, survived longer than with other EPN strains. Combination of Sf enema with PCL1391-gfp leaded to a faster death of larvae compared to the single Sf enema treatment. Log Rank test comparing the survival for each strain between single EPN and combined EPN x EPP treatments, shows no significance for any strain.

In the second repetition, survival in control and PCL1391-gfp treatments was lower than in the first repetition (Figure 18). One of the four larva from the control treatment died after 79 hours, which led to a survival of 75%. For the PCL1391-gfp treatment, survival reached 50% at the end of experiment. Larvae of all treatments containing EPN died latest 108 hours after infection. Larvae treated with Sf enema alone and in combination died later than the other EPN treatments. Larvae infected with Hb enema, Sf MG594, Sf RS5 and Sf MG608 alone or in combination died 36 hours after infection. Log Rank test, comparing the survival for each strain between single EPN and combined EPN x EPP treatments showed no significance for any treatment alike the first repetition.

3.5.2. Presence of pseudomonads in *G. mellonella* larvae after co-infection

To investigate, whether EPN are able to carry EPP into the hemolymph or if the penetration of the insect cuticula by EPN increases the concentration of PCL1391-gfp inside the host, bacterial concentration was determined as colony forming units (cfu) per larva 72 hours after infection with a combination of different EPN-strains and PCL1391-gfp. The single PCL1391-gfp treatment acted as a control. It was assumed that bacteria alone are not able to invade *G. mellonella* larva, however, cfu/larva were higher than expected in the PCL1391-gfp treatment in both repetitions (Figures 19 and 20). Combination with Sf RS5 had the highest concentration of pseudomonads in in the first repetition. On average, about 10^5 cfu/larva were present. Second highest concentrations were observed for Sf MG608 with an average of about 10^4 cfu/larva. Those two treatments were the only ones in which bacteria were present in all four tested larvae. Hb enema showed the lowest concentration with about 10 cfu/larvae and bacteria were present only in two out of four larvae. Sf enema and Sf MG594 were similarly colonized by bacteria and bacteria were present in three out of four larvae. Statistical analysis showed a significant difference in cfu/larva between Hb enema and Sf RS5 (Figure 19).

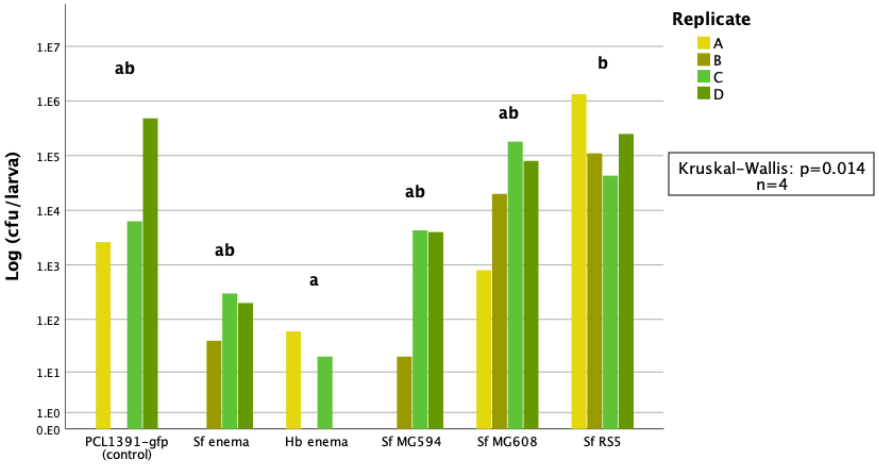


Figure 19: Concentrations of cfu/larva of PCL1391-gfp, 72 hours after infection of G. mellonella larva with combinations of EPN (1000IJ) and PCL1391-gfp (10^7cfu). Presented data derive from four replicate larvae for single PCL1391-gfp and combined treatments (first repetition).

In the second repetition, cfu/larva was highest for the PCL1391-gfp control treatment (Figure 20). Similar to the first repetition, concentrations of EPP were high for the Sf MG608 treatment and bacteria were found in all larva of this treatment. In combinations with Sf enema, Hb enema and Sf RS5, concentrations ranged between 10^2 and 10^4 cfu/larva. In larvae treated with Sf MG594, no bacteria were present in any of the four tested larvae. Therefore, cfu/larva was significantly different when comparing single PCL1391-gfp control treatment and the combination of Sf MG608 to the Sf MG594 combination.

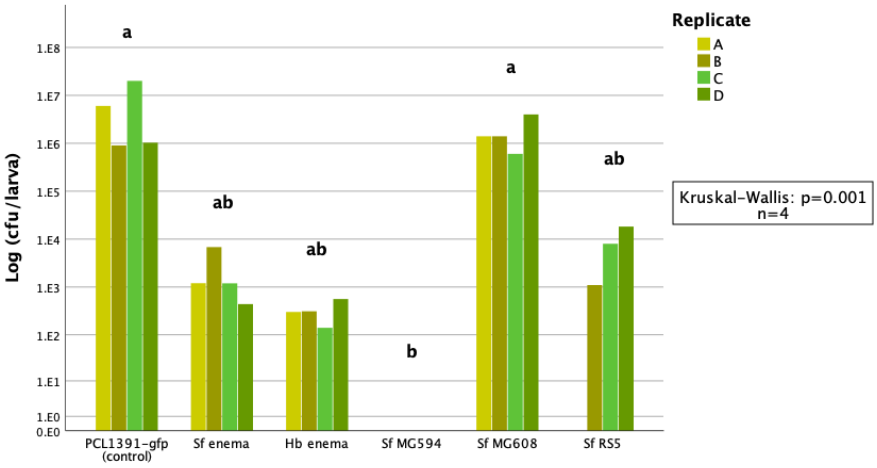
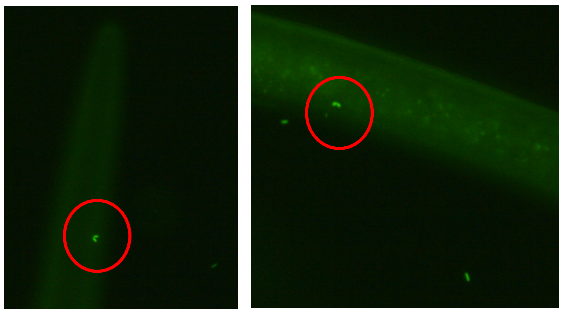


Figure 20: Concentrations of cfu/larva of PCL1391-gfp, 72 hours after infection of G. mellonella larva with combinations of EPN (1000IJ) and PCL1391-gfp (10^7cfu). Presented data derive from four replicate larvae for single PCL1391-gfp and combined treatments (second repetition).

After their death, larvae infected with combinations where placed on white traps. After EPN emerged from insect cadavers, they were collected and checked for PCL1391-gfp bacteria under the fluorescent microscope. In the combinations of PCL1391-gfp with Hb enema, bacteria were detected (Picture 13). It could not be determined, if these bacteria were in- or outside of EPN.



Picture 13: Nematodes from combined infections of Hb enema and PCL1391-gfp after they have emerged from G. mellonella larva under the fluorescent microscope.

3.6. *In vivo* assay to evaluate potential of nematodes as vectors for entomopathogenic pseudomonads

The possibility that nematodes can act as vectors for PCL1391-gfp was further investigated with this experiment. Two different approaches were used. For both approaches, larvae were either infected with five single EPN-strains or with the combination of EPN with PCL1391-gfp as in chapter 3.5. Survival of *G. mellonella* larvae and cfu/larva was assessed after first infection. All larvae infected with EPN and EPN x EPP combinations, died within 60 hours after infection. Control larvae treated with H_2O, survived until the end of experiment at 72hpi (Figure 21). Survival of control and PCL1391-gfp compared to all other treatments was significantly higher. Log Rank test comparing the survival for each strain between single EPN and combined EPN x EPP treatments showed no significance for any treatment, except for the Sf MG608 and Sf MG608 x PCL1391-gfp (p=0.016). Larvae treated with Sf MG608 x PCL1391-gfp died significantly faster than these with single Sf MG608 application.

