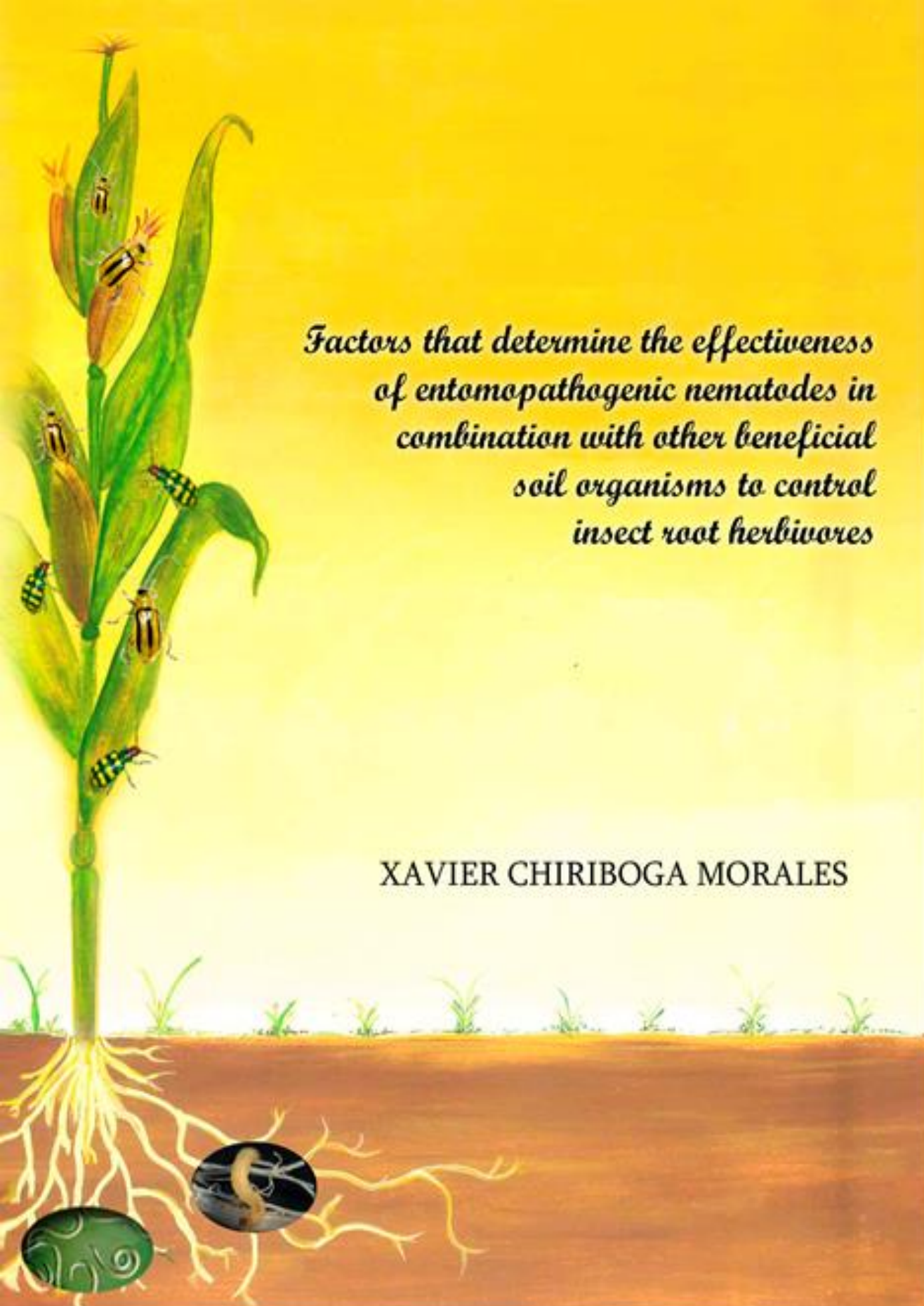

Factors that determine the effectiveness of entomopathogenic nematodes in combination with other beneficial soil organisms to control insect root herbivores

XAVIER CHIRIBOGA MORALES

UNIVERSITÉ DE NEUCHÂTEL

FACULTY OF SCIENCES

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Xavier Chiriboga M.

UNIVERSITÉ DE NEUCHÂTEL

Faculté des Sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel – Suisse
Tél : + 41 (0)32 718 21 00
E-mail : secretariat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Monsieur Xavier CHIRIBOGA M.

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sur le rapport des membres du jury composé comme suit:

- Prof. Ted Turlings, directeur de thèse, UniNE
- Prof. ass. Sergio Rasmann, UniNE
- Dr Raquel Campos Herrera, MeditBio, University of Algarve, Faro, Portugal
- Dr Monika Maurhofer, ETH Zürich

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Thesis Committee

Thesis Director: Prof. Ted C.J. Turlings, University of Neuchatel, CH

Co-Supervisor: Dr. Raquel Campos-Herrera, University of Algarve-MeditBio, POR

Other Members of the Committee:

Dr. Sergio Rasmann, University of Neuchatel, CH

Dr. Monika Maurhofer, ETH Zurich, CH

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To the memory of grandmom Beatriz (2009†)

and grandad Pedro (2015†)

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SUMMARY

Belowground ecosystems are inhabited not only by organisms that represent a threat to plants (e.g. root herbivores and pathogens), but also by organisms that provide protection to these antagonists (e.g. entomopathogenic nematodes, mychorrizal fungi and root associated bacteria). Herbivore-induced root volatiles, such as the sesquiterpene *E*-(β)-caryophyllene, are an indirect defense in maize plants that attract entomopathogenic nematodes to their host habitat. Entomopathogenic nematodes (EPN) readily infect and kill root herbivores, such as the voracious maize pest *D. virgifera virgifera*, thereby providing protection to the emitting the plant. Certain root-associated bacteria of the genus *Pseudomonas* also benefit plants by promoting growth, suppressing pathogens or inducing systemic resistance (ISR). Some *Pseudomonas* strains also have insecticidal activity. Arbuscular mycorrhizal fungi (AMF) are beneficial symbionts that colonize the roots of the majority of land plants, promote water and nutrients uptake in their host plant and contribute to enhance tolerance of their host plants to biotic and abiotic stresses.

This thesis explores how abiotic (e.g. soil texture and moisture) and biotic (e.g. root-associated bacteria) factors affect the emission and function of the herbivore-induced root volatile *E*-(β)-caryophyllene, an EPN attractant, in maize plants. Furthermore, we investigated how the single and combined application of EPN, *Pseudomonas* bacteria, and AMF can enhance wheat crop performance under realistic field conditions and traced their persistence after augmentation.

Using gas chromatography-mass spectrometry analyses, we found that *E*-(β)-caryophyllene diffuses best when humidity in agricultural soils is high (20%), which is the opposite from pure sand, where diffusion is best under drier conditions. We also found that this root-produced signal recruits the EPN *Heterorhabditis megidis* more efficiently in clay loam soils than in any other type of soil (Chapter 1). Furthermore, we discovered that root-colonization by the bacterium *Pseudomonas protegens* CHA0 enhances the emission of *E*-(β)-caryophyllene from *D. balteata*-infested maize roots. This was confirmed with qPRC analyses of the expression of the terpene synthase gene *Zm-tps*23 in maize roots. Rootworm larvae tended to gain more weight and cause more damage when feeding for three days on roots

colonized by *P. protegens* CHA0, whereas the opposite trend was found for larvae that fed on *P. chlororaphis* PCL-colonized roots, resulting in lower levels of root damage (Chapter 3).

In field trials with wheat, we found that, after application, EPN, *Pseudomonas* bacteria, and AMF persisted in the soil and persisted until the end of the cropping season, although populations declined considerably over time. Single applications of *P. protegens* CHA0 and *P. chlororaphis* PCL, as well as their application with the EPN *H. bacteriophora* improved seedlings survival in plots where the wheat plants were infested by larvae of the frit flies and hessian flies. Moreover, the combination of *P. protegens* CHA0, *P. chlororaphis* PCL and *H. bacteriophora* resulted in a significant increase of wheat seed productivity under frit-flies stress. Seedling survival, under insect attack, tended also to be higher in plots inoculated with the AMF *Rhizoglomus irregulare* (Chapter 2).

Finally, in pot experiments we tested the efficacy of certain combinations of EPN and root-associated bacteria to control *D. balteata* larvae in the rhizosphere of squash plants. We only found a marginal difference between treatments. The combination of *P. protegens* plus *H. bacteriophora*, the bacteria may display an antagonism against the EPN and/or its enteric bacteria. *H. bacteriophora* alone resulted in significant higher mortality than the bacteria *P. protegens* CHA0. The combination of *P. chlororaphis* PCL with the EPN *Steinernema feltiae* enhanced larvae mortality comparing with the treatment with *P. chlororaphis* PCL alone. But, larvae mortality in the combination *S. feltiae* plus *P. chlororaphis* was not different from mortality with *S. feltiae* alone (Chapter 2-Apendix).

RESUMEN

Los ecosistemas subterráneos están habitados no solamente por organismos que representan una amenaza para las plantas (ejem: herbívoros de raíz y patógenos), pero también por organismos que proveen las protejen de estos antagonistas (ejem: nemátodos entomopatógenos, hongos micorrízicos y bacterias asociadas de raíz). En plantas de maíz, los volátiles de raíz inducidos por herbívoros, como el (*E*)-β-cariofileno, son una defensa indirecta que atrae nemátodos entomopatógenos al hábitat de su hospedero. Los nematodos entomopatógenos (NEP) localizan a los insectos herbívoros de raíz, inmediatamente, infectan y matan, como a la voraz plaga del maíz *Diabrotica virgifera virgifera*, protegiendo a la planta emisora de daños posteriores. Ciertas bacterias asociadas a raíz del género *Pseudomonas* también benefician a la planta promoviendo el crecimiento, suprimiendo patógenos o induciendo Resistencia Inducida (RSI). Se sabe que algunas cepas de *Pseudomonas* también tienen actividad oral insecticida. Los hongos micorrízicos arbusculares (HMA) son simbiontes benéficos que colonizan la mayoría de plantas terrestres, facilitan la adquisición de agua y nutrientes a su planta hospedera y contribuyen a incrementar su tolerancia a estreses bióticos y abióticos.

En esta tesis exploramos cómo factores abióticos (ejem: textura de suelo y humedad de suelo) y bióticos (ejem: bacterias asociadas a raíz) afectan la función y emisión del vólatil de raíz inducido por herbívoro (*E*)-β-cariofileno, un atrayente de NEP en plantas de maíz. Además, investigamos cómo aplicaciones individuales y combinadas de NEP, bacterias *Pseudomonas* y HMA pueden fortalecer el desarrollo del cultivo de trigo en condiciones reales de campo y trazamos su persistencia en el suelo después de la inoculación.

Usando cromatografía de gases-espectrometría de masas, encontramos que (*E*)-β-cariofileno se difunde mejor cuando la humedad es alta en suelos agrícolas (20%), lo cual es opuesto a la difusión en arena pura, donde la difusión es mejor en condiciones de menor humedad. También encontramos que esta señal producida por raíces recluta el NEP *Heterorhabditis megidis* más eficientemente en suelos arcillo-limosos que en otros tipos de suelo (Capítulo 1). Además, descubrimos que la colonización de raíz por la bacteria *Pseudomonas protegens* CHA0 incrementa la producción de (*E*)-β-cariofileno en raíces de maíz infestadas

por larvas del insecto *D. balteata*. Esto fue confirmado mediante análisis de la expresión del gen *Zm-tps*23 en raíces de maíz con rt-PCR. Las larvas del insecto de raíz tendieron a ganar más peso y causar más daño cuando se alimentaron por 72 horas en raíces colonizadas por *P. protegens* CHA0; mientras la tendencia opuesta fue hallada para larvas que se alimentaron en raíces colonizadas por *P. chlororaphis* PCL resultando en niveles menores de daño de raíz (Capítulo 3).

En ensayos de campo en trigo, encontramos que después de la inoculación, NEP, bacterias *Pseudomonas* y HMA persistieron en el suelo hasta el final del ciclo de cultivo, aunque las poblaciones declinaron el transcurso del tiempo. Aplicaciones individuales de *P. protegens* CHA0 y *P. chlororaphis* PCL, así como su aplicación con el NEP *H. bacteriophora* mejoraron la sobrevivencia de plantitas de trigo en parcelas que infestadas por moscas del trigo y por moscas de los pastos. Además, la combinación de *P. protegens* CHA0, *P. chlororaphis* PCL y *H. bacteriophora* resultó en un incremento significativo de la productividad de semillas de trigo bajo condiciones de estrés por las moscas. La sobrevivencia de plantitas también tendió a ser más alta en parcelas inoculadas con el HMA *Rhizoglomus irregulare* (Capítulo 2).

Finalmente en experimentos en macetas probamos la eficacia de combinaciones de NEP y bacterias asociadas a raíz para el control de larvas de *D. balteata* en la rizósfera de plantas de zuquini. Encontramos solamente una diferencia marginal entre tratamientos. La combinación de *P. protegens* más *H. bacteriophora*, la bacteria podría mostrar un antagonismo en contra del NEP y/o su bacteria entérica. *H. bacteriophora* causó una mortalidad significativamente más alta que la bacteria *P. protegens* CHA0. La combinación de *P. chlororaphis* con el NEP *Steinernema feltiae* resultó en un incremento de la mortalidad comparando con el tratamiento individual con *P. chlororaphis*. Pero la mortalidad en la combinación de *Steinernema feltiae* con *P. chlororaphis* no fue diferente de la mortalidad causada por *S. feltiae* (Capítulo 2-APÉNDICE).

GENERAL INTRODUCTION

BELOWGROUND PLANT HERBIVORY

The belowground plant biomass in Earth's soils is tremendously vast and serves as food to members of at least seven insect orders that spend an important part of their life cycle as belowground feeders (Blossey and Hunt-Joshi, 2003; Hunter, 2001). Belowground insect herbivores can exert many negative effects on plants, also in agricultural systems. For example, root damage may result in inadequate uptake of water, nutrients, and minerals, and thereby reduce the growth of aboveground plant parts (Maron, 2001; Erb, 2009). In addition, tissue damage inflicted by root herbivores increases a plant's susceptibility to infections by pathogens (Adair and Mehta, 2001). Although the study of root herbivory and plant defenses has received less attention than insect feeding on aboveground plant parts, an increasing number of studies are focusing on belowground plant defenses, not only from a fundamental (Huber et al., 2016), but also from an applied point of view (Robert, 2012; Erb, 2009). The latter is particularly relevant because insects often inflict much more damage in agroecosystems than in natural settings, and crop losses due to insect feeding reach 15% or more worldwide. (Peterson and Higley, 2001).

PLANT DEFENSES AGAINST INSECT PESTS

To cope with insect herbivory, plants have developed a wide array of defensive strategies that can be classified as direct and indirect defenses. Direct defenses are mediated by plant characteristic's that that directly affect an herbivore's performance or behavior, such as cuticles, trichomes, thorns or toxic secondary metabolites. Indirect defenses improve the performance of the herbivore's natural enemies, for example through the provision of shelter, alternative food or infochemicals for host location (Dicke and Baldwin, 2010; Schoonhoven et al., 2005). In the latter case, carnivorous and parasitic organisms take advantage of plant-provided infochemicals to locate their prey or hosts (Turlings et al., 1990; Turlings and Wäckers, 2004). These defenses can also be either constitutive, when their presence is independent of insect damage, or induced, when they are activated in response to insect attack (Schoonhoven et al., 2005).

Furthermore, chemical cues that serve as cues for the natural enemies of herbivores are not only released by the aerial parts of plants after aboveground insect damage (Unsicker et al., 2009) or egg deposition (Fatouros et al., 2008), but also by roots after root herbivore attack (Van Tol et al., 2001; Boff et al., 2001; Aratchige et al., 2004; Rasmann et al., 2005; Ali et al., 2010; Tonelli et al., 2016).