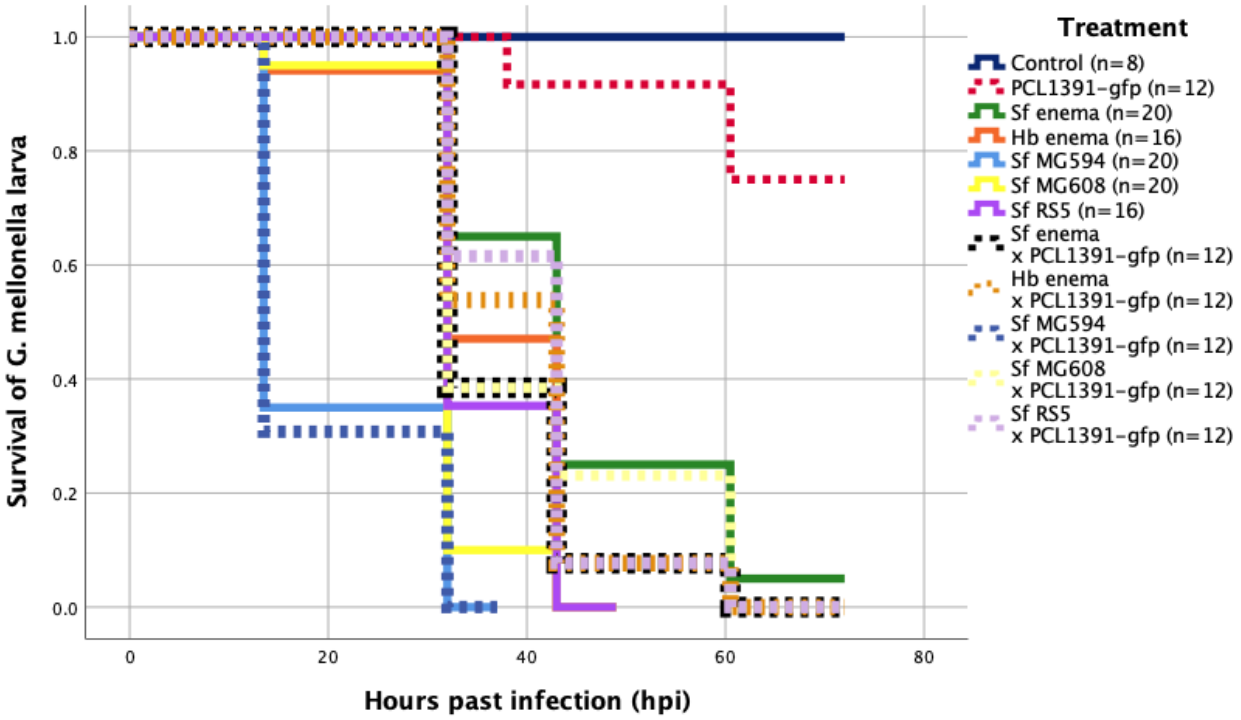


Figure 21: Survival of G. mellonella *larva over time after infection with different EPN-strains and their combination with PCL1391-gfp. Presented data derive from larvae infected with single EPN (1000IJ), single PCL1391-gfp (10^7cfu) or the combination of both (1000IJ plus 10^7cfu). For the control treatment, survival of eight replicates was assessed. For single EPN treatments Hb enema and Sf RS5 survival of sixteen replicates was observed, while for single Sf enema, Sf MG594 and Sf MG608, survival of twenty replicates was observed. For single PCL1391-gfp, survival of twelve replicates per treatment was observed. For combined treatments, survival of twelve replicates per treatment was observed (first infection).*

At 53 hours past infection, hemolymph of four infected larvae was extracted and checked for PCL1391-gfp bacteria. Afterwards, these larvae were blended, and cfu/larva was assessed. In the hemolymph, PCL1391-gfp bacteria were only present in one larva treated with Sf MG594 x PCL1391-gfp, in two larvae treated with Sf RS5 x PCL1391 and in three of the four tested larvae from the single PCL1391-gfp treatment. Where bacteria were present, concentrations ranged from 10^2 cfu/ml to $2x10^5$ cfu/ml. In all other treatments, no bacteria were present in the hemolymph. In blended larvae, only in the PCL1391-gfp treatment pseudomonads were present in all four larvae (Figure 22). In this treatment, concentrations reached on average about 10^5 cfu per larva. For Hb enema and Sf enema, no bacteria were present inside

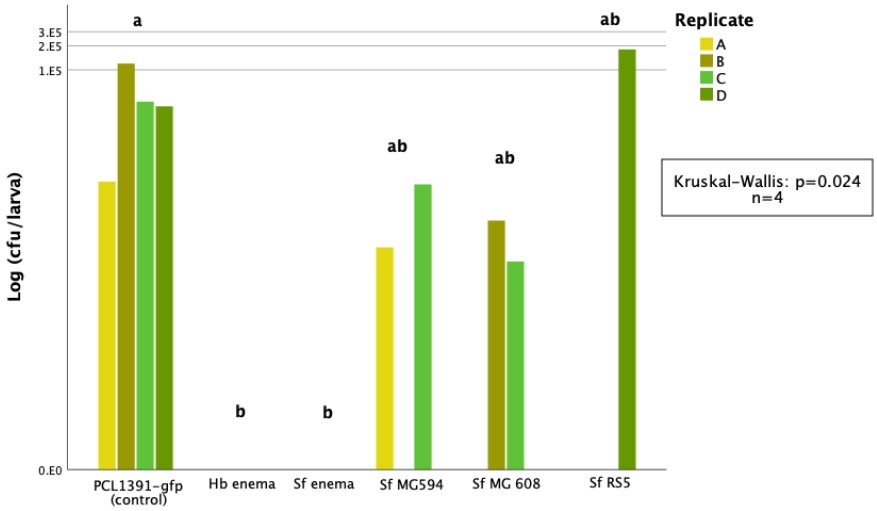


Figure 22: Concentrations of cfu/larva of PCL1391-gfp, 72 hours after infection of G. mellonella larva with combinations of EPN (1000IJ) and PCL1391-gfp (10^7cfu). Presented data derive from four replicate larvae for single PCL1391-gfp and combined treatments (first infection).

the larvae. For Sf MG594 and Sf MG608, bacteria were present in two out of four larvae at concentrations around 10^3 cfu/larva. Pseudomonads were only present in one Sf RS5 larva at a concentration slightly above 10^5 cfu/larva. An overall comparison showed a significant difference in bacteria concentration between the two treatments containing no bacteria (Hb enema and Sf enema) and the PCL1391-gfp treatment.

3.6.1. Transmission of pseudomonads into *G. mellonella* on soil

For the first approach to test whether nematodes can act as vectors, dead larvae were placed in containers on soil, covered with a petri dish and living larvae added. Pseudomonads and nematodes

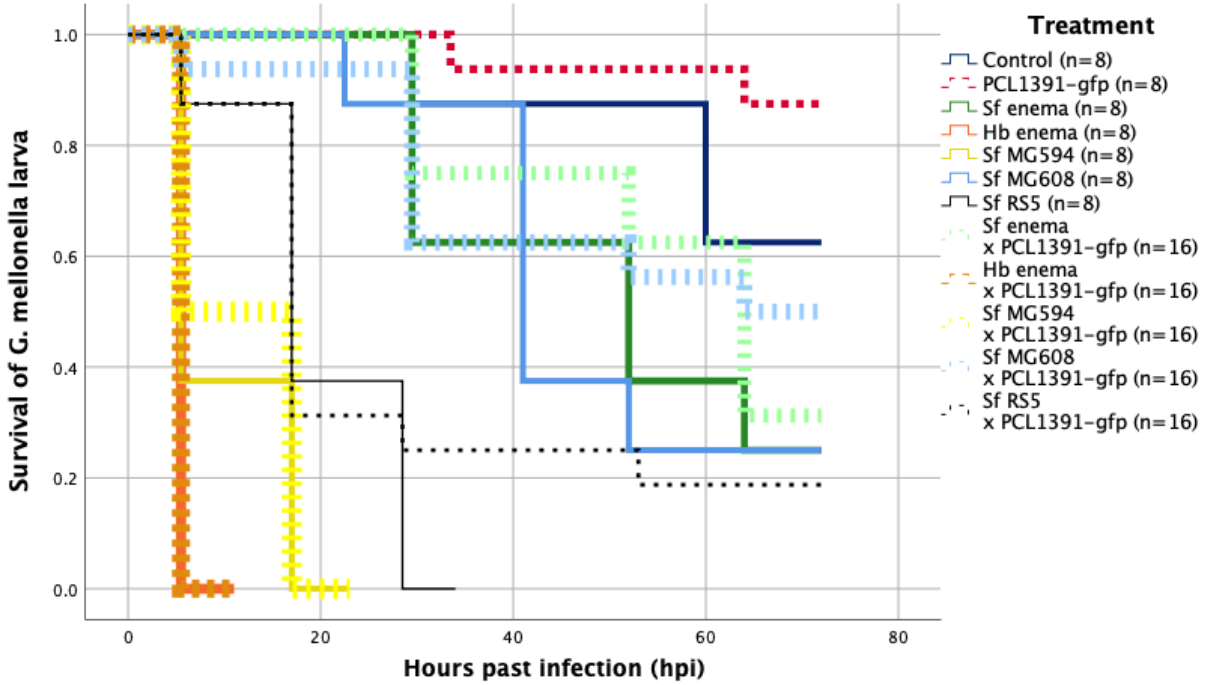


Figure 23: Survival of G. mellonella *larvae over time, after they were added to soil containers in which covered larvae from first infection of single EPN- or combined treatments of EPN and EPP were placed. For single EPN, single PCL1391-gfp and control treatments, data derive from survival of eight replicates per treatment. For combined treatments data derive from sixteen replicates per treatment.*

were present inside the carcass of *G. mellonella* from previous infection with combinations of EPN x EPP. To infest living larvae, nematodes and bacteria must move through soil. To get inside living larvae, bacteria were either carried by nematodes, accidentally taken up orally by the larvae or entered through natural wounds. Survival of larvae over time was recorded. Only 67,5% of control larvae survived to the end of the experiment at 72 hours after infection (Figure 23). 87,5% of larva larvae treated with PCL1391-gfp, survived until the end of experiment. Survival of all treatments, either single EPN or combinations of EPN and EPP, was significantly lower than for the single PCL1391-gfp treatment. Survival of Sf MG608, Sf enema x PCL1391-gfp and Sf MG608 x PCL1391-gfp was not significantly lower than survival in the control. No significant difference was found when comparing the survival of single EPN treatments to combinations of the respective EPN-strain with PCL1391-

gfp. For Sf MG608 and Sf enema, survival of combined EPN x EPP treatments was higher compared to the single EPN applications.