ROOT-MEDIATED INTERACTIONS

Belowground **herbivore-induced plant volatiles (HIPVs)** play an important role in mediating interactions in the rhizosphere and may help to control root herbivory and improve plant performance (Degenhardt et al., 2009; Hiltpold et al., 2009, Rasmann et al., 2011, Ali et al., 2012). So far, two root produced HIPVs have been identified: the sesquiterpene *E*-(β)-caryophyllene (*E*βc) in maize (Rasmann et al., 2005) and pregeijerene in the citrus hybrid Swingle citrumelo (Ali et al., 2010). Maize roots produce *E*βc in response to feeding by diabroticine larvae, such as *Diabrotica virgifera virgifera* and *Diabrotica balteata* Le Compte (Coleoptera: Chrysomelidae). This root signal is highly attractive to the entomopathogenic nematode (EPN) *Heterorhabditis megidis* and its production ensures higher infection rates of *D. virgifera virgifera* and lower root damage in maize plants (Degenhardt et al., 2009; Rasmann et al., 2005; Hiltpold et al., 2011) (Fig. 1). The production of *E*βc is highly variable at the genotype level (Gouinguené et al., 2001, Degen et al., 2004) in maize plants, and its is known that the emission has been lost in North-American maize varieties, possibly because this root volatile is also used by *D. virgifera virgifera* as an aggregation signal (Robert, 2012). Moreover, it is known that different root herbivores species induce distinct quantities of *E*βc in maize roots and different EPN species respond to the root signal in a different manner (Rasmann and Turlings, 2008). The emission of pregeijerene occurs broadly in different citrus genotypes. It attracts both plant parasitic and several species of EPNs (Ali et al., 2011, 2012) and even free living nematodes (Ali et al., 2013), which may act as hyperparasites. It has been suggested that with these signals and other root exudations plants can structure communities of nematodes in the rhizosphere (Rasmann et al., 2012).

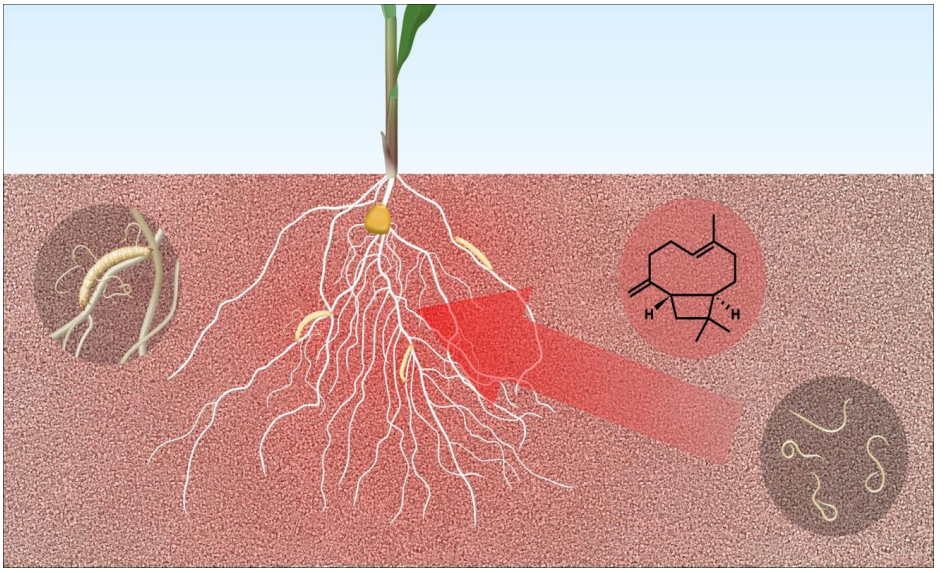

Figure 1. The sesquiterpene *E*-(β)-caryophyllene is produced by maize roots in response to rootworm feeding. Entomopathogenic nematodes use this volatile root signal to localize rootworm larvae, which they infect and rapidly kill.

ENTOMOPATHOGENIC NEMATODES: ALLIES WHEN A ROOT HERBIVORE ATTACKS

Entomopathogenic nematodes (EPNs) comprise the families Steinernematidae (genus *Steinernema* and *Neosteinernema*) and Heterorhabditidae (genus *Heterorhabditis*). They have lethal effects on insect pests, resulting from their association with a mutualistic enteric γ–Proteobacteria in the genera *Xenorhabdus* and *Photorhabdus*, respectively (Boemare, 2002). EPN reproduce inside their hosts and the third stage infective juvenile (IJ) is the only free-living stage that naturally occurs in the soil (Fig. 2), where they forage for new hosts. Once they find a host, they penetrate and release the symbiotic bacteria. The bacteria proliferate rapidly and release a toxin that kills the host within days. The nematodes feed on the degraded host tissues and the bacteria, develop and reproduce, until food is depleted (Boemare, 2002; Adams and Nguyen, 2002). Due to their shared characteristics with predators/parasitoids and microbial pathogens, and the facility of large-scale culturing, EPNs are considered highly suitable organisms for biological control of several insect pests, especially root feeders (Shapiro, 2013; Lacey et al., 2015).

Figure 2. Entomopathogenic nematodes.
Photo: Neil Villard

Various studies on the behavior and ecology of EPNs during the past decades have been conducted to optimize their use as efficient biocontrol agents (Lewis et al., 2006, 2015; Griffin, 2015). It is recognized that several factors can limit the efficacy of EPNs in controlling root pests under field conditions, such as the use of nematode strains or species that are not adapted to the target host or to local conditions (Georgis et al., 2006), a lack of alternative hosts in the soil (Susurluk; 2005), losses during or upon application (Smits, *et al*., 2004), unfavorable soil characteristics (Kaya, 1990), adverse environmental conditions (temperature and rainfall) (Georgis et al., 2006). Their success or failure is also dependent on the presence of other soil organisms that are part of the same food web, like nematophagous mites, nematophagous fungi, and free-living nematodes (Duncan et al., 2007, Campos-Herrera et al., 2012, Campos-Herrera et al., 2013).

The particular life cycle of EPNs places important constraints on the structure and dynamics of their populations. Once a new cohort of IJs emerge from the host cadaver, their ability to disperse and persist until they can locate a new host is crucial for their success in controlling insect pests. Occurrence, motility, distribution and persistence of EPNs is influenced by numerous interacting intrinsic factors (e.g., behavioral, physiological, and genetic characteristics) and extrinsic factors of abiotic (e.g., temperatures, soil moisture, soil texture, soil pH, and UV radiation) and biotic nature (host and non-host arthropods, intra and interspecific competitors, predators, parasites and pathogens) (Stuart *et al.*, 2006, 2015; Griffin, 2015; Koppenhöffer *et al.*, 2006). One of the most important soil factors is

moisture because nematodes need a water film for effective propulsion and dispersal (Koppenhöfer, 1995). In soil, IJs move through the water film that coats the interstitial spaces. When this film becomes too thin (in dry soil) or if the interstitial spaces are completely filled with water (in saturated soil), nematode movement can be restricted (Koppenhöffer *et al.*, 1995, 2006). Moreover, soil moisture in a given soil is closely linked with soil texture, which is defined mainly by soil particle size composition, and organic matter content (Barbercheck and Duncan, 2004). Generally, nematode motility decreases as soil pores become smaller (Kaya, 1990). Small soil pores, particularly in combination with high soil moisture, will also limit oxygen levels that affect activity and EPN survival (Burman & Pye, 1980; Kung *et al.*, 1990). Consequently, nematode efficacy against soil-dwelling insects should generally decrease in finer-textured, water-saturated soils and water-depleted soils.

To locate suitable hosts EPN can integrate different possible cues such as temperature, vibrations (Torr et al., 2004), electric potential (Shapiro-Ilan et al., 2012), and various organic and inorganic substances emanating from the hosts (Jones, 2002; Dillman et al., 2012). During the last 20 years it has become more and more evident that EPN also rely on cues provided by plant roots (Grewal et al., 1994; Wang and Gaugler, 1998; Boff et al., 2002; Turlings et al., 2012). These plant-derived odors, possibly in combination with ubiquitous gas CO_2, play an important role in EPN chemotaxis, the directed orientation of an organism towards or away of the source of stimulation (Rasmann et al., 2012; Turlings et al., 2012).

Belowground volatiles are expected to operate over much smaller scales than aboveground volatiles, because of their poor diffusion and interaction with soil particles, which inhibit their spread in the soil (van der Putten, 2003). Previous studies showed that diffusion of *E*βc occurs through the gaseous phase in sand rather than the aqueous phase, being favored by low water content. However, it has also been demonstrated that *E*βc diffusion is to some extend limited in an artificial sandy soil (Hiltpold and Turlings, 2008).

RHIZOSPHERE THE SCENARIO OF INDIRECT PLANT DEFENSE AGAINST ROOT HERBIVORES

The rhizosphere is a very complex habitat modeled by several factors: chemical and physical. The behavior and performance of EPN and other soil organisms are affected by these factors. For example, the three components that determine the physical properties of the soil: solid, liquid and gaseous, certainly affect mobility, foraging behavior, signaling, and interactions among organisms (Rasmann et al., 2012). Soil organisms are devoid of visual information, so they rather use chemical and tactile cues to communicate and orient themselves (Jones, 2002), and plants play an important role in providing such cues. Roots can produce and exude into the rhizosphere a great variety of compounds ranging from amino acids, complex polysaccharides, and proteins, to smaller more volatile lipophilic molecules, which directly or indirectly influence soil communities of organisms (Bais et al., 2012; Rasmann and Turlings, 2016). Hence, plant roots are not merely organs that serve to take up water and nutrients, but they also play a role in the synthesis, transport and exudation of defensive compounds that target herbivores direct, or indirectly by attracting natural enemies. The root-produced compounds serve not only to protect the roots, but are also transported to the aerial parts where they provide leaf resistance against aboveground insect and pathogens (Erb et al., 2009) Moreover, roots serve as storage organs for carbon-based metabolites that can be mobilized for regrowth after leaf attack (Erb, 2012). Finally, roots harbor numerous mutualistic micro-organisms that help shape belowground communities in the rhizosphere (Beredesn et al., 2012).

PLANT-BENEFICIAL SOIL INHABITANTS

Plant defensive strategies go beyond the combination of physical, chemical and developmental features, and soil not only holds beneficial EPN, but also an enormous diversity of microbes. Through evolutionary time, plants have allied with many of these belowground microorganisms to satisfy their nutritional needs and to protect themselves from harmful organisms. Microbial communities associated with roots are composed of tens of thousands of species and constitute a key determinant of plant health and productivity (Berendsen et al., 2012). Indeed, such is their importance that the plant microbiome has been considered as the second genome of the plant (Berendsen et al., 2012), and increasing evidence acknowledges that

rhizosphere microbial communities affect the plant and vice versa (Berendsen et al., 2012; Turner et al., 2013). In addition to direct effects on deleterious microbes on the rhizosphere (e.g. through competition for micronutrients, production of antibiotic compounds or enzymes, consumption of pathogen stimulatory compounds), many beneficial soil borne microorganism (e.g. *Pseudomonas* spp.) have been found to boost the defensive capacity in aboveground parts of the plant (Zamioudis and Pieterse, 2011). This induced systemic resistance (ISR) is a state in which the immune system of the plant is primed for accelerated activation of defense (Bakker et al., 2007).

Certain **root-associated bacteria of the genus *Pseudomonas***, besides their benefits for plant growth, pathogens suppression and induction of systemic plant defenses, also possess insecticidal activity against several herbivore species (Ruffner et al., 2013). Natural isolates of *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* have a high potential for application against various insect pests (Kupferschmied et al., 2013) (Fig. 3). Since many strains of the *P. fluorescens* group are adapted to live on plant roots, and because they show environmental persistence and are competitive and aggressive root colonizers, they may be ideal microorganisms for the long-term insect pest control (Lugtenberg and Kamilova 2009, Kupferschmied et al, 2013).

In addition to beneficial bacteria, several fungi, in particular **arbuscular mycorrhizal fungi**, have important symbiotic associations with the majority of plant species. They can greatly benefit plants, especially under conditions of phosphor limitation, and influence nutrient up-take, water relations and aboveground productivity. Arbuscular mycorrhizal fungi (AMF) also act as bioprotectants against pathogens and toxic stresses. Therefore, AMF are becoming important elements in low-input, organic or soil-free agriculture, and are incorporated in strategies such as bioaugmentation or inoculation of seedlings before transplanting (Fig. 4) (Jeffries et al., 2003). As proposed by Kupferschmied et al. (2013), future research should explore integrated pest management (IPM) strategies that combine the mentioned beneficial soil organisms. Such research may find potential synergistic effects of applying pseudomonads bacteria, EPN and/or AMF to optimally enhance plant health and the biocontrol of pest. Some studies have looked at the interactions between AMF and soil bacteria, but overall the underlying mechanisms behind these associations are very poorly understood (Artursson et al., 2006). One study suggests that certain nematodes that feed on bacteria can positively impact bacteria

colonization in wheat roots (Knox et al., 2004), but we are not aware of any study on combinations of soil bacteria and EPN and how they affect root colonization, plant health or root herbivore performance.

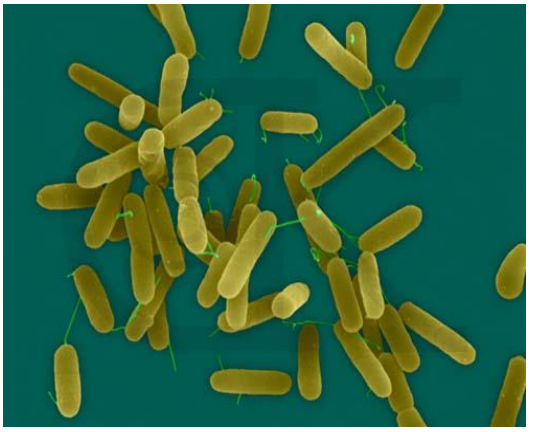

Fig 3. *Pseudomonas* sp.
Source: Pseudomonas genome database
http://v2.pseudomonas.com/

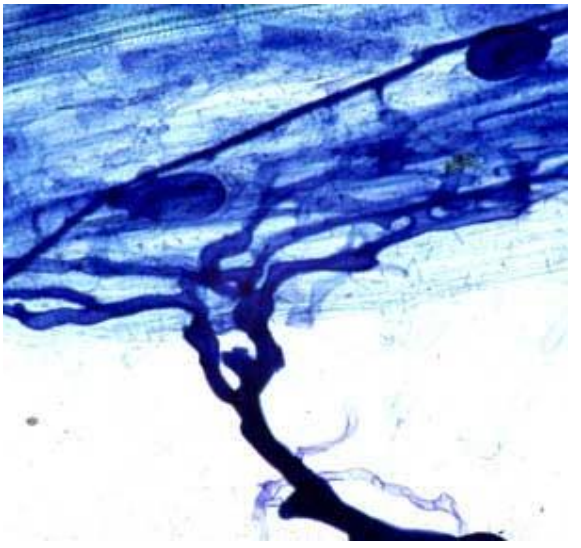

Fig 4. Arbuscular mychorrizal fungi
Source: Soil health website
http://www.soilhealth.com/soilhealth/biology/beneficial/fungi/index.htm

MAIZE AND WHEAT AS CROPS AND MODELS FOR RHIZOSPHERE STUDIES

It is noteworthy that 50 % of human food is obtained from only four crop species: maize, rice, wheat and potato (FAO, 2001). In 2013, maize and wheat were within the top five crops produced worldwide (FAO, 2013).

Maize *Zea mays* L. (Poales: Poacea) and its ancestor "teosinte" *Zea mays* spp. *parviglumis* Iltis and Doebley are original from Mesoamerica (Doebley, 1990). Modern maize varieties are adapted to different climatic regions and are cultivated worldwide, not only for human consumption, but also as food and fodder for cattle. Indeed, maize production has increased by 186 millions tons between 2010 and 2014 (FAOstat visualize, 2017) (Figure 3). However, maize crop is menaced by various oliphagous and polyphagous insect herbivores that feed on it aboveground and belowground. To date, one of the most important maize insect pests is the western corn rootworm *Diabrotica virgifera virgifera* Le Conte (Coleoptera: Chrysomelidae). It has spread from its origin in Mexico widely to the north in United States and Canada during the twentieth century. Through accidentally repeated introductions in Europe over the last two decades, this pest is now also an economic threat to maize production in many European countries (Toepfer et al., 2005). In Europe, *D. virgifera virgifera* extends over 11 countries, from Austria to Ukraine and from southern Poland to southern Serbia and several disconnected outbreaks have been reported in Italy, Switzerland, Eastern France, Belgium, United Kingdom, Netherlands and the Parisian region (France) since 1998 till 2005 (Ciosi et al., 2008). Another diabroticine rootworm that feeds on maize and other crops is *D. balteata* Le Conte (Coleoptera: Chrysomelidae), which is an important pest in Central and North America (Capinera, 2011). Because of this, maize has become an important biological model for the study of belowground plant defenses against insect herbivores, including their interactions with the natural enemies of these herbivores (Rasmann et al., 2005; Erb, 2009; Robert, 2012).

Fig. 5. *D. virgifera virgifera* larvae feeding on maize roots.
Photo: Neil Villard

Wheat *Triticum aestivum* L. and *T. turgidum* var. *durum* L. (Poales: Poacea) have been the staple food of the major civilizations in Europe, Western Asia and North Africa for over 8000 years. Wheat production is constantly growing worldwide without increasing the harvested areas (FAOstat visualize, 2017) (Figure 4), in part because of crop improvements to grow under diverse climatic conditions. As all crops, wheat is attacked by several species of insect pests (Miller and Pike, 2002) and numerous diseases of roots and shoots (Singleton, 2002). For example, the wheat bulb fly (WBF) *Delia coarctata* Fall., is an important pest in most of the major wheat-growing areas of England (Kowalski and Benson, 1978). It is a widespread Eurasian species that is also present in Switzerland with a restricted distribution (CABI, 2014) and it has also been recorded in Canada (McAlpine and Slight, 1981). Wheat is also affected by several diseases caused by fungi such as *Bipolaris sorokiniana*, *Fusarium culmorum*, *Fusarium graminearum*, *Gaeumannomyces graminis* var. *tritici*, *Phytium* spp. and *Rhizoctonia* spp. (Singleton, 2002). Several studies have addressed the use of beneficial soil (BeSo) microorganisms for the control of such fungal diseases and of insect pests (Tarasco, 2011; Lacey, 2015) and some studies have focused on the use of BeSo in the wheat crop (Pierson and Weller, 1994; Knox et al. 2004). For example, Pierson and Weller (1994) report an enhancement in wheat yield with the application of certain combinations of pseudomonas bacteria. In addition, Knox et al (2004) demonstrated that the use of a mixed nematode

community of *Acrobeloides* spp. and *Caenorhabditis elegans* enhance bacteria seed colonization by *Pseudomonas fluorescens* SBW25. Therefore, wheat crop is a suitable model to evaluate the use of BeSo organisms for enhancement of plant health in field conditions.

THESIS OUTLINE

The emission of the sesquiterpene *E*βC by maize plants has potential for crop protection against belowground insect pests. How this important EPN attractant diffuses in the complex matrix of the soil is as yet unknown, as is the influence of interacting abiotic and biotic elements that determine particular soil characteristics. In this thesis, I investigated the effects of selected abiotic and biotic factors on the diffusion, attractiveness and production of *E*βC. I hypothesized that: 1) *E*βC diffuses differently depending on soil texture and soil moisture, 2) the recruitment efficiency of the EPN *H. megidis* differs depending on soil texture 3) root colonization by Pseudomonas bacteria affect *E*βC release in maize plants.

In addition, I investigated the feasibility of combining EPN, with plant-beneficial bacteria, and AMF to enhance wheat protection and performance in field experiments, hypothesizing that 4) their combined application has a greater positive effect on wheat performance than their individual (or no) application. I also tested the efficiency of combining EPN and Pseudomonas bacteria in controlling rootworm larvae under laboratory conditions. Expecting that 5) that the combination of EPN and root colonizing bacteria acts synergistically to control the root pests.

- In the Chapter 1, I investigated the diffusion dynamics of synthetic *E*βc in different soil textures at different levels of water content. Furthermore, I studied the efficiency of the root produced maize volatile *E*βc in attracting the EPN *H. megidis* in different soil textures.

- In the Chapter 2, I investigated the seasonal persistence of several soil beneficial organisms (BeSO) that were applied in combination or singly in wheat plots, and I evaluated how these combinations affected wheat performance (field experiments) and mortality of root herbivore *D. balteata* (pot experiments).

- In the Chapter 3, I investigated how maize root-colonization by Pseudomonas bacteria affects the production of *E*βc and the expression of its terpene synthase gene TPS23. In this study, I further evaluated if root-colonization by *Pseudomonas* spp. affects root growth and the performance and mortality of root feeding larvae of *D. balteata*.

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-CHAPTER 1-

Diffusion of the maize root signal (*E*)-β-caryophyllene in soils of different textures and the effects on the migration of the entomopathogenic nematode *Heterorhabditis megidis*

Xavier Chiriboga M.[1], Raquel Campos-Herrera[1,2] Geoffrey Jaffuel[1], Gregory Röder[1] and Ted C.J. Turlings[1]

[1]Fundamental and Applied Research in Chemical Ecology-FARCE Lab. Institute of Biology, University of Neuchâtel, Emile-Argand 11, Neuchâtel CH-2000 Switzerland

[2] Centro para os Recursos Biológicos e Alimentos Mediterrânicos (MeditBio), Campus Gambelas, Edf. 8, FCT, Universidade do Algarve, 8005-191, Faro, Portugal

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ABSTRACT

Maize roots respond to feeding by larvae of the beetle *Diabrotica virgifera virgifera* by releasing (*E*)-β-caryophyllene (*E*βc). This insect-induced root volatile attracts entomopathogenic nematodes (EPN) and thereby helps to protect the roots against herbivore damage. Previous studies suggest that diffusion of *E*βc occurs through the gaseous rather than the aqueous phase in sand and its diffusion is best at low levels of humidity. However, it remains largely unknown how *E*βc diffuses in typical natural and agricultural soils. To fully understand the function and efficiency of root-produced *E*βc as a belowground signal it is important to know how it spreads in real soils and how soil properties affect its diffusion. Using gas chromatography-mass spectrometry analyses, the diffusion of two doses of synthetic *E*βc (200 ng and 20000 ng) injected in sand was compared with the diffusion of *E*βc injected in clay, clay-loam and sandy-loam soils, at 3 moisture levels (5, 10 and 20% water), and at two distances (5 and 10 cm) from the *E*βc injection point. The diffusion of the compound was measured with a Solid Phase Micro Extraction (SPME) fiber every 30 minutes over a period of 9 hours. We found that, in contrast to the pattern observed for pure sand, diffusion of *E*βc was best when humidity was high in the three agricultural soils. In subsequent experiments we used glass-trays to create two types of mesocosms to assess the effect of synthetic or root-produced *E*βc on the dispersal of the EPN *Heterorhabditis megidis* and its infection of sentinel hosts in the trays. The presence of synthetic *E*βc did not affect the ability of *H. megidis* to infect the sentinel host. However, under the test conditions, *E*βc released from maize roots influenced the migration behaviour of *H. megidis* depending on soil type. The results suggest that *D. virgifera*-damaged maize plants may recruit *H. megidis* more efficiently in clay loam soils than in other types of soil. These new insights into the diffusion dynamics and attraction efficiency of the root-produced signal *E*βc may help efforts to develop novel strategies for the sustainable management of the maize pest *D. virgifera*.

Key words: entomopathogenic nematode, *Heterorhabditis megidis*, belowground signaling, *Diabrotica virgifera virgifera*, (*E*)-β-caryophyllene, maize, soil, texture

INTRODUCTION

Plants produce herbivore-induced plant volatiles (HIPVs) in response to herbivory attack (Karban and Baldwin, 1997; Dicke and Baldwin, 2010). These volatiles, which are not only emitted from leaves (Dicke and Sabelis, 1988; Turlings et al., 1990), but also from roots (Rasmann et al. 2005; Ali et al. 2010) have been proposed to function as an indirect defense to attract natural enemies of the herbivore that attacks the plant. HIPVs mainly comprise terpenoids, fatty acid derivatives, phenyl propanoids and benzenoids (Mumm and Dicke, 2010; Dudareva et al. 2006). Recently, two terpenes have been described as herbivore-induced root signals, the sesquiterpene (*E*)-β-caryophyllene (*E*βc) in maize (Rasmann et al., 2005) and pregeijerene in citrus, specifically Swingle citrumelo (Ali et al., 2010). Both attract entomopathogenic nematodes (EPNs), which infect and kill the root herbivores feeding on the roots of the emitting plants, thereby reducing root damage (Degenhardt et al., 2009; Ali et al., 2012; Hiltpold & Turlings, 2012).

The role of *E*βc as an EPN attractant has been confirmed in laboratory and field experiments. For instance, in experiments with belowground olfactometers, the EPN species *Heterorhabditis megidis* Poinar, Jackson & Klein (Rhabditida: Heterorhabditidae) and *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) were significantly attracted to damaged maize roots when compared with the attractiveness of undamaged roots (Rasmann et al., 2005; Rasmann and Turlings, 2008). Furthermore, the EPNs *H. megidis* and *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae) are more efficient in reducing root damaged by *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) in *E*βc emitting maize than in non-emitting variety (Rasmann et al., 2005; Degenhardt et al., 2009; Hiltpold et al., 2010). It has been suggested that the diffusion properties of *E*βc make it a highly suitable belowground signal (Hiltpold & Turlings, 2008).

The diffusion of organic compounds in soil matrices is affected by several factors that are closely related to the sorption properties of the soil (Lindstrom et al., 1967; Steinberg and Kreamer 1993). Sorption of organic compounds in the soil largely depends on its chemical composition (e.g. mineral) and physical properties (e.g. particle size, density, porosity) (Ruiz et al., 1998). Water content (Porter & Kemper, 1960) and organic matter

also play key roles in sorption (Li & Werth, 2001). Previous studies with pure sand have demonstrated that *E*βc rapidly diffuses through the gaseous phase of the sand matrix (Rasmann et al., 2005; Hiltpold & Turlings, 2008), which was most evident from the easy and rapid horizontal diffusion of *E*βc at low moisture levels. However, a first attempt to characterize *E*βc diffusion in a sandy soil showed a clear decrease in relation to pure sand (Hiltpold & Turlings, 2008). It therefore remains unclear how *E*βc diffuses in different soil types, and to what extent diffusion depends on sand content. To broaden our knowledge on the signaling function and efficiency of *E*βc as a belowground signal, it is essential to identify key soil characteristics that affect its diffusion.

Soils are composed of particles of different sizes (such as sand, clay and silt) and their relative proportion defines soil texture, which in turn determines pore size. We therefore hypothesized that diffusion of *E*βc and EPN attraction towards the root volatile diminishes when sand content decreases.

The current study aimed to characterize the diffusion of *E*βc through soils of different textures and define how soil humidity affects this diffusion. We used a combination of fibre-based solid phase microextraction and gas chromatography-coupled mass spectrometry (GC-MS) to measure *E*βc diffusion in various soils. In subsequent experiments we used soil-filled glass trays to test how a point source of authentic *E*βc may help guide the EPN *H. megidis* towards sentinel insect hosts in different soil textures. In addition, we tested the attraction of *H. megidis* to insect-damaged maize varieties with distinctly different *E*βc emission rates in these soil types.

MATERIALS AND METHODS

Soils, nematodes, insects, plants, and general procedures

Experiments were performed with pure sand (Migros, Switzerland) and three agricultural soils of different textures (Table 1). We used a Gleyic Cambisol clay soil (C), a clay loam soil (CL) and sandy loam soil (SL) (IUSS Working Group WRB, 2006). Texture analyses were performed by Soil Conseil (Nyon, Switzerland). The soils were collected in the experimental fields of Agroscope, *Institut des Sciences en Production*

Végétale (46° 24' N, 6° 14' E, 430 m above sea level, Changins-Nyon, Switzerland) during 2013 and 2014. Following procedures described by Hiltpold & Turlings (2008), soils were ground, sieved in a 2 mm mesh and autoclaved (120°C) to obtain a sterile substrate. Soils were also ventilated for at least 24 hours to eliminate possible odours and volatiles that might interfere with the detection of *E*βc.

Table 1. Characteristics of soils used for diffusion and foraging behaviour experiments.

Nomenclature	Soil texture	%Sand †	%Clay †	%Silt †	pH †	% organic matter †	Field Capacity ‡
CA	Clay	17	48	35	6.7	4.1	25%
CL	Clay loam	29	42	29	8	2.3	21%
SL	Sandy loam	56	18	26	8	2.9	20%
S	Sand (pure)	100	-	-	-	0	10%

† Determined at Soil Laboratory (Soil Conseil, Nyon, Switzerland)
‡ Determined following Estimating Soil Moisture by Feel and Appereance, USDA (1998)

A commercial population of the EPN *H. megidis* (Andermatt Biocontrol AG, Switzerland) was used for the glass-tray experiments. The identity of the species was morphologically and molecularly confirmed (ITS rDNA region sequence, GenBank Accession number KJ938577) (Campos-Herrera et al., 2015). New generations of freshly emerged nematodes reared from *Galleria melonella* L. (Lepidoptera: Pyralidae) larvae were used no more than 15 days after emergence. Suspensions of infective juveniles (IJs) were prepared by counting IJs under a stereo-microscope and by adjusting the concentration in distilled water to 2000 IJs/mL. In each soil tray, 2000 IJs of *H. megidis* were inoculated at 10 cm distance from a capillary dispenser (2nd experiment, see below) or the maize plant (3rd experiment).

Larvae of *G. mellonela* (commercial stock, *Au Pêcheur* SARL, Neuchâtel, Switzerland) were used for the nematode rearing and also as sentinel hosts in the first tray experiments to quantify infection success. In the second tray experiments, we infested maize plants with second instar larvae of *Diabrotica virgifera virgifera* Le Conte (Coleoptera: Chrysomelidae) obtained from the North Central Agricultural Research Laboratory-USDA (Brookins, USA).

Two maize (*Zea mays* L., Poales: Poaceae) varieties were used: *i*) Graf as the high *E*βC emitting plant and *ii*) Pactol as the non-emitting plant (Gouinguené et al., 2001; Rasmann et al. 2005). Seeds were sown in plastic pots and plants grown in a climate chamber (Grow bank, 24°C, 14:10 hours light:dark photoperiod, 320 µmol m^{-2} s^{-1}) during 16 days until they reached the 4 leaf-stage.

Diffusion patterns of synthetic Eβc in various soil types, at different moisture levels, distances and concentrations

A solution of authentic *E*βc (Sigma Aldrich, > 98% pure) was prepared at a low (200 ng) and high concentration (20000 ng) by dissolving the compound in different amounts of pentane. Soil moisture was adjusted by weight/volume to obtain levels corresponding to 5, 10 and 20% with mQ water (Milli-Q Water System, Millipore S.A., Molsheim, France). For the diffusion experiments a Teflon box (12 cm x 10 cm x 4 cm) was filled with one of the soil treatments. For each treatment, we used a soil moisture-soil texture combination with a constant mass of moistened soil or sand, thereby maintaining a homogenous density among all replicates (Supplementary data 1).