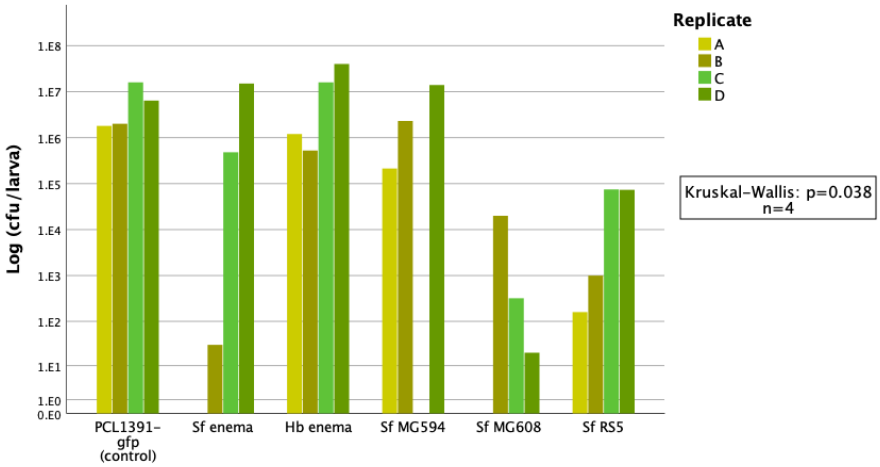


Figure 24: Concentrations of PCL1391-gfp bacteria in G. mellonella *larvae which were added to containers with larvae from first infection. Larvae from first infection had been killed by combined treatments of EPN and EPP. Cfu/larva was assessed 72 hours after living larva were added in the container. Presented data derive from four replicate larvae per treatment.*

Concentrations of PCL1391-gfp bacteria at 72 hours past infection were assessed for all combined treatments as well as for the single PCL1391-gfp control treatment. PCL1391-gfp bacteria were either present in all four larvae (PCL1391-gpf control, Hb enema, Sf RS) or in three larvae (Sf enema, Sf MG 594, Sf MG608) (Figure 24). The single PCL1391-gfp control treatment shows the highest concentration of cfu/larva. In all tested larvae of this treatment, at least 10^6 cfu/larva were present. Concentration of pseudomonads was only at equal levels for Hb enema (p=1.0). In all other treatments, concentration varied a lot between replicates. For the combinations with Sf enema and Sf MG594, concentrations ranged from zero to 10^7 cfu/larva. In the two combinations with Sf MG608 and Sf RS5, bacteria concentrations were all below 10^5 cfu/larva. An overall comparison showed significant differences in bacterial concentration across treatments, but pairwise comparison did not show significant differences after the Bonferroni correction for multiple tests.

3.6.2. Transmission of pseudomonads by surface sterilized nematodes

Larva that died from first infection of single EPN and combined EPN x EPP application, were placed on white traps after death. Emerging nematodes were collected, surface sterilized and new *G. mellonella* larva were infected with sterilized and non-sterilized nematodes as controls. Survival and cfu/larva are measured after the 2nd infection.

Survival was assessed for the first 39 hours after infection (Figure 25). Most treatments with single EPN applications died within 32 hours after infection. Only for the single Sf enema treatment no larva died within 39 hours while for the single Hb enema treatment, survival of larva was 75% at 39 hpi. All larvae from control and single PCL1391-gfp treatment survived until the end of experiment. Compared to the control, and the PCL1391-gfp treatment, survival of all other treatments, except the Hb enema and Sf enema x PCL1391-gfp treatment, was significantly lower. Comparing survival of each

combined treatment with the related single EPN application shows no significant difference. After 39 hours, no PCL139-gfp were present neither in the hemolymph, nor in the larvae of any treatment.

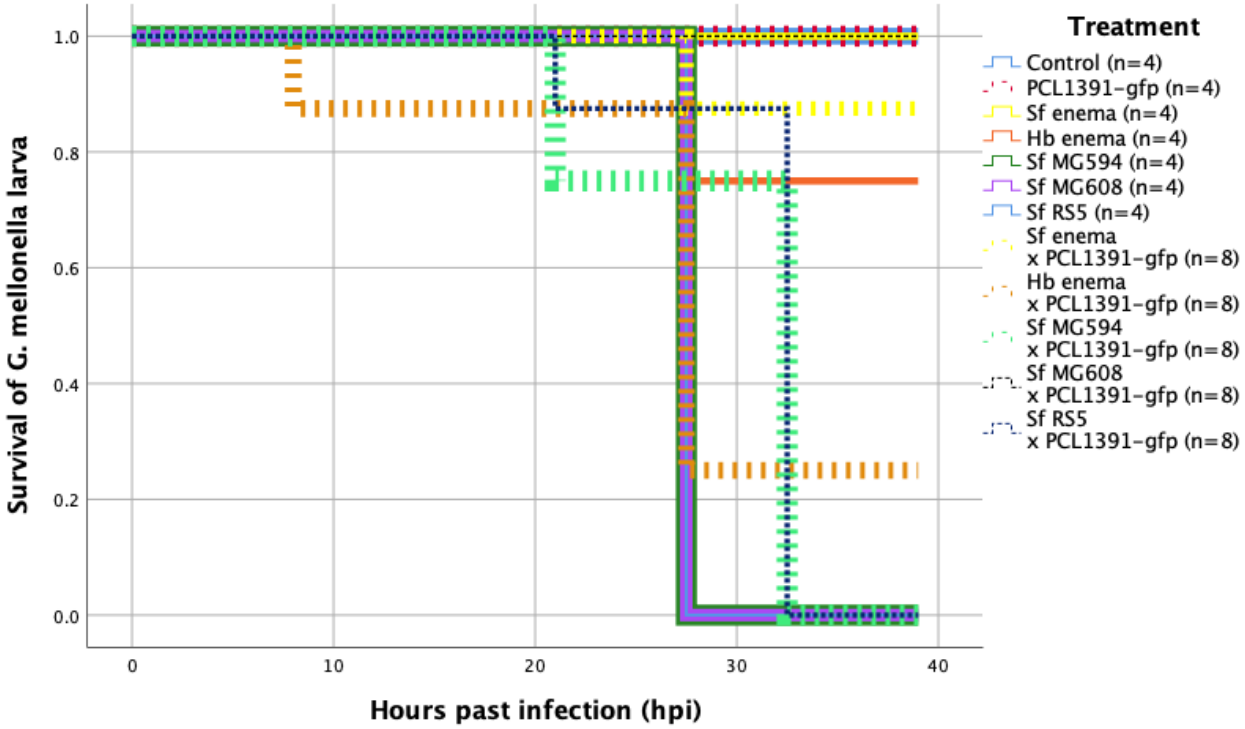


Figure 25: Survival of G. mellonella *larva over time after they were infected with surface sterilized EPN from first infection. For single EPN, PCL1391-gfp and control treatments data derive from four replicate larvae per treatment. For combined applications data derive from eight replicate larvae per treatment.*

4. Discussion

4.1. Challenges when evaluating effects on development of *D. radicum*

Assessing effects of pseudomonads on development of *D. radicum* was challenging because in most experiments, control groups showed low pupation rates. In greenhouse and field trials from Chen et al. (2003) and Ratzinger et al. (2014), pupation rates of *D. radicum* were low due to low egg hatch rates. When rearing *D. radicum* under controlled conditions, normally only about 70% of eggs hatch (Chen *et al.*, 2003; Razinger, 2014). After hatching, larvae are known to be very sensitive and mortality up to 30% in control treatments is not unusual (Nielsen, 2003). Taking this into account, pupation rates of 50% in the present study are similar to pupation rates in previously performed experiments with *D. radicum*. The very low pupation rates in some climate chamber experiments around 30% in the present study might be caused by bad egg quality and inbreeding in the *D. radicum* population.
For the greenhouse experiments, pupation rates were on average around 30% in this study. Conditions in the greenhouse might not have been optimal for larval development. Experiments were carried out during the summer in a greenhouse without sufficient cooling. Temperatures in the sun were measured above 36°C. Temperatures below plants, where *D. radicum* eggs were placed, reached up to 27°C. It is known that eggs are very susceptible to drought and in nature often only 10% develop to flies (Crüger, 2002). High temperatures and resulting dry conditions in the top layer of soil might have influenced development negatively. Additionally, *D. radicum* larvae are known to undergo aestivation at soil temperatures above 22°C (Crüger, 2002), which might have influenced their development in the greenhouse experiments. Some larvae had not yet pupated at timepoint of evaluation in the last repetitions, possibly due to slower development under elevated temperatures. Also, pupae were found to be buried in soil up to 10cm deep. In the first experiments this was not considered and from some pots only 5cm of the topsoil were sieved in search for pupae. This way, some pupae might not have been detected. Increasing the number of eggs added per pot might reduce the variability in pupation rates within treatments and carrying out the experiments in greenhouse cabinets with better cooling system or at cooler outside temperatures might result in better pupation rates.