Either a low or high concentration of *E*βc was injected in a tray with one of the substrates at different levels of humidity (5%, 10% and 20%) and at different distances from the sampling fibre (10 cm or 5 cm). Each combination of soil, humidity and distance was replicated five times. The Teflon box was placed on a thermal tray, maintaining the temperature at 12 °C. A 0.2 mm diameter cylinder made of ultrafine metal mesh (2300 mesh, Small Parts Inc., USA) was inserted into the soil, creating a hole in which a Solid Phase Micro Extraction (SPME) fibre (100 µm polydimethylsiloxane, Supelco, Buchs, Switzerland) was introduced at a depth of 3 cm from the soil surface. Automated sampling was done over a

total period of 9 or 12 hours with a multipurpose sampler (MPS2, GerstelGmbH & Co. KG, Germany) (Hiltpold & Turlings, 2008). After the first 30 min sampling period, the *E*βc solution was injected at 3 cm deep into the soil. Every 30 min, the fibre was retracted automatically (multi-purpose sampler MPS2, Gerstel GmbH &Co. FG, Germany) from the soil matrix and inserted for 3 min into the injector of an Agilent 680 Series gas chromatograph (G1530) coupled to a quadrupole-type mass-selective detector (Agilent 5973, transfer line 230°C, source 230°C ionization potential 70 eV). The injector was kept at 230°C and the desorbed compounds were separated on a polar column (HP1-MS, 30 M, 0.25 MM id, 0.25 µm film; Agilent Technologies, USA) using helium as a carrier gas (constant pressure of 127.9 kPa). Following injection, the temperature of the column was maintained at 40°C for 1 min and then increased 20 °C min -1 to 250 °C, where it was held for 12 min more.

Statistical analysis. For soils at 10% of humidity, the curves were fitted according to a Linear Mixed Effects Model (LME) and the parameters for intercept and slope were compared. We exclude sand from the comparison because its diffusion did not fit a linear model. For soils at 20% of humidity, values obtained for abundance of *E*βc were used to model a curve according to the diffusion equation (see equation A.1) (Eqworld, 2015). The parameters of the curve were compared with a Non-linear mixed effects model (NLME). We used R environment (version 3.1.2, 2015) for both analyses.

Equation A.1

$$Abundance(t) = \frac{A}{\sqrt{t}} \exp\left(-\frac{1}{B*t}\right) \quad (1)$$

Where: A represents the slope and B the peak

Effects of soil texture on host infection by *Heterorhabditis megidis* in the presence or absence of synthetic EβC

Soils of three different textures were prepared as described above. For each substrate one kg of each substrate was adjusted to 20% humidity (weight/volume) and placed in a glass tray (Pyrex, France, 23 cm x 15 cm x 6 cm) (n = 3 per treatment). Four larvae of *G. melonella* were caged in a cylindrical metallic mesh cage (6.5 cm x 2.7 cm, diameter), which was

buried at 3 cm distance from one of the edges of the glass container filled with soil. EβC dispensers were made of 1 ml vials containing 10 mg of cotton wool treated with 200 µL of authentic EβC. The vials were closed with a screw cap with a Teflon-covered septum through which a 100 µL glass capillary (Hirschmann Laborgerate ringcaps Duran, GmbH & Co. KG, Germany) was inserted (as described by Hiltpold et al., 2010). The dispensers were inverted and the capillaries inserted into trays with the substrate, behind the cage with larvae and 2 cm from the edge (Fig. 1A). Soil trays without dispensers were used as controls (Fig. 1B). Experiments were conducted at room temperature (22 ± 2 ºC, 33% relative humidity) and larval mortality was recorded every 12 hours during 4-5 days. The experiment was repeated three times, each time using fresh nematode inoculum, insect larvae and newly prepared soils.

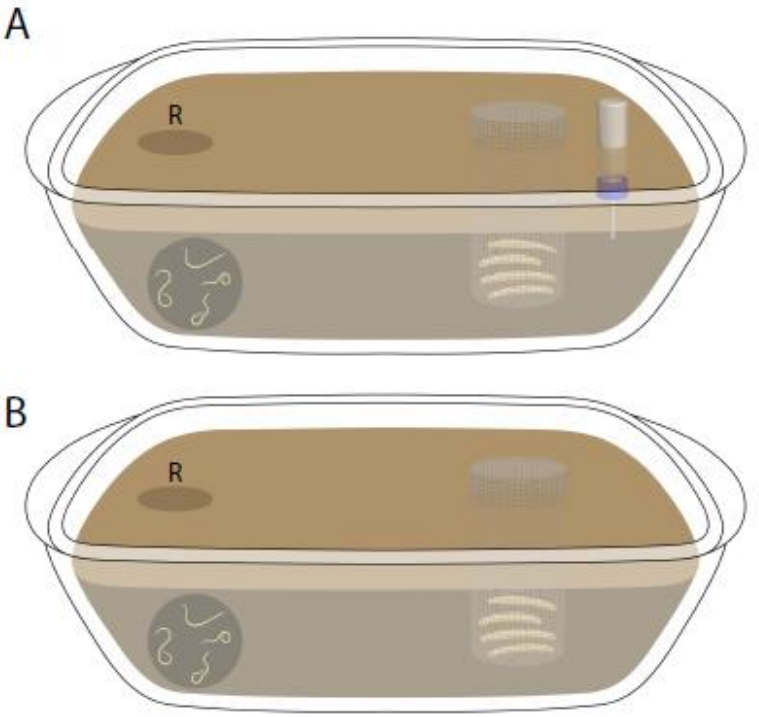

Fig. 1. Set-up used to test the effects of soil type on host infection by *Heterorhabditis megidis.* (A) Four *Galleria melonella* larvae caged next to an (*E*)-β-caryophyllene releasing capillary dispenser, (B) Four *Galleria melonella* larvae caged without capillary dispenser (control).

Data of larval mortality from the three experiments were pooled after testing homogeneity of the results. To compare mean times of death between different treatments statistical analyses were performed with a Generalized Linear Mixed-Effects model, with gamma distribution and replicate as a random effect in R environment (version 3.1.2., 2015). In the second and third experiments, numbers of nematodes that infected each sentinel larva were recorded by dissecting cadavers and digesting the tissues with a pepsin solution 72 hours after larval death (Mauleon et al., 1993). Data from these experiments were pooled. The proportion of nematodes that succeeded to infect one larva at each time-point of evaluation was calculated and these data were square root transformed in order to normalize their distribution. Data were analysed with a Two-Way Anova in R environment (version 3.1.2., 2015).

Effects of soil texture on migration of *Heterorhabditis megidis* in the presence or absence of naturally produced EβC

The effect of plant-produced *E*βC on EPN attraction in different soil types was tested. To provide optimal water conditions for the plants, water content for each soil was adjusted to achieve field capacity based on values provided by the USDA (1998). MilliQ water was added to each substrate in different proportions: C (25%), CL (21%) and SL (20%). In each tray we placed a 16-day old (four leaves) maize plant at 1 cm distance from one of the edges of the tray and the root system was infested with six late second instar *D. virgifera virgifera* larvae. The larvae were allowed to feed on the roots for 48 hours before releasing the EPNs (Fig. 2). Trays without plants were used as controls. Three replicates per treatment were done simultaneously and the experiment was repeated two times (n=6). Infective juveniles of *H. megidis* were released 48 hours after insect infestation, 12 cm away from the maize plant. Fifty-six hours after this release, the numbers of individuals of *H. megidis* were estimated by sampling four positions within each soil tray: 1) 12 cm away from the inoculation point, in the plant/no-plant (P), 2) in the middle of the tray, at 5 cm distance from the inoculation point (M), 3) in the release point (R) and 4) in the sides of the trays (L) (Fig. 2). A sample consisted of two cores of soil of 19.6 cm^3 each one (approximately 50 g of soil), taken with a cylindrical metallic sampler (2.6 cm, diameter). To recover the EPNs from the samples they were placed in Baermann funnels (Hass et al., 1999). Each time the sampler was cleaned with distilled water. After 24 hours, the

numbers of *H. megidis* individuals that had fallen from the funnel trap were decanted in 10 mL of water and were counted under a stereo-microscope. The experiment followed a split-plot design with four factors: Replicate, Soil type, Plant presence and Location within the tray. The numbers of nematodes counted at each location were transformed with (log+1) to normalize the data. Data were analysed with a Mixed Procedure with fixed factors (Soil, Plant, Location, Replicate) and random factors (Replicate x Soil, Replicate x Soil x Plant in SAS (9.2. Cary, NC, USA). Differences between treatments were obtained by Least Square Means.

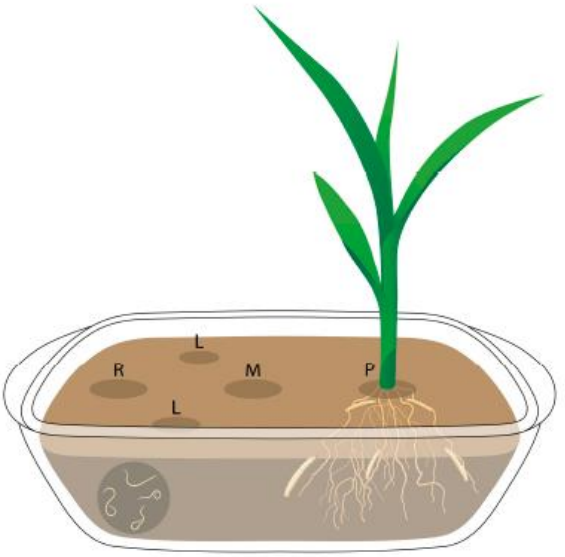

Fig. 2. Set-up used to test the foraging success of *Heterorhabditis megidis* in different soil types. Schematic layout of a tray with a maize plant, rootworm larvae and nematodes: nematode release point (R), lateral (L), middle (M) and plant (P). Control trays did not include maize plants

RESULTS

Diffusion patterns of synthetic Eβc in various soil types, at different moisture levels, distances and concentrations

The diffusion of *E*βC was strongly reduced in soils with high clay content and 10% moisture level, but markedly improved when soil moisture was increased to 20%. At 10% moisture and 10 cm distance from the source, *E*βC (in low concentrations) diffused readily in sand, as expected (Hiltpold & Turlings, 2008), but was not detected in any of the soils we tested **(**Supplementary data 2). When the concentration of *E*βC was increased 100-fold, *E*βC rapidly diffused in sand, and we could also detect it at 10 cm distance in clay loam (CL), but not in sandy loam (SL) and clay (C) soils (Supplementary data 3). When reducing the distance from the fibre to the source to 5 cm, with the high dosage of *E*βC and 10% of humidity in the substrates, EβC was detected in considerable amounts immediately after injection in sand, while in all soils a slower but increasing gradient of detection was found over the 9 hours of measurements (Fig. 3). The slope and intercept for the gradients of diffusion varied among the soil types. The soils C and CL showed differences in both slope ($P = 0.03$) and intercept ($P = 0.03$), whereas C and SL only differed in slope ($P = 0.02$) and CL and SL differed marginally in the intercept ($P = 0.09$) (Fig. 3).

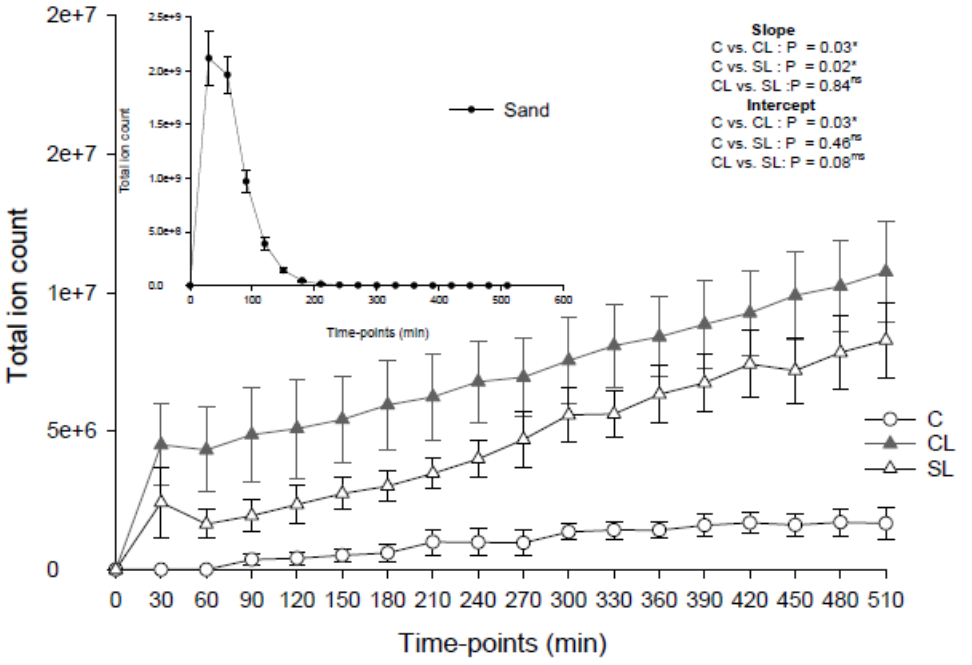

Fig. 3. Diffusion of (*E*)-β-caryophyllene injected at a high concentration (20,000 ng) in pure sand (S) and three different soil types: clay (C), clay loam (CL) and sandy loam (SL) at 10% humidity and at 5 cm distance from the sampling fibre. Data are average ±SE

Interestingly, when soils were tested at 20% humidity, detection of *E*βC improved dramatically (Fig. 4). The highest detection was recorded in sandy loam (SL) within one and half hours after injection. Much lower and similar amounts of *E*βC were measured both in clay loam (CL) and clay (C) within 120 minutes and 60 minutes after injection, respectively. Differences between slope/peak in each of the comparison between soils were not significant (P >0.1). In contrast, in soils at 5% of humidity the diffusion of EβC was marginal and variable in the three soil textures (Supplementary data 4).

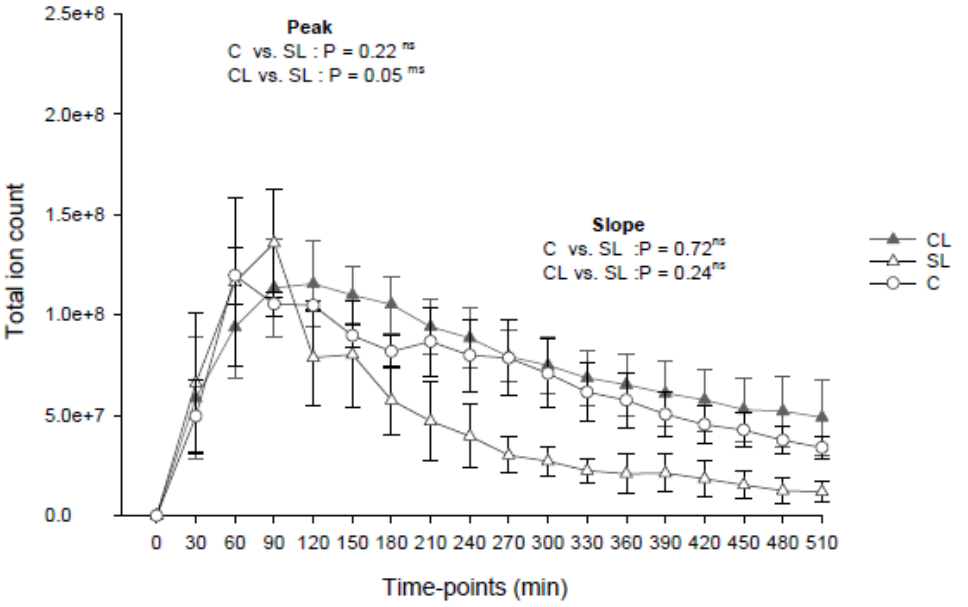

Fig. 4. Diffusion of *E*-(β)-caryophyllene injected at a high concentration (20,000 ng) in three different soil types: clay (C), clay loam (CL) and sandy loam (SL) soil at 20% humidity and at 5 cm distance from the sampling fibre.

Effects of soil texture on host infection by *Heterorhabditis megidis* in the presence or absence of EβC

Overall, the mean time of death after release of *H. megidis* was affected by soil type ($P < 0.01$; Fig. 5). Mean time of death was also affected by the presence or absence of an *E*βC-releasing capillary dispenser ($P < 0.05$; Supplementary data 5) but this effect only accounted for 27% of the variation in the experiment. The interaction between soil and *E*βC was not significant ($P > 0.1$) and we therefore used an additive model to analyse the data. The model showed that the observed mean times of death are lower for sand, sandy loam and clay loam soils compared with clay soil (Table 2). Numbers of IJs that succeed to infect one larvae of *G. melonella* were different between soil types ($F_{3,163} = 10.7$, $P < 0.01$) and the presence of a *E*βC-capillary dispenser had a marginal effect ($F_{1,163} = 2,7$, $P = 0.09$). The interaction between soil type and presence of *E*βC was not significant (Supplementary data 6a and 6b).

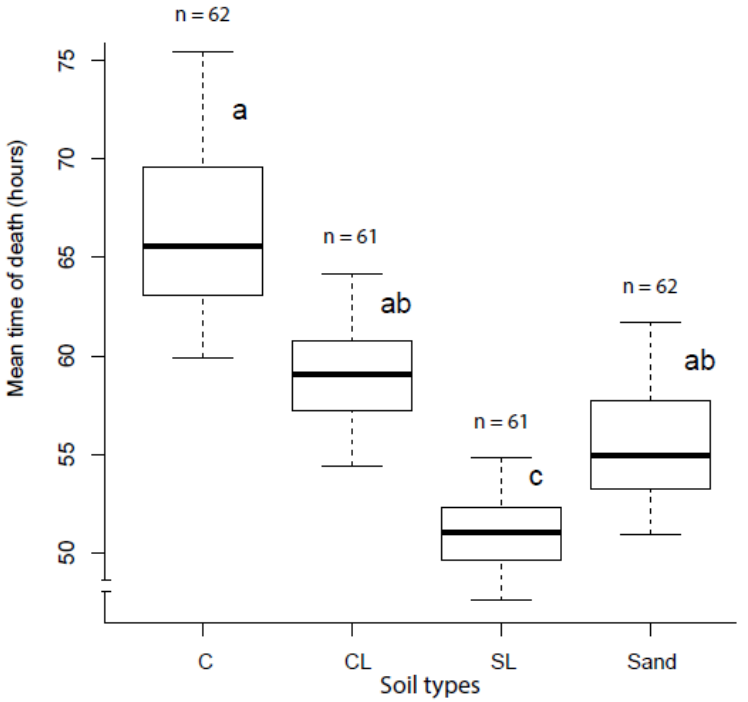

Fig. 5. Mean times (hours) until the death of *Galleria melonella* larvae infected by *Heterorhabditis megidis* that foraged in: sand (S), clay (C), clay loam (CL) and sandy soil (SL). Values represent means in presence of an *E*βC-capillary dispenser or absence of an *E*βC-capillary dispenser. Different letters represent significant differences. Data are averaged and the box plots represent the SEM.

Table 2. Observed means time of death for *G. melonella* larvae in each type of soil

Type of Soil	Observed mean time of death (hours)	Predicted mean time of death (hours)
Sand	55,5	55.4
Sandy loam	51,6	51,4
Clay	66,8	66,3
Clay loam	60,0	59,7

Effects of soil texture on migration of *Heterorhabditis megidis* in the presence or absence of naturally produced EβC

The migration of IJs of *H. megidis* was different for the two maize varieties ($F_{2,84} = 10.42$, $P < 0.0001$) but was not affected by soil type ($F_{3,84} = 0.67$, $P = 0.67$). However, there was a significant interaction Plant x Soil ($P = 0.002$) affecting EPN migration in the trays. In trays with pure sand and in trays with clay loam soil more IJs of *H. megidis* were recruited in the rhizosphere of maize plants var. Graf (high emitter of *E*βC) as compared to var. Pactol (low emitter of *E*βC) (Fig. 6A and B). This was not the case for trays with sandy loam and clay soil, where there were no differences between the nematodes recruited by the two maize varieties (Fig. 6C-D). In all cases, many more EPN were recovered near the plants than in the same place in control trays without plants (Fig. 6). The numbers of nematodes that were recovered from the other sampling spots in the trays showed not clear patterns (Fig. 6C).

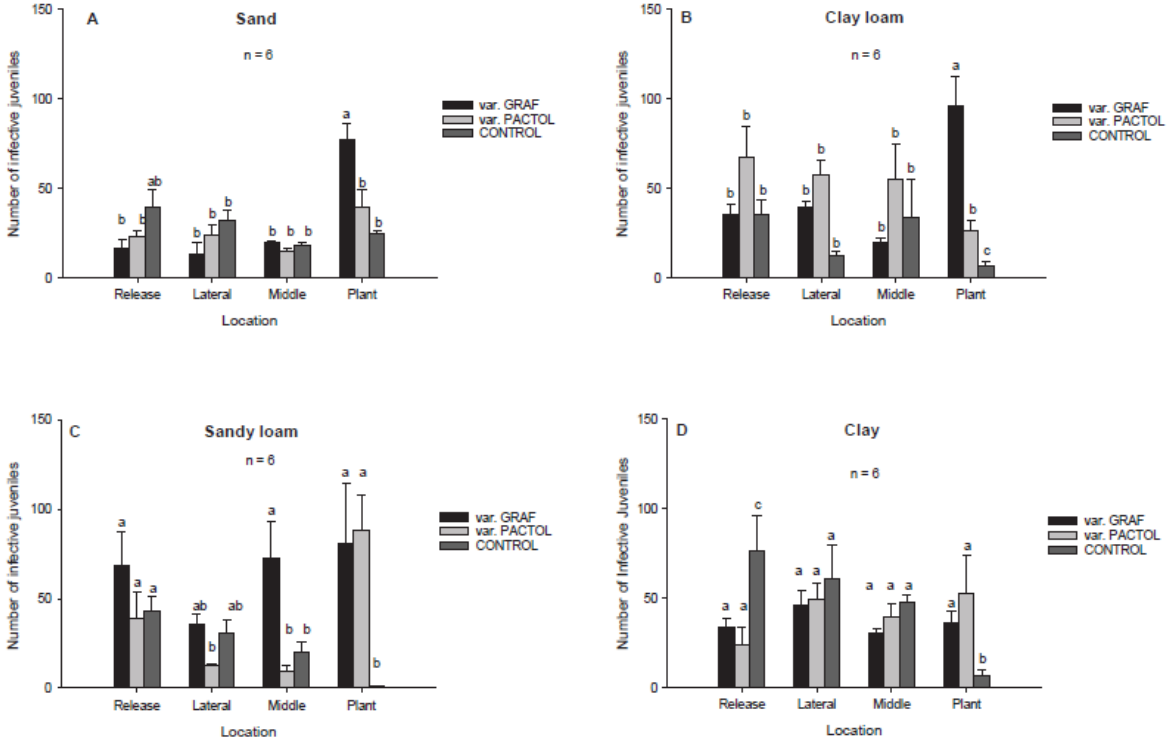

Fig. 6. Numbers of infective juveniles (IJs) of *Heterorhabditis megidis* in samples taken from different spots in the experimental tray (Fig. 2) in: A. pure sand B. clay loam C. sandy loam D. clay. Data are average ± SEM and pooled for two experiments. Different letters show significant differences.

DISCUSSION

Diffusion of E-(β)-caryophyllene in different soil textures

Our findings confirm the hypothesis that sand content in a soil affects the diffusion of *E*βC. In soils with 10% moisture, *E*βC diffused better in both a clay loam soil and a sandy loam soil (29.2 and 55.9% sand) compared to a clay soil (17% sand). A previous study by Hiltpold & Turlings (2008) showed that diffusion of *E*βC was significantly decreased in a sandy loam soil comparing to diffusion in pure sand. Moreover, when studying *E*βC diffusion in relation to water content in pure sand, diffusion of *E*βC was almost two-fold larger at 1% of humidity than at 10% humidity (Hiltpold & Turlings, 2008). In sharp contrast, in our study the best diffusion of *E*βC occurred in soils at 20% of humidity, which is around the field capacity of these soils (see Table 1). The quantity of *E*βC (100-fold higher than in the Hiltpold & Turlings (2008) study) used in our experiments was far outside of the natural range of *E*βC production by maize roots, but it allowed us to detect the volatile with the employed methodology and to reveal how *E*βC behaves in different soils and under different humidity conditions. In soils with 10% water, *E*βC was detected in clay loam soil, but not in sandy loam and clay soils, which is in agreement with the pattern observed in soils at 20% moisture. However, in soils with 5% water, *E*βC diffused better in sandy loam than in clay loam soil. This may be explained by the fact that pores in clay loam soils are easily saturated with small quantities of water in contrast to sandy loam soils.

*E*βC is a non-polar compound that dissolves poorly in water and is assumed to disperse through the gaseous phase of the soil (Hiltpold & Turlings, 2008; Turlings et al., 2012). It has been well documented that diffusion of volatile organic compounds in soils is highly dependent on soil adsorption properties (Lindstrom et al 1967; Steinberg and Kreamer 1993), and is largely determined by the mineral composition of soils (Ruiz et al. 1998). It has been suggested that since the water molecule is a strong dipolar molecule, it competes for adsorption sites in the soil mineral particles and may displace non-polar organic molecules (Ruiz et al., 1998). Our results are in agreement with this hypothesis and imply that in soil water prevents the interaction of *E*βC with soil particles of high adsorption capacity (i.e. clay). It is also interesting to note that the detectability of the

*E*βC in a sandy loam soil decreased rapidly in comparison to a clay loam or clay soil (Fig. 4), this might be explained by the fact that adsorption is relatively low in sand (Ruiz et al. 1998) and as a result the root volatile may be partially lost by vertical diffusion, as shown by Rasmann et al. (2005).

Soil porosity also plays a role in the diffusion of volatile organic compounds. Pore size affects and is affected by several factors, such as the movement of water, air and other fluids, the transport and reaction of chemicals, and the residence of roots and other biota (Nimmo, 2004). In general, sandy soils have a larger particle and pores sizes (Plant & Soil Sciences elibrary, 2014), which may favour the diffusion of root produced volatiles through the gaseous phase. Indeed, we found better diffusion of *E*βC in clay loam and sandy loam soils, which have a relatively higher content of sand and larger pores in comparison to a clay soil.

Overall, the results confirm that EβC is a suitable belowground signal in real cropping conditions, but different soil types may differ in the action-radius of *E*βC for EPN attraction.

Effects of soil texture on host infection by *Heterorhabditis megidis* in presence or absence of synthetic EβC

Our results support previous conclusions about the effects of soil texture on virulence and infectivity of EPNs. We recorded earlier mortality of *G. melonella* larvae in pure sand and sandy loam than in clay and clay loam soils, probably due to reduced motility of the *H. megidis* in the clay-rich soils, as Kaya (1990) suggests that nematode motility generally decreases as soil pores become smaller. Indeed, the rates of movement and infection by nematodes are strongly correlated with the amount of soil pore openings of dimensions similar to or greater than the diameters of the nematodes (Portillo-Aguilar et al., 1999). Small soil pores, particularly in combination with higher soil moisture also limit oxygen levels and with that activity and survival (Kung et al., 1990) of EPNs. Indeed, motility and persistence are influenced by numerous interacting intrinsic (e.g. behavioural, physiological and genetic characteristics) and extrinsic factors [e.g. temperatures, soil moisture, soil texture, relative humidity and UV radiation (Kaya, 1990; Smits, 1996; Stuart et al. 2006, 2015).

The *E*βC releasing capillary had only a small effect on the infectivity and virulence of *H. megidis*. This result may have several explanations. Possibly, under the experimental conditions, the CO_2 expelled by *G. melonella* larvae was more readily detected by the IJs of *H. megidis*, before they were able to detect *E*βC. Turlings et al. (2012), showed that CO_2 works in synergy with the EβC and propose CO_2 predominantly serves as a response activator that alerts EPNs to the general presence of living organisms and may enhance their responsiveness to more specific and more reliably inducible plant cues. In the current study we also did not find any differences between the number of IJs that succeed to infect one *G. melonella* larvae in treatments with an *E*βC releasing capillary and without it, in contrast to when we used *E*βC-releasing maize plants.

Effects of soil texture on migration of *Heterorhabditis megidis* in presence or absence of natural EβC

In agreement with observations in pure sand by Rasmann at el. (2005) and Hiltpold et al. (2009), IJs of *H. megidis* were significantly more attracted to maize plants that release *E*βC than to plants with low production or controls without plants. We also confirmed the important role of root cues for EPNs attraction (Wang and Gaugler, 1998), independently of the fact that roots produce *E*βC or not, they recruited more EPNs than empty trays, possibly because of root-produced CO_2, which may serve as a universal host cue (Gaugler et al., 1980; Dillman et al., 2012; Turlings et al., 2012). The attraction towards the roots is also evident from the fact that fewer nematodes remained at the original point of release in trays with plants.

The migration of *H. megidis* toward *E*βC producing plants was dependent on soil type and was most effective in clay loam soil. In this soil, IJs migrated more toward *E*βC producing plants (var. Graf) than toward non-producing maize plants (var. Pactol). This result is in agreement with a Hungarian field trial in clay loam soil, where plants that produced *E*βC were found to attract more entomopathogenic nematodes than plants that did not produced *E*βC (Rasmann, 2006). In our tray experiments with non-releasing Pactol plants, the majority of nematodes remained at the release point or in the middle of the tray, which was not the case for the trays with Graf plants (Fig. 6). In contrast, in sandy loam soil,

at the time we evaluated the experiment, there was not difference between Graf and Pactol plants in the number of nematodes that reached the plant area. However, in the middle of the tray we found a significantly higher number of IJs in the trays with Graf plants, suggesting that IJs were moving toward the plant releasing the EPN attractant. If this is the case, we can conclude that recruitment of *H. megidis* by *E*βC producing plants takes a longer time in a sandy loam soil than in a clay loam soil. This corresponds well with the diffusion of *E*βC in the different soils: *E*βC detection after applying it in the diffusion experiments reached its maximum level at the same time for both soils. However, the detection of the *E*βC was more sustained over time in the clay loam soil than in the sandy loam soil (Fig. 4). This suggests that *E*βC may be perceived more easily by the EPN *H. megidis* in clay loam soil and may be rapidly lost in a sandy loam soil due to upward volatilization.

In clay soil, the migration of H. megidis toward EβC producing and non-producing plants was much less than in the other soil types. The diffusion of EβC in clay soil was comparable to its diffusion in clay loam soil. Yet, the IJs took longer to reach the roots. This suggests that, even though the IJs might readily detect the EβC signal, their movement is impaired in this type of soil because of low porosity (Kaya, 1990; Stuart et al., 2006, Stuart, et al., 2015).

CONCLUSIONS

The diffusion of the root signal *E*βC was found to strongly depend on soil texture and soil humidity. At low water content (10%), its diffusion was reduced specially in clay soil but it was improved when water content was increased to 20% in clay loam, sandy loam and clay soils. Moreover, the gradient of CO_2 produced by insect-caged hosts may be established faster than the one of the synthetic *E*βC, favouring the location of the hosts by the nematodes independently of the presence of other cues in soil-filled glass trays. However, in soil-filled glass trays with insect induced maize plants, the production of *E*βC by maize roots was found to influence the migration behaviour of *H. megidis* depending on the soil texture. Under real agricultural conditions, clay loam soils may facilitate the recruitment of *H. megidis* IJs towards the rhizosphere of *E*βC-producing plants. Orientation by EPN in sandy loam soils may be less efficient and in clay soils the *E*βC signal may not at all help maize plants to recruit IJs. Further

research on the dynamics of the *E*βC root signal in relation to other soils factors such as: pH, organic matter and temperature is needed to elucidate how to better exploit this HIPV to control *D. virgifera virgifera* and other soil-borne pests.