4.2. Single entomopathogenic *Pseudomonas* application to control *D. radicum*

Currently, only Spinosad and insecticides based on organophosphates as active ingredients are available for the control of *D. radicum* on the European market (Börner, 2009). Besides their negative effect on the environment and concerns about public health, efficiency for the control of *D. radicum* was high for organophosphates in a laboratory assay performed by Joseph and Zarate (2015). Organophosphates showed high mortality of *D. radicum* larvae with mortality up to 90%. One problem of organophosphates is inconsistent efficiency as they are for example leached out when precipitation is high, or fields are irrigated (Joseph and Zarate, 2015). Leaching can drastically reduce their

concentration over time in the area where larvae are feeding (Joseph and Zarate, 2015). For organic farming, the application of Spinosad is the only available control measure for *D. radicum*. Spinosad did not perform well under laboratory conditions and showed only moderate mortality of 50% (Joseph and Zarate, 2015). In a field study by Ratzinger et al. (2014), treatment with Spinosad decreased the number of pupae and larvae about 66% in Germany and 50% on Slovenian fields. As Spinosad is only active upon oral uptake, a treatment of the soil might not be sufficient. Parts of plants like roots and tubers might have to be treated with Spinosad to have a sufficient effect on larvae (Herbst *et al.*, 2017).

Compared to these two available control measures, the two *P. chlororaphis* strains PCL1391 and 64 showed to have a comparable effect on the development of *D. radicum* as Spinosad in the present study. The strain PCL1391 reduced average pupation rates up to 42.6% and fly emergence up to 54% compared to the control treatment. The fluorescent Pseudomonas strain 64 reduced pupation rate only 34.4%, but reduced fly emergence by 64%. These results are comparable to previously observed experiments performed in the climate chamber (Spescha et al., unpublished). The strong reduction in fly emergence by the strain 64 supposes a toxicity more towards pupae than larvae. Additionally, the strains PCL1391 and CHA0 were able to colonize bulbs of radish plants and soil well under greenhouse conditions. This might be an advantage compared to Spinosad and synthetic insecticides, as both are threatened by elution (Joseph and Zarate, 2015). Applied at the right timepoint before second or third generations emerge, fluorescent *Pseudomonas* might avoid an epidemic outbreak of the pest insect. Second and third generations would be strongly depleted. Whether an application would reduce the feeding damage on bulbs needs to be investigated. As PCL1391 and CHA0 have oral toxicity to a wide range of insect pests, this colonization might protect bulbs from damage. In previous studies it was shown that low concentrations of pseudomonads on plant leaves are sufficient to induce high mortality of insects feeding on them (Kupferschmied *et al.*, 2013). Spraying concentrations of only 1000 *Pseudomonas* bacteria per millilitre on leaves were sufficient to kill 70-80% of *Spodoptera littoralis* and *Heliothis virescens* larvae feeding on them. Bacterial concentrations on radish bulbs were about $1x10^5$ cfu/g bulb which should therefore be sufficient to kill larva feeding on them. However, results show that the development of *D. radicum* larvae was not inhibited significantly on radishes colonized by pseudomonads. A tendency of pseudomonads in reducing the development of *D. radicum* could be observed. So far, not much research has been carried out on the relationship between fluorescent *Pseudomonas* and insects. The pseudomonads strategy of interaction with the insect plays an important role for pathogenicity (Flury *et al.*, 2017). It has been suggested that fluorescent *Pseudomonas* are either pathogenic, using insects as food source and therefore cause lethal infections, or commensal and live inside the insect host without causing obvious damage, exploiting it as vector for disposal. For the *P. protegens* strain CHA0, experiments already showed a more commensal than pathogenic relationship with *D. radicum* (Flury *et al.*, 2019). CHA0 affected pupal size, but not pupation rate and fly emergence. This indicates

that bacteria persist throughout different developmental stages of the insect without pathogenic activity, maybe using it as a vector. In the present study, CHA0 did also not have an effect on pupation rate and fly emergence of *D. radicum*, but on pupal size. The suggestion by Flury et al. (2019), that CHA0 seems to follow a commensal or an opportunistic pathogenic strategy with *D. radicum* can be supported by present results.

4.3. Single entomopathogenic nematodes application to control *D. radicum*

Several studies have shown susceptibility of *D. radicum* larvae to entomopathogenic nematodes, but the degree of susceptibility depends on the insect stage, nematode strains used end experimental conditions (Nielsen, 2003; Chen *et al.*, 2003; Royer *et al.*, 1996). In the present study, the potential of the five tested entomopathogenic nematode strains to infest *D. radicum* larvae was also observed in a direct infection assay. Single applications of EPN did influence the development of *D. radicum* inconsistently across repetitions. In some repetitions, *D. radicum* performed even better in the pots treated with EPN compared to the control. Even though all strains were shown to be able to infest *D. radicum* larvae, no significant reduction in pupation rate or fly emergence was observed. Previously conducted experiments showed also variable results (Nielsen, 2003). The degree of susceptibility of *D. radicum* larvae seems to depend on the larval stage (Royer *et al.*, 1996). First instar larvae have a very low or no mortality and are probably not very attractive for EPN due to their small size (Nielsen, 2003; Royer *et al.*, 1996). Second instar larvae showed to be the most susceptible larval stage with infestations of 60% (Nielsen, 2003). Therefore, application should coincide with the occurrence of the most destructive and susceptible second and third instar larvae for efficient control (Bal and Grewal, 2017). The timepoint of applying EPN might be a reason for inconsistent effects on development *of D. radicum* in the present study. Nematodes were applied at the same time as eggs of *D. radicum* and not at the timepoint when second and third instar larvae occurred. Another fact that favors a later timepoint of applying nematodes is nematode's attraction to cues of larvae, but not to eggs, which was already proven for the species *S. carpocapsae* (Royer *et al.*, 1996). Infective juveniles of *H. bacteriophora* and *S. feltiae* are also strongly attracted by cues emitted from potential hosts (Hurley, 2018; Grewal *et al.*, 2008). When applied simultaneously with eggs, nematodes might waste energy in search for food the first days after application. Starvation can cause dispersion and declined infectivity of nematodes (Beck *et al.*, 2014). This might be a reason for the low effect on development of *D. radicum* observed in some repetitions.

Additionally, *D. radicum* larvae are known to tunnel into belowground plant parts which might create a physical protection from entomopathogenic nematodes. In the case of Brassicacean plants, larvae might additionally be protected by chemicals emitted from plant roots. The larvae might have evolved protective mechanisms against pathogens, possibly with the aid of glucosinolate compounds (Vänninen *et al.*, 1999). When ingested and metabolized, these compounds may protect *D. radicum* larvae from infection by nematodes. Decomposition products of glucosinolates are known to be toxic

for the free living nematode *Caenorhabditis elegans* and plant parasitic nematodes (Nielsen, 2003; Dutta *et al.*, 2019). So far, the role of glucosinolates and its metabolites for entomopathogenic nematodes is not yet known in detail. At least, nematodes of the species *S. feltiae* were shown to be capable of following *D. radicum* larvae into roots of Brassicacean plants and infest them (Royer *et al.*, 1996). To which extent glucosinolates and its metabolites inhibit infestation needs more detailed research.

When sprayed onto soil surface, nematodes are influenced by UV-rays, temperature fluctuations and varying moisture conditions which can have adverse effects on their pathogenicity (Smits, 1996; Chen *et al.*, 2003). In the present study, most pots were dry when evaluating the experiment. Even though humidity was set to 80%, the sand dried quickly in climate chambers. Sand was checked for living nematodes once, by adding living *G. mellonella* larvae. No signs of nematode infection or nematodes itself were observed. This indicates, that EPN might have died or become inactive due to desiccation. To avoid desiccation, the experimental setup should be optimized by either spraying water regularly on pots or by placing pots in trays containing water.

Although effects on *D. radicum* of tested EPN strains in this study were inconsistent, the *S. feltiae* strains Sf enema, Sf MG594 and Sf RS5 had the largest effects on the development. These findings are in accordance with previous experiments where nematodes from the species *S. feltiae* were shown to reduce development of *D. radicum* most (Royer *et al.*, 1996; Chen *et al.*, 2003; Royer *et al.*, 1996). In one greenhouse assessment conducted by Schroder et al. (1996), *S. feltiae* even caused a significant reduction in the number of *D. radicum* larvae. In comparison to the species *H. bacteriophora,* pathogenicity of *S. feltiae* is not temperature dependent, which increases the potential of this species for the control of *D. radicum* (Chen *et al.*, 2003). Although nematode applications did not eradicate larvae, treatments can reduce the amount of deep feeding scars caused by *D. radicum* significantly (Schroder *et al.*, 1996). These findings and observations in the present study emphasize the potential of entomopathogenic nematodes for the control of *D. radicum* as they are able to infest and kill larvae when applied at the right timepoint under favorable conditions.

4.4. Potential of a combined entomopathogenic nematode - *Pseudomonas* application for the control of the cabbage root fly *D. radicum*

In the present study, the combined application of EPN Sf enema and EPP PCL1391 reduced the development of *D. radicum* in both repetitions. Single BCA applications showed effects as well but were not consistent over repetitions. In the combined application of Sf enema and PCL1391, reduction of pupation rate and fly emergence seems to be less inconsistent compared to single BCA applications. So far, not many experiments on the combined application of entomopathogenic nematodes and pseudomonads have been conducted. In a study by Imperiali et al. (2017), a combined application of arbuscular mycorrhizal fungi, entomopathogenic nematodes (*H. bacteriophora, S. feltiae)* and pseudomonads (*P. chlororaphis, P. protegens*) showed beneficial

effects in wheat fields when a pest outbreak occurred. It was suggested that the combination of beneficial soil organisms can lead to a positive effect under certain circumstances, but it mainly has similar effects as single applications. Under conditions with high biotic stress, combinations of nematodes and pseudomonads produced the highest yields (Imperiali *et al.*, 2017). In another study, the combined application of entomopathogenic nematodes (*H. bacteriophora, S. feltiae*), fluorescent *Pseudomonas* (*P. protegens, P. chlororaphis*) and arbuscular mycorrhizal fungi in maize against the western cotton rootworm reduced root damage caused by the pest in one out of three years (Jaffuel *et al.*, 2019). In these experiments, soil moisture was variable over the years which might have influenced especially the performance of nematodes. Single nematode application showed to be most successful in the year with highest precipitation. Meyer and Roberts (2002) already suggested, that the consistency over a wider range of environmental conditions is a potential advantage of biocontrol agents applied in combination. Results of the present study showed that a combination might increase the consistency. However, only two repetitions were carried out in the present study and therefore, the experiment needs to be repeated to get representative results.