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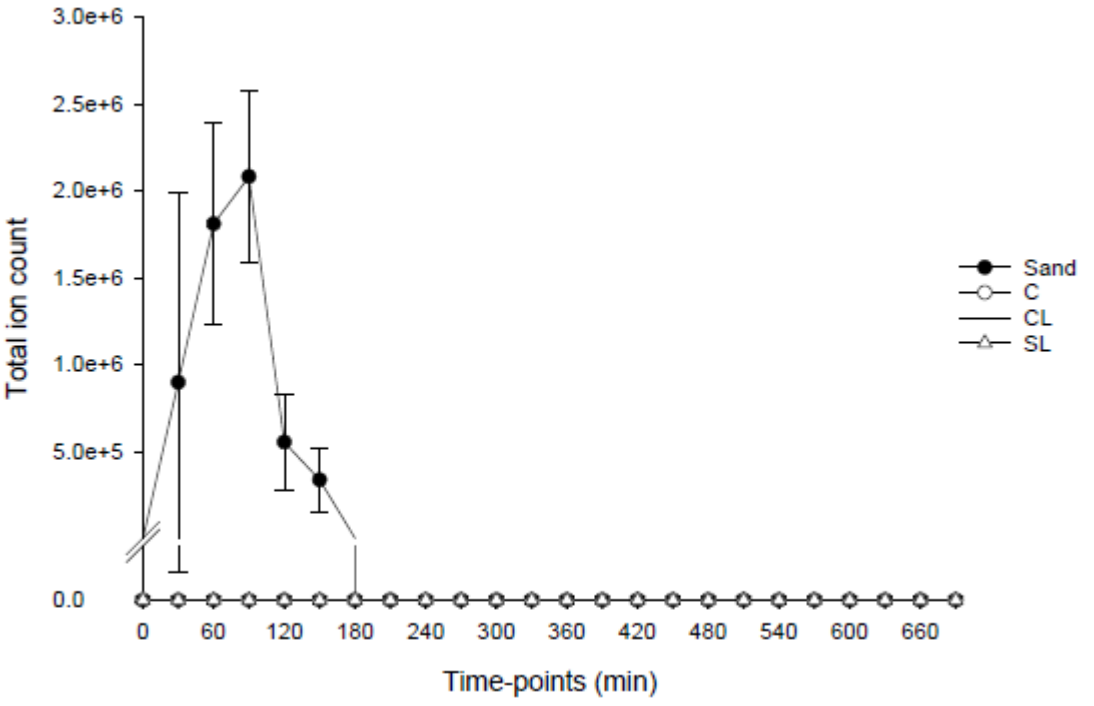
SUPPLEMENTARY DATA

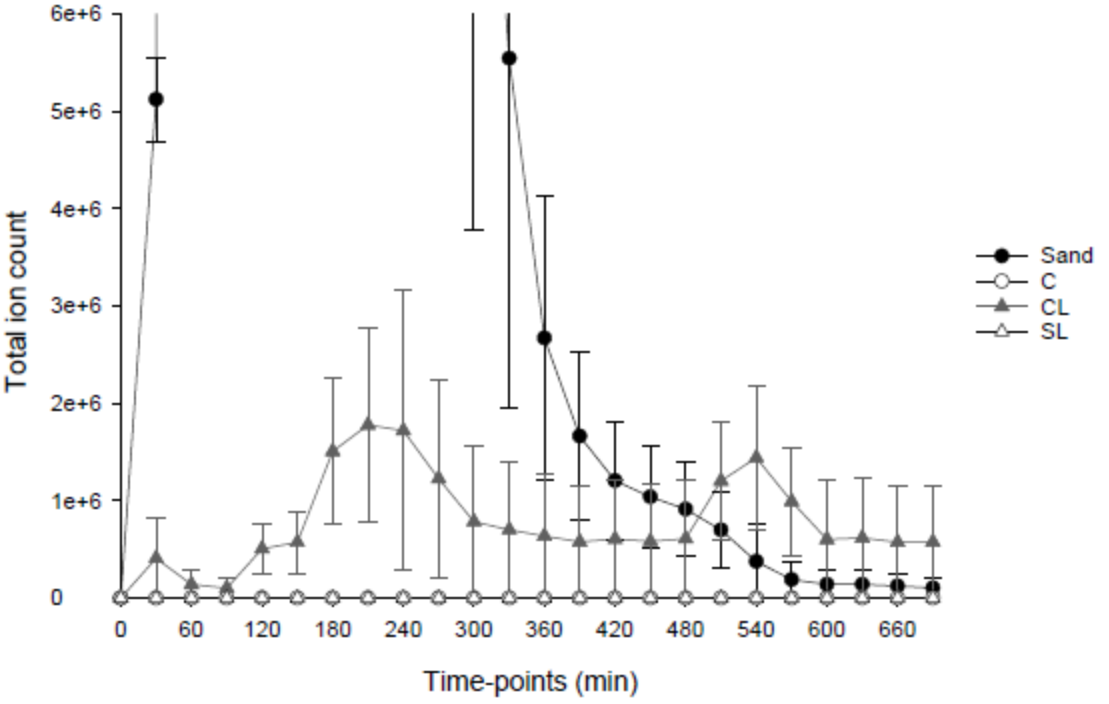
Supplementary data 1. Mass (weight) of moistened soil or sand and the substrate densities used to perform the diffusion experiments with synthetic *E*βC.

Type of Soil	Moisture Level	Mass Soil + Water (g)	Density ($g*cm^{-3}$)
Sand	20 %	494	1.03
Sand	10%	405	0.84
Sandy loam	20 %	493	1.02
Sandy loam	10%	404	0.84
Clay	20 %	391	0.81
Clay	10%	450	0.93
Clay loam	20 %	500	1.04
Clay loam	10%	457	0.95

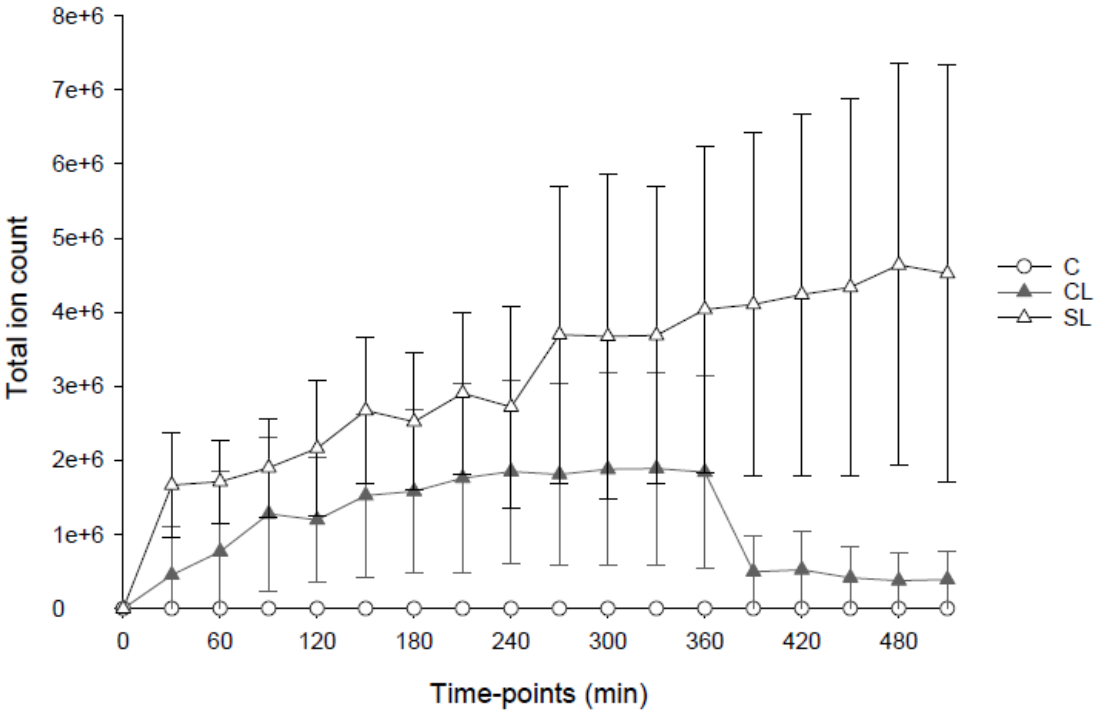
SUPPLEMENTARY FIGURES



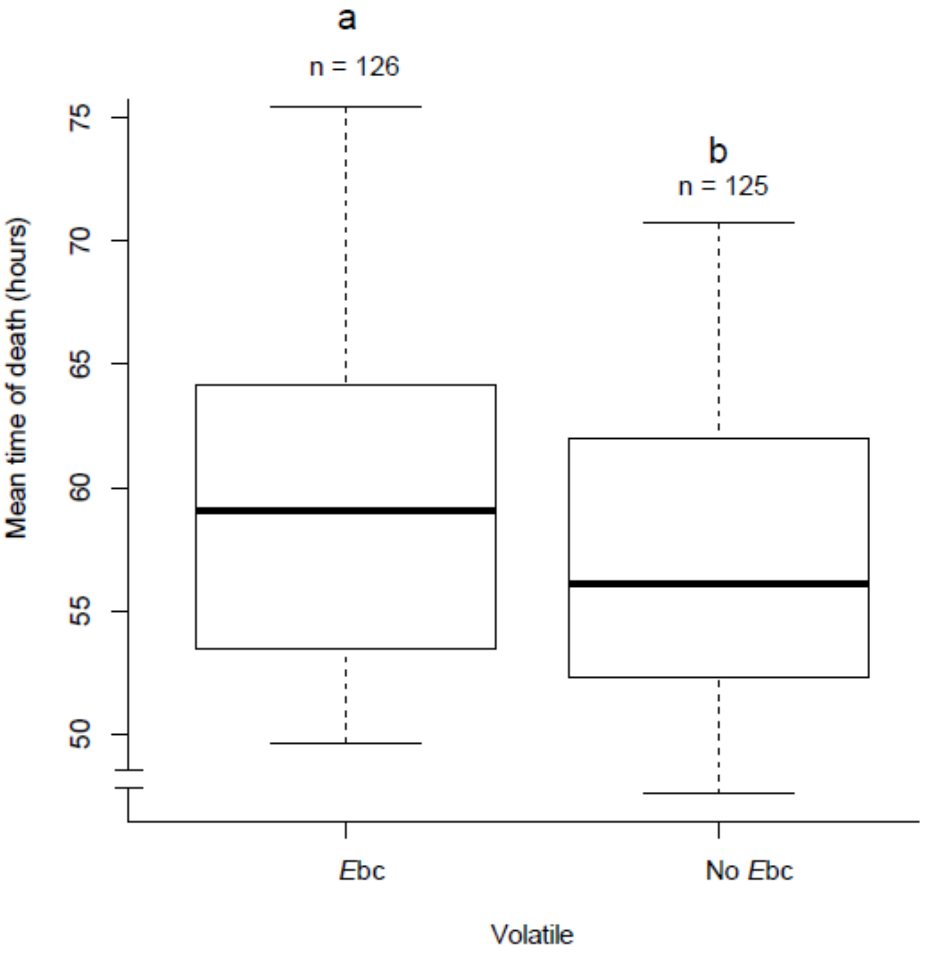
Supplementary data 2. Diffusion of *E*βC injected at a low concentration (200 ng) in pure sand (S) and three different soil textures: clay (C), clay loam (CL) and sandy loam (SL) at 10% humidity and at 10 cm distance from the sampling fibre. Data are average ± SEM.



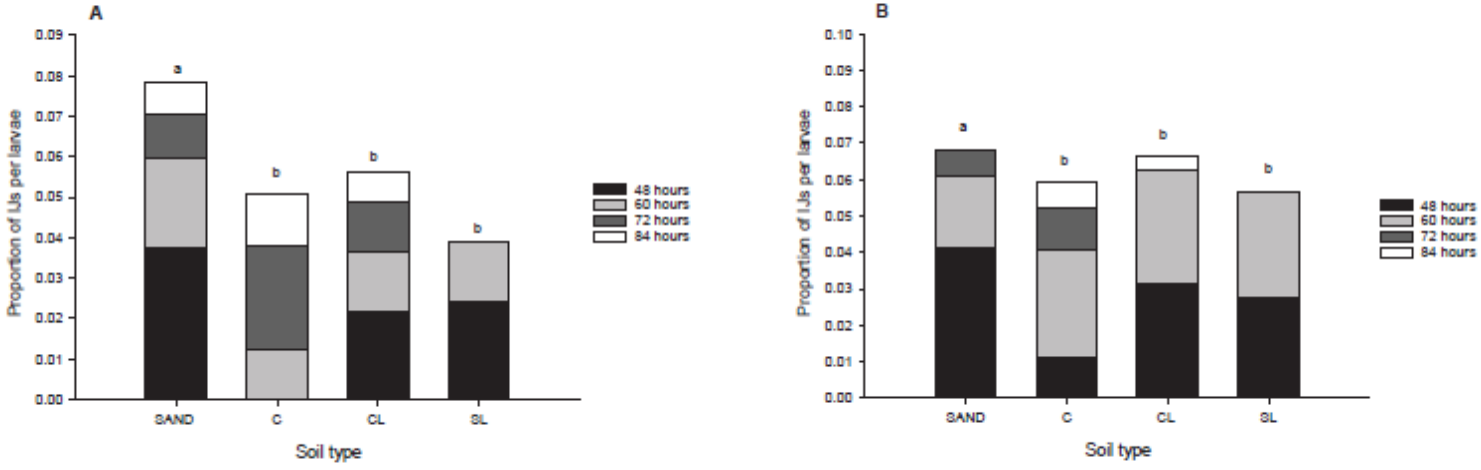
Supplementary data 3. Diffusion of *E*βC injected at a high concentration (20000 ng) in pure sand (S) and three different soil textures: clay (C), clay loam (CL) and sandy loam (SL) at 10% humidity and at 10 cm distance from the sampling fibre. Data are average ± SEM.



Supplementary data 4. Diffusion of *E*βC injected at a high concentration (20000 ng) in three different soil textures: clay (C), clay loam (CL) and sandy loam (SL) at 5% humidity and at 5 cm distance from the sampling fibre. Data are average ± SEM.



Supplementary data 5. Mean time of death (hours) of *Galleria melonella* larvae infected by *Heterorhabditis megidis* in A. pure sand (SAND) B. clay (C) C. clay loam (CL) and D. sandy loam (SL). All graphs represent values in: presence or absence of an *E*βC-releasing capillary dispenser. Data are average ± SEM. Different letters show significant differences.



Supplementary data 6. Proportion of infective juveniles (IJs) that succeed to infect one larvae of *Galleria melonella* in: pure sand (SAND), clay (C), clay loam (CL) and sandy loam (SL). A. Values correspond to treatments in presence of synthetic *E*βC and B. absence of synthetic *E*βC. Data pooled from 2 experiments and are average ± SEM. Different letters show significant differences.

-CHAPTER 2-

Combined field inoculations of *Pseudomonas* bacteria, arbuscular mycorrhizal fungi and entomopathogenic nematodes and their effects on wheat performance

Nicola Imperiali[1†], Xavier Chiriboga[2†], Klaus Schlaeppi[3], Marie Fesselet[4], Daniela Villacrés[4], Geoffrey Jaffuel[2], S. Franz Bender[5,6], Francesca Dennert[7], Ruben Blanco-Pérez[2,8], Marcel G.A. van der Heijden[5,7,9], Monika Maurhofer[7], Fabio Mascher[4], Ted C.J. Turlings[2], Christoph Keel[1], and Raquel Campos-Herrera[2, 8]

[1]Department of Fundamental Microbiology, University of Lausanne, Switzerland, [2]FARCE Laboratory, University of Neuchâtel, Switzerland, [3]Plant-Soil-Interactions, Department of Agroecology and Environment, Agroscope, Reckenholz, Zurich, [4]Plant Breeding and Genetic Resources, Institute for Plant Production Sciences, Agroscope, Nyon, Switzerland, [5]Department of Evolutionary Biology and Environmental Studies, University of Zurich, 8057 Zurich, Switzerland, [6]Department of Land, Air, and Water Resources, University of California, Davis, USA, [7]Institute of Integrative Biology, ETH Zurich, Switzerland,[8]Centro para os Recursos Biológicos e Alimentos Mediterrânicos (MeditBio), Universidade do Algarve, Faro, Portugal, [9]Plant-Microbe Interactions, Institute of Environmental Biology, Faculty of Science, Utrecht, University, 3584 CH Utrecht, The Netherlands

[†]These authors have contributed equally to this work.