The compatibility of EPN and EPP was assessed *in vitro* in 96-well plates and in *G. mellonella* larvae in the present study. *In vivo c*o-infection of *G. mellonella* larvae with EPN and EPP did not have an effect on entomopathogenic *Pseudomonas* inside the larvae. These findings are in accordance with Hurley (2018), who successfully recovered *P. fluorescens* F113 from *G. mellonella* larvae after a combined infection. Fluorescent *Pseudomonas* successfully colonized infective juveniles (Hurley, 2018). In the present study, *P. chlororaphis* PCL1391 bacteria were observed together with entomopathogenic nematodes after nematodes escaped from the insect cadaver. EPP were shown to survive in *G. mellonella* larvae when co-infected with EPN. These findings indicate some level of mutualistic relationship between nematodes and pseudomonads. Presence of entomopathogenic *Pseudomonas* had no significant effect on the virulence of *H. bacteriophora* and *S. feltiae* towards *G. mellonella*. Experiments conducted by Hurley (2018), also showed no significant effect of fluorescent *Pseudomonas* on nematode's virulence towards *G. mellonella* and *Otiorhynchus sulcatus*. But, the effect on virulence of entomopathogenic nematodes varied between nematode and bacterial strains. Even though entomopathogenic *Pseudomonas* were recovered from co-infected larvae in the present study, bacterial concentrations inside larvae varied. Some larvae did not contain any *Pseudomonas*. It is known, that the bacterial symbionts of nematodes produce a wide range of antimicrobial organic compounds and bacteriocins active against other *Photorhabdus* and *Xenorhabdus* species as well as closely related bacteria (Gaugler, 2002; Boemare *et al.*, 1992). In *G. mellonella* larvae, these compounds might outcompete or even kill EPP. However, some bacteria like *Paenibacillus spp*. seem to be resistant to theses antimicrobials (Enright and Griffin, 2004) which gives hope for a resistance of entomopathogenic *Pseudomonas* as well. To determine whether antibiotics have an influence, the interaction of bacterial symbionts of nematodes and certain entomopathogenic *Pseudomonas* strains needs to be investigated more detailed.

On the other side, pseudomonads produce a wide range of antibiotics as well (Gallagher and Manoil, 2001). *P. aeruginosa* was shown to paralyze and rapidly kill the free-living nematode *Caenorhabditis elegans*. Cyanide is responsible for the toxicity and is a typical secondary metabolite of pseudomonads. Cyanide is a potent poison against most eukaryotic species and inhibits fungal growth. It accounts for the suppression of several plant pathogens by fluorescent *Pseudomonas* (Gallagher and Manoil, 2001). Another antibiotic compound produced by fluorescent *Pseudomonas* is DAPG (2,4-diacetylphloroglucinol) which was shown to have a negative effect on plant parasitic nematodes *(Globodera pallida* and *Meloidogyne javanica)*, but not on entomopathogenic nematodes *H. bacteriophora* and *S. feltiae* (Hurley, 2018). Effect of compounds produced by entomopathogenic *Pseudomonas* should be evaluated for their toxicity towards entomopathogenic nematodes.

In *in vitro* experiments carried out in the present study, survival of nematodes was not significantly influenced when mixed with entomopathogenic *Pseudomonas*. These findings are in accordance with results from Hurley (2018). *P. fluorescens* F113 was shown to have no influence on survival, biology and behavior of entomopathogenic nematodes (Hurley, 2018). Still, some improvements on the experimental setup of the present study are necessary. Survival of nematodes was assessed by counting moving nematodes as living. But some nematode species adopt quiescent postures where they stop moving which may be confused with dead nematodes (Grewal *et al.*, 2008). This might have been the reason for no survival of nematodes in the tested strain Sf MG594. Therefore, motionless nematodes should be agitated by adding a drop of hydrogen peroxide prior to counting. Another problem was the survival of entomopathogenic *Pseudomonas* in *in vitro* mixtures. Most bacteria were already dead after 48 hours especially in wells with a concentration of 10^6 cfu/ml. Because no single PCL1391 control well was checked for the survival of bacteria, it could not be shown whether the presence of nematodes has an influence on the survival of pseudomonads. The survival of bacteria might be improved by using saline instead of autoclaved water, but further experiments with single PCL1391 treatments as control need to be conducted to investigate the susceptibility of pseudomonads to entomopathogenic nematodes. Additionally, the number of replicates must be increased to gain statistically correct statements and the assessment of living IJs must be improved. As adjusting concentrations to 100IJ/well seems to be difficult, determining the ratio of living IJs to dead IJs might be more suitable.

Other experiments showed that nematodes have a close relationship with fauna in the soil ecosystem and indicate a possible mechanism of transporting bacteria (Hurley, 2018). Unfortunately, experiments to determine whether EPN can act as vectors for EPP did not show clear results in the present study. Although *G. mellonella* larvae used for experiments were in a non-feeding stage and EPP are not able to penetrate the cuticular, bacterial concentrations inside larvae were high when infected with single EPP treatments. Most probably, bacteria entered the larvae through the mouth as they used filter paper or soil to build a cocoon. Inside the larva, they entered the hemolymph through the gut. Because EPP were able to invade *G. mellonella* larva, it could not be shown whether

nematodes can act as vectors for EPP. In order to avoid bacterial uptake by larva, the experimental setup should be optimized. As larvae seem to take up any material provided with their mouth, infection might be more successful when *G. mellonella* larvae are infected in empty petri dishes. Another solution could be to bath them in bacterial suspension containing nematodes instead of infecting larvae directly.

When leaving the *G. mellonella* cadaver, entomopathogenic *Pseudomonas* escaped together with EPN. By surface sterilization it should have been determined whether pseudomonads are carried inside or outside the nematode. After surface sterilization, EPP were not present with nematodes anymore. However, EPN showed lower virulence towards *G. mellonella* after surface sterilization. As surface sterilization was done with highly toxic $HgCL_2$, both bacteria, pseudomonads and EPN symbionts, inside and outside of nematodes might have been killed. This would explain the lower virulence of EPN after surface sterilization. Therefore, another less toxic surface sterilization method might help to prove whether EPN can carry EPP inside and transmit them into insect hemolymph.

Another challenge that should be taken into account when evaluating experiments of combined BCA applications is the statistical analysis and interpretation of the results. Statistically, it is often not obvious whether a combination has a synergistic, additive or antagonistic effect compared to the effect of the single biocontrol organism. For an adequate analysis, Xu *et al*. (2001) suggest that Bliss independence should be used when BCAs act through different modes of action, while Loewe additivity is more suitable when both agents are the same or have the same mode of action. None of this analysis methods were used in the present study. Bliss independence seems to fit for testing combinations of EPN and EPP and should be considered for analysis. Still, the combination was so far only tested under controlled conditions with only two repetitions. Performance in the greenhouse and in the field should be evaluated before drawing a conclusion on the potential of a combined application for the control of *D. radicum*. Based on the results of this study, entomopathogenic nematodes and entomopathogenic *Pseudomonas* are compatible and show a high potential as a combined product for insect control of soil borne pests.

5. Conclusion

In this study it was shown that two *Pseudomonas* strains with oral toxicity against insects, PCL1391 and CHA0, have the ability to colonize bulbs of radish plants very well under greenhouse conditions. An effect on the development of the cabbage root fly *D. radicum* could be detected, even though it was not significant. The strain PCL1391 reduced development of *D. radicum* most, with an average reduction of up to 42.6% in pupation rate, while the *P. chlororaphis* strain 64 was able to reduce fly emergence by 64% compared to the control in one repetition. In general, tested pseudomonads were able to inhibit the development of *D. radicum* on bulbs in the greenhouse. The lack of significant results might be due to high variability in the control treatments which could be solved by increasing the number of replicates in future experiments. Still, the results of the present study show a promising potential of EPP for the control of *D. radicum*. In previous experiments, conducted in the climate chamber, EPP already showed the ability to inhibit development of *D. radicum* (*Spescha* et al., unpublished). This potential could be confirmed for greenhouse conditions in the present study. The additionally proven ability to colonize roots and soil turns certain EPP strains into promising candidates for the control of *D. radicum* and other soilborne pests.

All tested entomopathogenic nematode strains were able to infest *D. radicum* larvae when applied directly. Testing the effect of five different EPN strains on the development of *D. radicum* larvae showed inconsistent results over four repetitions. Inconsistent performance might be due to an early timepoint of applying the nematodes and dry conditions in the pots. Experimental setup should be improved to evaluate the potential of EPN strains for the control of *D. radicum* better.

Combination of PCL1391 and Sf enema against *D. radicum* in the climate chamber showed more consistent reductions than single BCA treatments. On average, pupation rate was reduced by 59% in the first repetition and by 44% in the second one by this combination compared to the control. Single BCA applications reduced development stronger, but only in one out of two repetitions. In general, combinations seem to have a higher consistency in reducing the development of *D. radicum* larvae, but more repetitions need to be done to verify this hypothesis. Still, it is an exciting finding for the combination of entomopathogenic nematodes and entomopathogenic *Pseudomonas* for the control of *D. radicum* in the field and could present a new control method to fight belowground pests in general.

Compatibility of EPP and EPN was assessed *in vivo* and *in vitro*. EPP and EPN were compatible when *G. mellonella* larvae were infected simultaneously. As EPN kill *G. mellonella* in a short period of time, no additive or synergistic effect in mortality could be seen in combined applications. At the same time, no antagonism between EPP and EPN was observed. However, concentrations of EPP in dead larva seemed to be slightly lower when infected in combination compared to larvae infected only with EPP. To explain this phenomenon, interactions between entomopathogenic *Pseudomonas*, entomopathogenic nematodes and its symbiotic bacteria have to be investigated in more detail. In the

present study, it could not be revealed whether EPP use EPN as vectors, but new insights into the interaction of entomopathogenic nematodes and entomopathogenic *Pseudomonas* were gained. EPP were detected together with EPN emerging from dead *G. mellonella* larvae after a combined infection with EPP and EPN. However, the failure of both approaches does not exclude EPN as vectors for EPP.

Future experiments to investigate the interaction of EPN and EPP should focus on the effect of different metabolites of pseudomonads on symbiotic bacteria of EPN and vice versa. The effect of these metabolites plays an important role for the compatibility of EPN and EPP as a plant protection product. For the potential as a plant protection product, the question whether the interaction of EPN and EPP is commensal or pathogenic when attacking the same host, needs to be answered. Even though the present study suggests that EPN and EPP are compatible, experiments on the molecular level are necessary to uncover their direct and indirect interactions.

As certain EPP and EPN strains showed higher potential for the control of *D. radicum* than others, most promising candidates should be selected to carry out experiments with a combined application in the climate chamber. As conditions in the climate chamber seem to be important for nematodes, keeping the pots moist during the experiment, as well as a later timepoint of applying EPN should be taken into consideration. Applying EPP at the same time as eggs of *D. radicum* to givie them time to colonize radish bulbs and adding EPN a few days later might be an option to optimize the effect of a combined application. EPP were shown to colonize bulbs and have an oral toxic effect on larvae and therefore can form the first line of defense against feeding larvae. As EPN are able to actively follow larvae, they could prevent further damage by *D. radicum* larvae after they have entered the bulbs without being killed by EPP. To investigate, whether EPN might follow the larvae into roots or bulbs, their interaction with Brassicacean plants and their root exudates should be investigated in more detail. When applying EPN and EPP at the same time, a simultaneous application with the infection by *D. radicum* eggs might be optimal. After application, pots should be humidified to ensure survival of EPN. Method optimization and experiments with a higher number of replicates might compensate the high variability in pupation rates and fly emergence rates.

In the present study, the effect of a combined EPN-EPP application was indicated to be more consistent compared to single BCA applications. To confirm this assumption of a synergistic effect, further repetitions need to be done with more replicates to compensate the high variability in pupation rates and fly emergence rates. Additionally, to investigate a possible higher consistency, experiments under different environmental conditions should be conducted. Therefore, greenhouse experiments with most promising EPN and EPP strains under different controlled environmental conditions might be suitable to investigate a higher consistency. As the lack of consistency is a general problem of BCA's under field conditions, performance under different controlled conditions might show limits and possibilities of a combined EPN-EPP application.

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7. Appendix

Protocol 1: Protocol for PCR confirmation with a Fit primer pair specific to P. chlororaphis and P. protegens.

PCR protocols

Primer list

Target / gene	Primer name	Sequence (5'-3')	Annealing temp. (°C) Length (bp)	Reference
P. fluorescens FitD	FitD_66F_DEG	CTA TCG GGT SCA GTT CAT CA	60°C	Francesca
	FitD_308R_DEG	TTC TTG TCG GSA AAC CAC T	+- 240 bp	
Pseudomonas spp. 16S	Pse435F	ACT TTA AGT TGG GAG GAA GGG	60°C	Bergmark, 2012
	Pse686R	ACA CAG GAA ATT CCA CCA CCC	+- 250 bp	
P. protegens	DGPf_4F_Protegens	CGCTGATCCTCTCGTTGTCTGC	64°C	Garrido-Sanz, 2017
	DGPf_4R_Protegens	ACGCCCTTGTCCACATCG	1072 bp	
P. chlororaphis	DGPf_8F_Chlororaphis	CCCACCGACAGCCAGCAACG	67 °C	
	DGPf_8R_Chlororaphis	CGGTCTTGTCGCTGATGCCG	661bp	

Colony PCR protocol

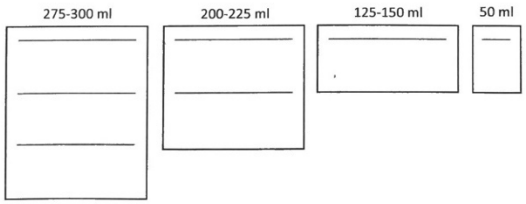
- Grow single colonies in 200µl LB in cell culture plate for 8h at 24°C / 16h at 18°C
- Transfer 25µl in a PCR plate; freeze both plates until further use
- Put the PCR plate in the thermocycler at 96°C for 5 mins
- Prepare the PCR mix and add 5µl heated DNA to the mix
- Run the colony PCR program on the thermocycler
- Store the remaining 20µl DNA in the freezer
- Run a 1% Agarose gel in 1xTAE
- Add 3µl gel Red per 100ml TAE
- Loading dye 6x: Add 4µl loading dye to PCR product
- Run 12µl on gel and freeze rest (in case you have to repeat gel)
- Add 5µl ruler (1kb plus) to each row

***Colony PCR** (12.5µl reaction)*

µl	component	µl (x)	µl (x)
2.5	DNA (~10ng)	412,5	
0.625	dNTP's (0.5µM)	103,125	
0.625	Primer F (0.5µM)	103,125	
0.625	Primer R (0.5µM)	103,125	
1.25	Buffer 10x (1x)	206,25	
0.175	DreamTaq	28,875	
6.7	ddH_2O	1.105,5	
12.5	total	2062,5	

°C	t	
95	5 min	
95	30s	35x
x	1min	
72	1 min	
72	5 min	
14	∞	

1x TAE 1% Agarose gel I GelRed I 6x loading dye I 80-100V for nice bands, 120V bands okay



Protocol 2: Surface sterilization protocol for EPN.

Surface sterilisation Protocol:

Ringers solution: *1L*

- calcium chloride (C24-131) 0.12 g/L
  = Calciumchlorid entwässert mittel gekörnt rein
- potassium chloride (C24-143) 0.105 g/L
- sodium bicarbonate (C24-222) 0.05 g/L
  = Natriumhydrogencarbonat
- sodium chloride (C24-212) 2.25 g/L
  = Natriumchlorid

=> Auflösen in 1l Wasser (dH2O) mit Magnet- Rührer
=> Autoklavieren (Wet 15min)

0.1 % HgCl2 solution: *250ml*
-0,25g Quecksilber(II)-chlorid (99%) (C24-332)
=> Auflösen in 250ml H2O
=> Autoklavieren (Wet 15min)

0.001% HgCl2 solution: *275ml*
- 25ml von 0.1% HgCl2 solution
=> Auflösen in 225ml H2O
=> Autoklavieren (Wet 15min)

Sterile dH2O: *200ml* autoklaviertes Hahnewasser

1. Add *5 ml* of a dense EPN suspension to a *15 ml* centrifuge tube
2. wash IJ in *7,5 ml* of sterile Ringer's solution for *5 minutes*, shake by hand for *5 minutes*
3. pellet IJ by centrifugation at *2500 rpm for 5 minutes at 15°C*
4. remove and discard resulting supernatant
5. wash IJ-pellet in *7,5 ml* of a 0.01 % HgCl2 solution for shake by hand for *3 minutes*
6. pellet IJ by centrifugation at *2500 rpm for 5 minutes at 15°C*
7. remove and discard HgCl2
8. wash surface sterilized IJ-pellet in 7,5ml Ringer's solution once, shake by hand for *5 minutes*
9. pellet IJ by centrifugation at *2500 rpm for 5 minutes at 15°C*
10. remove and discard resulting supernatant
11. wash surface sterilized IJ-pellet in dH2O, shake by hand for *5 minutes*
12. pellet IJ by centrifugation at *2500 rpm for 5 minutes at 15°C*
13. remove and discard resulting supernatant
14. wash surface sterilized IJ-pellet in autoclaved tap water, shake by hand for *5 minutes*
15. pellet IJ by centrifugation at *2500 rpm for 5 minutes at 15°C* remove and discard resulting supernatant
16. re-suspense IJ-pellet in *10ml* autoclaved tap water