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ABSTRACT
In agricultural ecosystems, pest insects, pathogens and reduced soil fertility pose major challenges to crop productivity and are responsible for significant yield losses worldwide. Management of belowground pests and diseases remains particularly challenging due to the complex nature of the soil and the limited reach of conventional agrochemicals. Boosting the presence of beneficial rhizosphere organisms is a potentially sustainable alternative and may help to optimize crop health and productivity. Field application of single beneficial soil organisms (BeSO) has shown satisfactory results under optimal conditions. This might be further enhanced by combining multiple BeSO, but this remains poorly investigated. Here, we inoculated wheat plots with combinations of three BeSO that have different rhizosphere functions and studied their effects on crop performance. Plant beneficial *Pseudomonas* bacteria, arbuscular mycorrhizal fungi and entomopathogenic nematodes were inoculated individually or in combinations at the time of seeding, and their effects on plant performance were evaluated throughout the season. In addition, we used traditional and molecular identification tools to monitor BeSO persistence over the cropping season in augmented and control treatments, and to estimate the possible displacement of native populations. In three separate trials, BeSO were successfully introduced into the native populations and readily survived the field conditions. Various *Pseudomonas*, mycorrhiza and nematode treatments improved plant health and productivity, while their combinations provided no significant additive or synergistic benefits compared to when applied alone. EPN application temporarily displaced some of the native EPN, but had not significant long-term effect on the associated food web. The strongest positive effect on wheat survival was observed for *Pseudomonas* and arbuscular mycorrhizal fungi during a season with heavy natural infestation by the frit fly, *Oscinella frit*, a major pest of cereals. Hence, beneficial impacts differed between the BeSO and were most evident for plants under biotic stress. Overall, our findings indicate that in wheat production under the test conditions the three BeSO can establish nicely and are compatible, but their combined application provides no additional benefits. Further studies are required, also in other cropping systems, to fine-tune the functional interactions among beneficial soil organisms, crops and the environment.

Key words: *Pseudomonas* bacteria, arbuscular mycorrhizal fungi, entomopathogenic nematodes, wheat, biological control, insect pest, pathogen, plant growth promotion

INTRODUCTION

In addition to poor soil fertility, soil pests and pathogens pose major threats to the health and productivity of crops in agricultural ecosystems resulting in important yield losses every year (Oerke et al., 2006; Kupferschmied et al., 2013). The use of fertilizers, fungicides, nematicides and insecticides to counter these problems can have important negative consequences, such as the persistence of these agrochemicals in the soil, water and food with potential negative impacts on the environment and consumers (Bale et al., 2008; Lichtfouse et al., 2009; Kupferschmied et al., 2013; Johnson et al., 2016). Hence, new and more sustainable pest and disease control strategies need to be explored for a next-generation agriculture and the application of beneficial soil organisms (BeSO) presents a promising alternative for maintaining crop health and productivity (Bommarco et al., 2013; Bender et al. 2016).

Various BeSO are known to enhance plant performance, e.g. by directly promoting plant growth, by stimulating plant defenses, by facilitating nutrient acquisition by the plant, or by protecting the plant from pathogens and pests (Philippot, et al. 2013; Rasmann and Turlings 2016; Venturi and Keel 2016). The three groups of BeSO investigated in the present study fulfill one or several of these beneficial functions, i.e. plant-growth promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF) and entomopathogenic nematodes (EPN). Root-colonizing bacteria belonging to the *Pseudomonas fluorescens* group are well-characterized PGPR that have the ability to induce systemic plant defenses and ward off soil-borne pathogens, in particular pathogenic fungi and oomycetes, including *Gaeumannomyces*, *Thielaviopsis*, *Rhizoctonia*, *Fusarium oxysporum*, and *Pythium* (Cook et al., 1995; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Vacheron et al., 2015). Moreover, certain subgroups, in particular the two species *Pseudomonas protegens* and *Pseudomonas chlororaphis* exhibit potent oral insecticidal activity notably against Lepidopteran pests (Kupferschmied, et al., 2013; Ruffner et al., 2013; Flury et al., 2016). To date, several biocontrol products that are based on PGPR pseudomonads are on the market (Berg, 2009; Kupferschmied et al., 2013).

AMF are well-known beneficial symbionts that colonize the roots of the majority of land plants (Schueßler et al., 2001; van der Heijden et al.

2015). AMF form extensive hyphal networks that provide water and nutrients to their host plant. AMF are key actors in processes such as the mineralization of phosphorus and nitrogen and enhancing the nutrient uptake by plant roots (Jakobsen et al., 1992; Mäder et al., 2000; Smith et al., 2004; van der Heijden et al., 2006). AMF primarily improve plant nutrition, but they can also contribute to enhance the tolerance of their host plant against biotic and abiotic stresses (van der Heijden et al., 2015). Numerous AMF species like, e.g. *Rhizoglomus irregulare*, are commercialized as inoculum to improve soil fertility (Lekberg and Koide, 2005; Pellegrino et al., 2015) and plant productivity (Hijri 2015; Köhl et al., 2016). Today, the agronomic use of AMF includes the direct augmentation or inoculation of seedlings in nurseries before transplanting to the field (Jeffries et al. 2003) and seed coating (e.g. Ijdo et al. 2011).

Finally, EPN of the genera *Steinernema* and *Heterorabditis* are well-known bio-control agents that selectively search their insect hosts and kill them within two or three days with the aid of mutualistic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively (Kaya et al., 2006; Georgis et al., 2006; Dillman et al., 2012; Lacey et al., 2015; Campos-Herrera, 2015). Their wide distribution in soils throughout the world (Adams et al., 2006) and the availability of commercial products (Lacey et al., 2015) make them excellent products in integrated pest management (IPM) programs or in organic production, both for augmentation or restoration of naturally occurring EPN (Campos-Herrera, 2015). However, their performance and activity are affected by biotic and abiotic factors, and hence, their efficacy depends on soil characteristics, agricultural management practices, and competition within the food web (Stuart et al., 2015).

The three groups of organisms - *Pseudomonas*, AMF and EPN - occur naturally in most arable soils and commercial formulations are available for agronomic use (Berg, 2009; Kupferschmied et al., 2013; Lacey et al., 2015; Stockwell and Stack, 2007). Previous greenhouse and field studies have reported varying effects on plant health and growth when combining inoculants of these BeSO groups. For example, combinations of certain *Pseudomonas* strains provided better control of the wheat disease take-all than did the individual strains alone (Pierson and Weller, 1994). Positive effects have been also recorded when combining bacteria, such as *Pseudomonas* or *Azospirillum* strains, with fungi, including the AM fungus *Glomus* (Frey-Klett et al., 2007; Walker et al., 2012; Couillerot et al., 2012), *Fusarium* or *Trichoderma* (Lemanceau and Alabouvette, 1991;

Fogliano et al., 2002). Similarly, EPN have been combined with other BeSO, with differing results. For example, the combination of *Steinernema kraussei* with the entomopathogenic fungus (EPF) *Metarhizium anisopliae* resulted in a synergistic effect in the control of *Otiorhynchus sulcatus* in strawberry (Ansari et al., 2010), while the combination of *Steinernema ichnusae* with the EPF *Beauveria bassiana* resulted in clear antagonism and competition for the host under controlled laboratory conditions (Tarasco et al., 2011).

Field applications of single BeSO have shown to greatly enhance plant growth and health in various crops (Jeffries et al. 2003; Berg, 2009; Kupferschmied et al., 2013; Lacey et al., 2015; Campos-Herrera, 2015), but the putative positive effect of combining various BeSO remains poorly predictable. The Swiss National Research Programme 68 (NRP 68) "Sustainable use of soil as a resource" (www.nrp68.ch) provided the framework for our multidisciplinary investigations into BeSO and their possible role in novel strategies for sustainable soil management. As part of this, we evaluated, for the first time, the simultaneous application of *Pseudomonas*, AMF and EPN inoculants in field experiments, using wheat as the model crop. We hypothesized that the combined application of these BeSO would show greater benefits for the crop than their individual application.

MATERIALS AND METHODS

Beneficial Organisms

Selected species of BeSO, all known to naturally occur in Swiss soils (Campos-Herrera et al., 2015a, Jaffuel et al., 2016; Schlaeppi et al., 2016; Imperiali et al., 2017), were applied depending on the objective and design of each field experiment (Figure S1). The BeSO that were used included two species of the genus *Pseudomonas*, three AMF species and four EPN species and they were applied as inoculants either individually or in various combinations in the different experiments (Table 1; Table 2).

To monitor the bacteria following field application, the bacterial inoculants, i.e. *Pseudomonas protegens* strain CHA0 (Stutz et al., 1986) and *Pseudomonas chlororaphis* strain PCL1391 (Chin-A-Woeng et al.,

1998) were tagged with a spontaneous resistance to rifampicin following previously described protocols (Natsch et al., 1994). Briefly, spontaneous rifampicin-resistant derivatives were obtained following plating concentrated cell suspensions of each parental strain on King's medium B agar (KMB) (King et al., 1954) supplemented with 100 µg/ml of rifampicin. A CHA0-Rif derivative and a PCL1391-Rif derivative (**Table 1**), which stably maintained rifampicin resistance and displayed wild-type growth and antifungal and insecticidal activities, were selected. For the preparation of the bacterial field inocula, the selected rifampicin resistant strains were grown overnight at 25°C in lysogeny broth (LB) (LB) (Bertani, 1951) containing 100 µg/ml of rifampicin. Aliquots of 200 µl of each culture were spread on KMB plates without antibiotics. After incubation at 27°C for 16 h, bacterial cells were harvested and washed in sterile distilled water. The optical density at 600 nm (OD_{600}) of the bacterial cell suspensions was adjusted to 0.15 corresponding to a cell density of 8×10^7 CFU ml^{-1}. These bacterial stock suspensions were maintained on ice until final dilution and use on the field sites.

The AMF strains *Claroideoglomas claroideum* SAF12, *Funneliformis mosseae* SAF11 and *Rhizoglomus irregular*e SAF22 were selected from the Swiss Collection of Arbuscular Mycorrhizal Fungi (SAF) at Agroscope (Reckenholz, Zurich, www.agroscope.ch/saf; Table 1). The inocula were prepared as described by Schlaeppi et al. (2016). Briefly, AMF were propagated over six months in the greenhouse in autoclaved sand:soil (85:15%; v/v) as substrate and using *Plantago lanceolata* as host. The final inoculum contained pieces of plant roots mixed with the substrate containing AMF hyphae and spores. In addition, a "mock" inoculum consisting of *Plantago* roots and substrate free of AMF propagules was prepared following the same protocol. AMF inocula as well as the mock-inoculum were mixed in separate plastic bags and stored at room temperature until use. In addition, the commercial AMF inoculum *Rhizoglomus irregulare* TOP (INOQ GmbH, Schnega, Germany, www.inoq.de) was used as obtained from Otto Hauenstein Samen AG (Rafz, Switzerland, www.hauenstein.ch). For the second trial (PERFORMANCE-2) autoclaved commercial inoculum was used as control treatment.

For the EPN, infective juveniles (IJs) of fours species were prepared in adjusted suspensions. *Heterorhabiditis* species were obtained from a commercial source (Andermatt Biocontrol, Grossdietwil, Switzerland, www.andermattbiocontrol.com), whereas *Steinernema*

species were propagated from field collected populations under laboratory conditions following protocols described by Campos-Herrera et al. (2015a) (Table 1). All nematodes were received or harvested within two weeks prior to field application. The day before application, the EPN inoculant suspensions were prepared in sterile water. To this end, infective juveniles (IJs) were counted and their density was adjusted to deliver the required field concentration per experimental unit (Table 2) by using separate containers. Containers were kept at 5°C overnight and transported in coolers to the field. In addition, laboratory infections of *Galleria mellonella* larvae by the inoculant EPN at field concentrations were used to verify their infectivity for each experiment (Jaffuel et al., 2017).

Experimental Designs

From spring 2014 to summer 2015, three field experiments were conducted in wheat plots and the applications of beneficial soil organisms were adapted for each experiment. All the experiments were carried out with the commercial spring wheat variety 'Rubli' in the experimental plots, whereas the commercial triticale variety 'Trado' was seeded in the buffer zones. Fields were bordered by strips of non-managed grassland. The three experiments were named as follows: COMBINATION (2014), PERFORMANCE-1 (2014) and PERFORMANCE-2 (2015) (Table 2). The selection of the applied organisms and combinations of treatments were adapted on results of the preceding trial. The first experiment (COMBINATION) was set up to test various species of each group of beneficials and first combinatory treatments. The second experiment was designed to evaluate wheat yield effects after combining bacteria and EPN (PERFORMANCE-1). In this experiment, the AMF treatment was not included due to limitations in scaling the production of inoculum for the large plot size. Finally, the PERFORMANCE-2 experiment consisted of the full bacteria-AMF-EPN combinations during the subsequent season (Table 2).

All the experiments were conducted in neighboring experimental field sites located near Prangins, Switzerland (see Table 2 for coordinates). The sites belong to Agroscope, research center of Changins, Nyon, Switzerland) and have documented crop and management sequences for

the last 30 years. The field sites chosen for the experiments had no overlapping areas to avoid cross-contaminations with inoculants. None of the experiments had irrigation systems. The soil type was sandy loam for the COMBINATION and PERFORMANCE-1 trials and loam for the PERFORMANCE-2 trial (Table 2). General agronomic preparations for all the experiments included tillage (15 cm deep) and harrowing about four days before seeding. The seeding machine 'Hege Seedmatic' (Hege Maschinen, Eging am See, Germany) allowed the customized seeding for each plot size and arrangement (Table 2; **Figure S1**) and was modified to keep the seed furrows open after placing the seeds. After seeding, the plots were marked for the corresponding treatments (see **Figure S1** for the exact field design of each of the three experiments) and inoculated on the same day with the beneficial soil organisms. In combination treatments, the application followed the order bacteria, EPN and AMF.

TABLE 1 | Beneficial soil organisms applied individually or in combinations in the field experiments.

Beneficial group / species	Strain	Treatment code	Application type	GenBank accession no.	Reference or source
Pseudomonas bacteria					
Pseudomonas chlororaphis	PCL1391 [a]	B2	Aqueous	NZ_LFUT01000004	Chin-A-Woeng et al. (1998)
Pseudomonas protegens	CHA0 [a]	B1	Aqueous	NC_021237	Stutz et al. (1986)
Arbuscular mycorrhizal fungi					
Claroideoglomus claroideum	SAF12 [c]	F4	Substrate	n.a.	Swiss Collection of Arbuscular Miychorrizal Fungi (SAF)
Funneliformis mosseae	SAF11 [c]	F3	Substrate	n.a.	SAF
Rhizoglomus irregulare [b]	INOQ Top	F1	Substrate	n.a. [d]	Inog GmbH, Schnega Germany
Rhizoglomus irregulare [b]	SAF22 [c]	F2	Substrate	DQ377990	SAF
Entomopathogenic nematodes					
Heterorhabditis bacteriophora	Andermatt	N2	Aqueous	KJ938576	Andermatt Biocontrol AG, Grossdietwil, Switzerland
Heterorhabditis megidis	Andermatt	N1	Aqueous	KJ938577	Andermatt Biocontrol AG, Grossdietwil, Switzerland
Steinernema carpocapsae	D-83	N3	Aqueous	KJ818295	Jaffuel et al. (2016)
Steinernema feltiae	RS-5	N4	Aqueous	KJ938569	Jaffuel et al. (2017)

[a] Rifampicin-resistant variants of strains CHA0 and PCL1391 were used as inoculants in the field trials (see Materials and Methods).