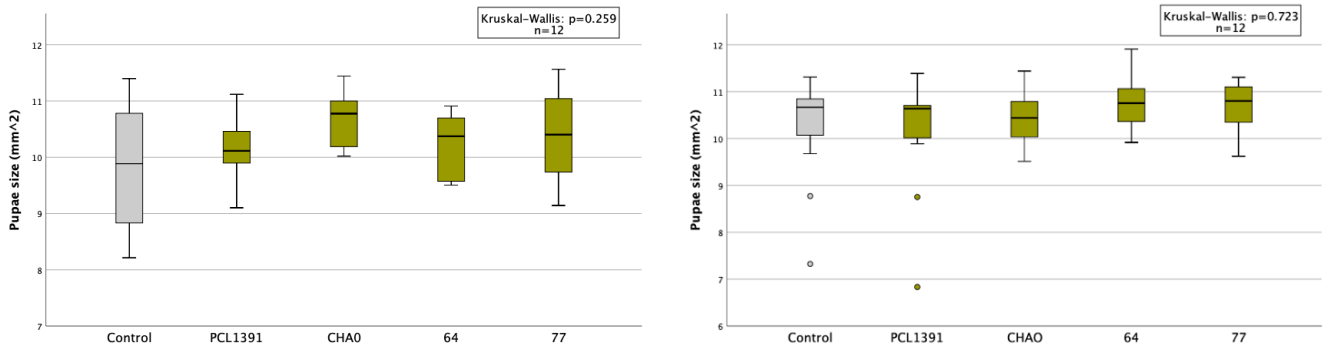


Figure 26: Pupal size when testing the effect of different EPP strains on development of D.radicum *in the greenhouse (second repetiton left and third repetition right). Presented data were derived from twelve pots per treatment. Per pot, ten eggs were added in the first repetition and fourteen eggs in the second repetition.*

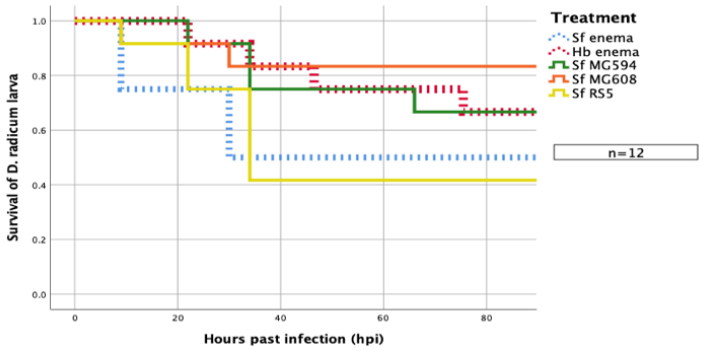


Figure 27: Kaplan-Meier survival plot of D. radicum *larvae direcly infected with different EPN strains.*

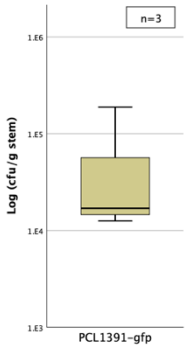


Figure 28: Stem colonization ability of P. chlororaphis PCL1391-gfp eleven days after second inoculation when testing root colonization ability of different fluorescent Pseudomonas isolates in the greenhouse.

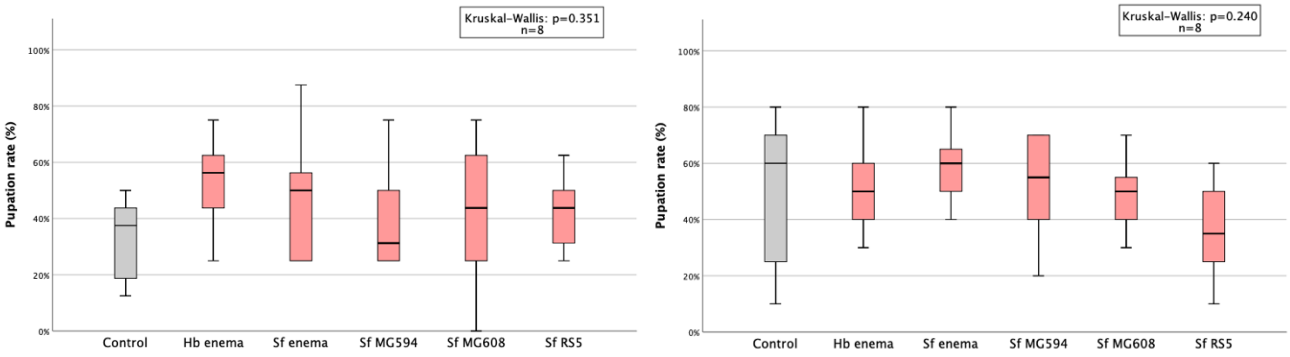


Figure 29: Pupation rate of D. radicum *eggs in the climate chamber assay to evaluate nematode strains for the control of* D. radicum *(first repetition left and third repetition right).*

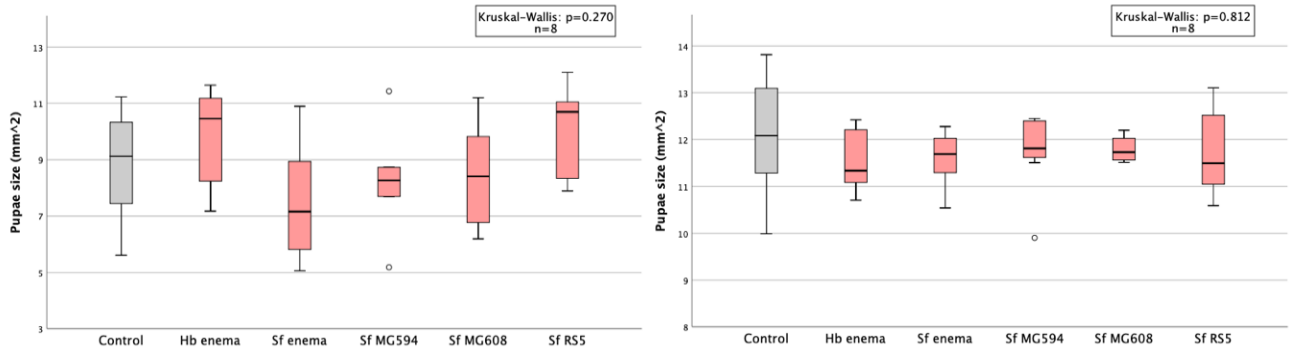


Figure 30: Pupal size of D. radicum *eggs in the climate chamber assay to evaluate nematode strains for the control of* D. radicum *(second repetition left and third repetition right).*

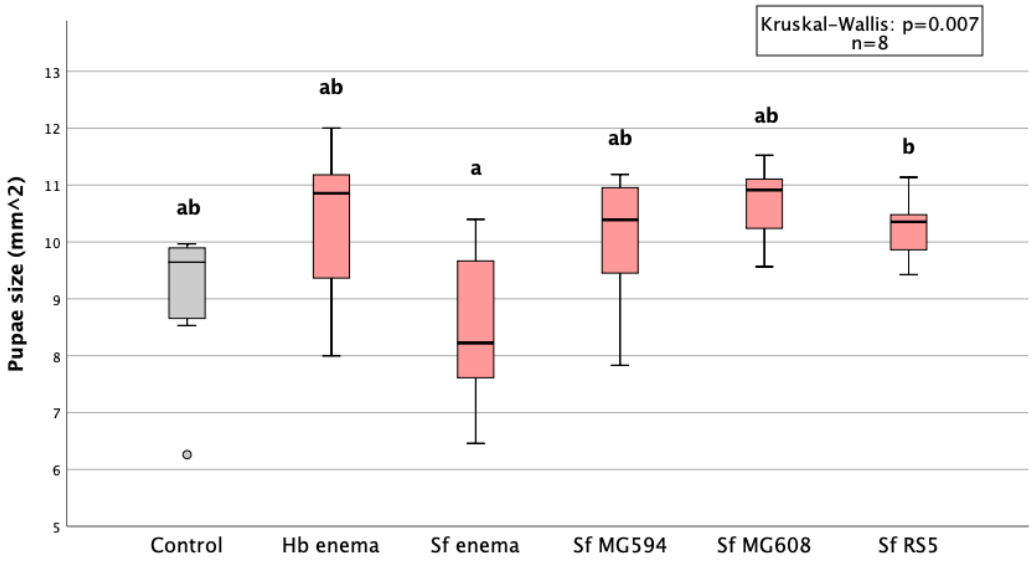


Figure 31: Pupal size of D. radicum *eggs in the climate chamber assay to evaluate nematode strains for the control of* D. radicum *(fourth repetition).*

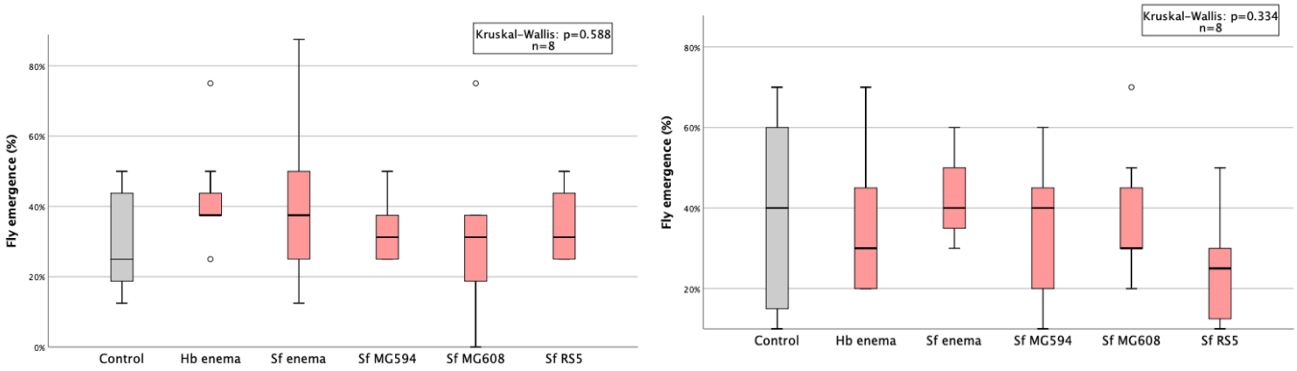


Figure 32: Fly emergence rates of D. radicum *in the climate chamber assay to evaluate nematode strains for the control of* D. radicum *(first repetition left and third repetition right).*

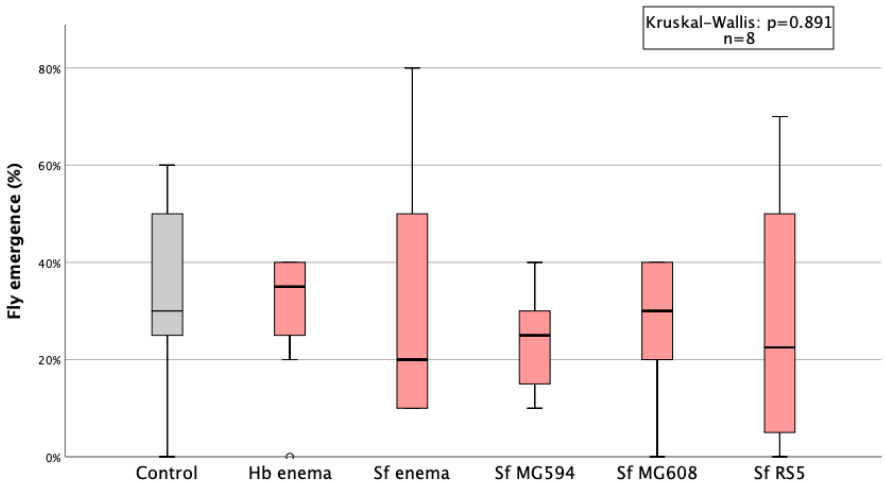


Figure 33: Fly emergence rates of D. radicum *in the climate chamber assay to evaluate nematode strains for the control of* D. radicum *(fourth repetition).*

Table 4: Ingredients used for LB medium.

water	**1 liter**	**2 liter**	**3 liter**	**5 liter**
Tryptone (Difco)	10g	20g	30g	50g
Bacto Yeast Extract (Difco)	5g	10g	15g	25g
$MgSO_4$ * $7H_2O$ (C24-163)	0.25g	0.5g	0.75g	1.25g
NaCl (C24-212)	8g	16g	24g	40g

pH 7.0

Table 5: Ingredients used for King's B (KB+++ or KB++^{G}) medium.

water	**1 liter**	**3 liter**	**5 liter**
Proteose peptone No. 2 (Difco)	20g	60g	100g
Glycerol 87% (→ 7.3ml/l of 100% Glycerol)	10g / 8.4ml	25.2ml	42ml
$MgSO_4$ * 7 H_2O (C24-163)	1.5g	4.5g	7.5g
K_2HPO_4 * 3 H_2O (C24-151)	1.5g	4.5g	7.5g
Agar → for SOLID medium only!	12-15g	12-15g/l	12-15g/l
Cycloheximide (100mg/ml stock → 100mg/l)	1ml	3ml	5ml
Chloramphenicol (100mg/ml stock → 13mg/l)	130**µl**	390**µl**	650**µl**
Ampicillin (100mg/ml stock → 40mg/l)	400**µl**	1.2ml	2ml
Gentamycin (50mg/ml → 10mg/l)	200**µl**	600**µl**	1ml

gfp-tagged strains: add Gentamycin (10mg/l final) *¡replace Ampicillin with Gentamycin!*

pH 7.2-7.4

Table 6: Average pupation rates and fly emergence rates compared to the control treatment when testing the effect of different EPP strains on the development of D. radicum *in the greenhouse.*

Second repetition		
	Pupation rate	Fly emergence
Control	100,0%	100,0%
PCL1391	86,5%	122,2%
CHA0	83,8%	116,7%
64	94,6%	66,7%
77	100,0%	111,1%
Third repetiton		
	Pupation rate	fly emergence
Control	100,0%	100,0%
PCL1391	57,4%	46,0%
CHA0	72,1%	64,0%
64	65,6%	36,0%
77	85,2%	72,0%

Table 7: Survival of D. radicum larvae, 100 hours after direct infection with different EPN strains.

Treatment	Total N	N of dead larvae	N of living larvae	Survival (Percent)
Sf enema	12	6	6	50.0%
Hb enema	12	4	8	66.7%
Sf MG594	12	4	8	66.7%
Sf MG608	12	2	10	83.3%
Sf RS5	12	7	5	41.7%
Overall	60	23	37	61.7%

Table 8: Average pupation rates and fly emergence rates compared to the control treatment when testing an EPN-EPP combination for the control of D. radicum *in climate chamber.*

First repetition		
	Pupation rate	Fly emergence
Control	100,0%	100,0%
PCL1391-gfp	75,0%	127,8%
Sf enema	20,5%	22,2%
Sf MG594	59,1%	38,9%
PCL1391-gfp x Sf enema	56,8%	55,6%
PCL1391-gfp x Sf MG594	81,8%	127,8%
Second repetition		
	Pupation rate	Fly emergence
Control	100,0%	100,0%
PCL1391	37,5%	28,1%
Sf enema	52,1%	43,8%
Sf MG594	77,1%	68,8%
PCL1391 x Sf enema	41,7%	25,0%
PCL1391 x Sf MG594	45,2%	17,9%

Table 9: Pairwise comparisons of the fly emergence rates of the second repetition in the climate chamber assay to evaluate the combination of nematodes and pseudomonads for the control of D. radicum.

Pairwise Comparisons of Treatment					
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.a
Sf enema-Sf MG594	-4.062	6.806	-.597	.551	1.000
Sf enema-Sf enema x PCL1391-gfp	-8.062	6.806	-1.185	.236	1.000
Sf enema-Control	15.000	6.806	2.204	.028	.413
Sf enema-Sf MG594 x PCL1391-gfp	-18.875	6.806	-2.773	.006	.083
Sf enema-PCL1391-gfp	19.625	6.806	2.883	.004	.059
Sf MG594-Sf enema x PCL1391-gfp	-4.000	6.806	-.588	.557	1.000
Sf MG594-Control	10.938	6.806	1.607	.108	1.000
Sf MG594-Sf MG594 x PCL1391-gfp	-14.812	6.806	-2.176	.030	.443
Sf MG594-PCL1391-gfp	15.563	6.806	2.286	.022	.333
Sf enema x PCL1391-gfp-Control	6.938	6.806	1.019	.308	1.000
Sf enema x PCL1391-gfp-Sf MG594 x PCL1391-gfp	-10.812	6.806	-1.589	.112	1.000
Sf enema x PCL1391-gfp-PCL1391-gfp	11.563	6.806	1.699	.089	1.000
Control-Sf MG594 x PCL1391-gfp	-3.875	6.806	-.569	.569	1.000
Control-PCL1391-gfp	-4.625	6.806	-.680	.497	1.000
Sf MG594 x PCL1391-gfp-PCL1391-gfp	.750	6.806	.110	.912	1.000
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.					
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.					
a Significance values have been adjusted by the Bonferroni correction for multiple tests.					

Table 9: Average pupation rates and fly emergence rates compared to the control treatment in the climate chamber assay to evaluate nematode strains for the control of D. radicum.

1. Repetition		
Average compared to control	pupation rate	fly emergence
Control	100,0%	100,0%
Hb enema	161,9%	142,1%
Sf enema	142,9%	136,8%
Sf MG594	119,0%	110,5%
Sf MG608	128,6%	105,3%
Sf RS5	128,6%	115,8%
2. Repetition		
Average compared to control	pupation rate	fly emergence
Control	100,0%	100,0%
Hb enema	89,7%	81,0%
Sf enema	27,6%	28,6%
Sf MG594	55,2%	47,6%
Sf MG608	62,1%	57,1%
Sf RS5	48,3%	47,6%
3. Repetition		
Average compared to control	pupation rate	fly emergence
Control	100,0%	100,0%
Hb enema	102,5%	90,3%
Sf enema	117,5%	109,7%
Sf MG594	105,0%	90,3%
Sf MG608	97,5%	96,8%
Sf RS5	72,5%	62,9%
4. Repetition		
Average compared to control	pupation rate	fly emergence
Control	100,0%	100,0%
Hb enema	105,1%	88,9%
Sf enema	102,6%	92,6%
Sf MG594	69,2%	70,4%
Sf MG608	74,4%	81,5%
Sf RS5	92,3%	83,3%

8. Declaration

I hereby declare on oath that I have created the present

□ seminar paper

□ bachelor`s thesis

■ master`s thesis

□ dissertation

Without any outside help and without the use of any sources and aids other than those specified, and that I have indicated as such passages taken from the sources I used either verbatim or in substance. I have never submitted this work in its present or a similar form to any other examiner as a graded work.

I am aware that breaches of conduct will be sanctioned (“use of unauthorized aids”) and may lead to further legal action. In addition to the printed version, this paper has been submitted as a digital file to the examiner in charge for verification of the above statement.

Zurich, 30.09.2019 Michael Brunner _____

Signature