[b] *Rhizoglomus irregulare* was previously referred to as *Rhizophagus irregularis* and earlier as *Glomus intraradices* (Sieverding et al., 2015).

[c] Strain ID referring to the Agroscope AMF strain collection, http://www.agroscope.ch/saf.

[d] n.a., not available.

TABLE 2 | Details on the characteristics of the three field experiments used to assess effects of inoculation of beneficial soil organisms (pseudomonads, entomopathogenic nematodes, and arbuscular mycorrhizal fungi) on growth, health and yield of spring wheat.

Field trials	COMBINATION	PERFORMANCE-1	PERFORMANCE-2
Coordinates SN / EW	46.397676 / 6.260763	46.397455 / 6.260166	46.39502 / 6.260444
Sowing/inoculation day	18th March 2014	18th March 2014	27th March 2015
Treatments* (Treatments codes refer to Table 1)	Control (no inoculants) B1: *P. protegens* CHA0-Rif B2: *P. chlororaphis* PCL1391-Rif N1: *H. megidis* Andermatt N2: *H. bacteriophora* Andermatt N3: *S. carpocapsae* D83 N4: *S. feltiae* RS5 F1-H: *R. irregulare* INOQ Top (high dosage, 250 ml/row) F1-L: *R. irregulare* INOQ Top (low dosage, 50 ml/row) F2: *R. irregulare* SAF22 F3: *F. mosseae* SAF11 F4: *C. claroideum* SAF12 AMF control (substrate only) B1+ N2 B1+ F1 N2 + F1 B1+ N2 + F1	Control (no inoculants) B1: *P. protegens* CHA0-Rif B2: *P. chlororaphis* PCL1391-Rif N2: *H. bacteriophora* Andermatt BM: B1 + B2 B1 + N2 B2 + N2 BM + N2	Control (no inoculants) BM: B1 + B2 NM: N1 + N2 + N4 F1-L AMF control (substrate only) BM + NM NM + F1-L BM + F1-L BM + NM + F1-L
Number of treatments	17	8	9
Number of replicates (plots) per treatment	4 replicates in randomized complete block design	4 replicates in randomized complete block design	9 replicates in randomized complete block design
Size of plots	1.5 m^2	6.75 m^2	9 m^2
Number of plant rows per plot	5	5	5
Wheat seeds per m of row	~ 80	~ 140	~ 140
Bacterial inoculum (CFU in 400 ml H_2O per meter	B1: 1.19 x 10^9 B2: 1.21 x10^9	B1: 1.42 x 10^9 B2: 3.37 x 10^9	B1: 4.875 x 10^8 B2: 8.25 x 10^8

of row)			
AMF inoculum	F1-H: 80 F1-L: 16 F2: 250 F3: 250 F4: 250 AMF control: 250	Not contributed	F1-L: 16 AMF control: 16
Nematode inoculum	50 infective juveniles/cm^2/ 4 L	50 infective juveniles/cm^2/ 8 L	50 infectice juveniles/cm^2/ 8 L
Pest insect stress	Heavy natural infestation with *Oscinella frit*	Heavy natural infestation with *Oscinella frit*	No relevant *Oscinella frit* infestation
Soil type and texture	Sandy loam (clay, 25.5%; silt, 34.3%; sand; 40.2%)	Sandy loam (clay, 25.5%; silt, 34.3%; sand, 40.2%)	Loam (clay, 14.5%; silt, 26.8%; sand, 58.7%)

Bacteria were applied as a cell suspension to the seed furrows (plant rows) using treatment-specific watering cans. Final cell suspensions were prepared directly on the field from bacterial stock suspensions (OD_{600} 0.15; i.e., 8×10^7 CFU ml^{-1}) by adjusting with water to obtain the required volumes (400 ml per meter of row) and bacterial cells (8×10^8 CFU per meter of row) (Table 2). Similarly, EPN were applied in variable volumes depending on plot size using treatment-specific watering cans. They were applied to entire plots (not just the rows), and in all the cases, the final concentration was 0.5 Mio. IJs/m^2 (equivalent to 50 IJs/cm^2, Grewal and Peters 2005). Finally, AMF inocula were applied manually employing 250-ml glass beakers. The material was applied directly over the seeds in the furrows, thereby gently mixing seeds and inoculum with a small hoe. AMF control plots received the same quantity of AMF-free substrate. Control plots were treated with the same volumes of BeSO-free water. Immediately after treatment application, the seeds were covered with soil using hoes to close the seed furrows. All equipment used for inoculant application was thoroughly cleaned and disinfested between manipulations using 70% ethanol.

Weed control included the application of the herbicides Azur (Omya AG, Switzerland) against monocots 2 weeks after seeding and Apell (Syngenta AGRO SA) against dicots shortly before earing (BBCH 45-50). When necessary, some persistent weeds (Galium spp., Setaria spp.) were controlled manually. No fungicides nor nematicides were applied during any of the experiments. The insecticide Karate Zeon (Lambda-Cyhalotryne, Syngenta Agro GmbH) was applied in the PERFORMANCE-2 experiment against cereal mining dipterae such as the frit fly and hessian fly conducted in 2015, but not in the 2014 COMBINATION and PERFORMANCE-1 experiments. Plots were fertilized once by supplementing nitrogen in liquid (Lonza-sol N liquid, Basel, Switzerland) and, at 62 kg ha^{-1} of to reach 155 units N and potassium (K_2O) at 30.6 kg ha^{-1}. The PERFORMANCE-2 trial was covered with a black hail net during the first two to three weeks to protect the seeds and young plants from cold conditions and predation by birds and small mammals.

Sampling of Beneficial Organisms and Measuring of Plant Traits

Pseudomonas bacteria. The presence of *P. protegens* CHA0-Rif and *P. chlororaphis* PCL1391-Rif was evaluated in both inoculated plots and non-inoculated control plots, as well as in the buffer zone around the experimental plots, and in the border zone (grassland) around the field site to control for possible cross contamination. This analysis was conducted four times during the growing season (i.e. at seeding, end of the winter, at earing and maturity) in selected experiments (**Table 3**). For this, the root systems from four wheat plants per plot (triticale plants and grass for the buffer and border zones, respectively) were collected, pooled, washed and gently dried with paper towels. Roots were weighed, cut into small pieces (about 15 cm long), placed in 50-ml Falcon tubes (Greiner Bio One, Germany) containing 40 ml of sterile water and kept overnight at 4°C. All sampling equipment was cleaned with 70% ethanol between samples to avoid cross-contaminations. Samples were vigorously agitated on a rotary shaker at 180 rpm for 20 min, and roots were removed and dried at 80°C for three days to obtain the dry weight. The remaining suspensions were transferred to fresh sterile Falcon tubes on ice and centrifuged at 8500 rpm and 9300 *g* at 4°C. The supernatant was discarded and the pellet was re-suspended in 1 ml of sterile water. Each sample was then serially diluted and dilutions spread on KMB supplemented with 100 µg/ml of cycloheximide

and 100 µg/ml of rifampicin (Scanferlato et al. 1990). The colonies were counted and the results were expressed as colony forming units (CFU) per gram of dry root weight.

Arbuscular mycorrhizal fungi. The inoculation success of the different AMF inocula was traced by quantitative PCR comparing their abundance in wheat roots sampled from inoculated, non-inoculated or mock-inoculated plots (**Table 3**). At harvest, the root systems of four plants per plot were pooled to become one sample. The fine roots (deeper than ca. 3 cm in soil) were cut from the root system using scissors, hackled into small pieces (1–2 cm long) with a scalpel and thereby homogenizing all root fragments of the four plants. The root samples were lyophilized and then ground to a fine powder using a Retsch Ball Mill (model MM301; settings 30 s at 30 Hz using one 1-cm steel ball). DNA was extracted from approximately 200 mg of fine root powder utilizing the NucleoSpin® Plant II kit from Macherey-Nagel following the instructions. DNA concentrations were determined on a Varian Eclipse Fluorescence plate reader using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and Herring Sperm DNA (Invitrogen) as standard solution. The *R. irregulare* strains INOQ Top and SAF22 were quantified by qPCR utilizing primers developed by Alkan et al. (2006) and Bender et al. (under review), respectively. The AMF signals were expressed relative to a plant signal obtained with qPCR primers targeting the wheat ADP-ribosylation factor (Giménez et al. 2010). Triplicate amplifications were performed in 20 µl reactions using the HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) from Solis Biodyne (www.sbd.ee, Estonia) on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (www.bio-rad.com, USA). Reactions contained 4 µl qPCR mix (5X), 1 µl of each primer (10µM), 9 µl distilled sterile water and 5 µl template (5 ng DNA). The cycling program consisted of a 15 minutes initial denaturation step at 95°C followed by 40 cycles (95°C for 15 seconds, 63°C for 40 seconds for both *R. irregulare* primer sets or 60°C for 10 seconds for the wheat reference primers, 72°C for 20 seconds) and a 10 minutes final extension step at 72°C. Melting curve analysis consisted of a gradient from 65°C to 95°C, increasing by half degrees/per 10 seconds to determine the uniformity of the amplicons. Raw data were imported from the qPCR cycler into the LinRegPCR program to determine the Ct and efficiency (E) values using a common fluorescence threshold for all samples (Ruijter et al., 2009). *Funneliformis mosseae* and *Claroideoglomus claroideum* were quantified with species-specific TaqMan probes following the protocols developed by Thonar et al. (2012). Template amounts were calculated for each reaction using the individual E, averaged among the replicates of each sample and expressed relative to the plant signal. Of note, for samples of the COMBINATION trial, we determined also the whole AMF community by amplicon sequencing (Schlaeppi et al. 2016).

Entomopathogenic nematodes, soil food web and post-application activity. A total of 18 soil organisms were identified and quantified before application (baseline) and post-augmentation (**Table 3**) to detect possible trophic cascade effects due to EPN augmentation (Campos-Herrera et al., 2013). These organisms comprised seven EPN species (all previously described for the area, Campos-Herrera et al., 2015a), four free-living nematodes (FLNs) that compete with EPN for the insect cadaver (Campos-Herrera et al., 2012, 2015b), six nematophagous fungi (NF) (Campos-Herrera et al., 2015a) and one nematode surface-associated bacterium (Enright and Griffin, 2005; Campos-Herrera et al., 2011a) (**Table S1**). Briefly, a composite soil sample composed of several cores (2.5 cm diameter, 20 cm depth, see **Table 3** for exact quantities per experiment) were taken per plot and kept on ice for transportation to the

laboratory. The nematode community and other soil organisms were extracted from aliquots of 300-400 g of fresh soil by sucrose-centrifugation (Jenkins, 1964), concentrated in 1.5 ml tubes and stored at -80°C until processed, following Campos-Herrera et al. (2015a, 2015b). Briefly, DNA was extracted from soil samples as well as from pure cultures for the generation of standard curves (when living material was available) with the Power Soil DNA Isolation Kit (MO BIO laboratories, Inc.). If no living material was available for a target organism, we employed plasmids with the whole sequence of interest to establish our positive control (**Table S1**; Campos-Herrera et al., 2015a). The quality and quantity of each DNA sample was analyzed prior to use (1 µl per duplicate, Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA).

Species-specific primers and probes were employed in real time qPCR assessment of the 18 soil organisms (Atkins et al., 2005; Campos-Herrera et al., 2011a, 2011b, 2012, 2015a, 2015b; Pathak et al., 2012; Zhang et al., 2006), following the MIQE procedures (Bustin et al. 2009). All samples were run in duplicates (unknown, positive and negative controls) employing optical 100-well gene disc reaction plates (Biolabo, scientific instruments, Switzerland) on a Corbett Research real time PCR machine. Final reactions, concentrations, and protocols were used as previously described (Campos-Herrera et al., 2015a, 2015b). Nematode quantification from the soil samples was done with a 10-fold dilution of the DNA, whereas the identification and quantification of NF and surface-associated bacteria required the use of total DNA without dilutions (see details in Campos-Herrera et al., 2015a). A correction factor was derived from the dilution series to transform qPCR data to numbers of IJs. Finally, a sub-sample of fresh soil was dried to allow quantification per 100 g of dry soil.

In addition to the EPN soil food web, we evaluated the EPN activity at post-application sampling times (**Table 3**), as previously described by Campos-Herrera et al. (2015a) and Jaffuel et al. (2016). Briefly, two aliquots of 200 g of fresh soil per sample were baited with larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) to test the suppressive potential of the soil. Following a modified procedure as described by Bedding and Akhurst (1975), each subsample (from augmented or not augmented plots) was baited with five final instar *G. mellonella* larvae (commercial stock, Au Pêcheur SARL Neuchâtel, Switzerland) in two independent rounds. After exposure for four days, the cadavers were recovered from the soil, thoroughly rinsed with tap water, and individually placed in White traps (White 1927). Under a stereoscope, we checked for nematode emergence every 2 to 3 days to determine the organisms responsible for larval mortality. We recovered the nematodes in tap water upon emergence. The cadavers for which no obvious cause of death could be determined after one month of incubation were discarded after dissection. The DNA of the progeny leaving the cadavers was extracted using the QIAamp DNA mini kit (Qiagen), purity checked (Nanodrop system), adjusted to the range of 0.5-1 ng/µl, and species identity assessed by qPCR as described above (Campos-Herrera et al., 2015a; Jaffuel et al., 2016).

Plant traits. A total of eight measurements recorded the evolution of plant growth, productivity and health. They were: average plant height per plot, plant density per plot, chlorophyll activity (N-tester), seed yield, thousand-seed weight, plant weight, plant protein content, and presence of pest insects and pathogens (**Table 3**). Regular monitoring of the experiments ensured the status of development into each phenostage. Most of the agronomical traits presented herein were measured at harvest (**Table 3**).

TABLE 3 | Description of the type of measurements and methods employed and timing in each of the field experiments.

Organisms	Type of measurement	Method	Reference	COMBINATION	PERFORMANCE-1	PERFORMANCE-2
Bacteria	Sample type	Composite samples of wheat roots	Authors	Wheat roots	Wheat roots	Wheat roots
	Quantification at seeding	CFU counting on selective media	Authors	28.03.2014	28.03.2014	27.03.2015
	Quantification during wheat growth	CFU counting on selective media	Authors	03.06.2014	03.06.2014 25.06.2014 22.07.2014	27.04.2015 18.05.2015 29.06.2015
	Tracing presence in buffer zones	CFU counting on selective media	Authors	Not done	03.06.2014	Not done
	Tracing in non agricultural soil of border zone	CFU counting on selective media	Authors	Not done	25.06.2014 22.07.2014	Not done
AMF	Sample type	Composite samples of wheat roots	Authors	Wheat roots	Not contributed	Wheat roots
	Quantification of inoculum	Real-time qPCR using primers targeting the inocula	Authors	At harvest	Not contributed	At harvest
	Determination of AMF community	AMF community sequencing	Schlaeppi et al. (2016)	At harvest	Not contributed	Not done
EPN	Sample type	Composite soil sample		12 soil cores/plot	15 soil cores/plot	15 soil cores/plot
	EPN presence: pre-inoculation (baseline)	Species-specific primers/probes and real time qPCR	Campos-Herrera et al. (2015a)	27.03.2014 (Baseline)	27.03.2014 (Baseline)	27.03.2015 (Baseline)
	EPN presence: post-augmentation	Species-specific primers/probes and real time qPCR	Campos-Herrera et al. (2015a)	25.06.2014	25.06.2014	17.06.2015
	EPN activity: insect-baits	*Galleria* bait	Bedding and Akhurst (1975)	25.06.2014	25.06.2014	17.06.2015

	Soil food web assemblage (nematophagous fungi, free-living nematodes and ectophoretic bacteria)	Species-specific primers/probes and real time qPCR	Atkins et al., (2005); Campos-Herrera et al. (2011b, 2012, 2015b); Pathak et al., (2012); Zhang et al. (2006)	27.03.2014 (Baseline) 25.06.2014	27.03.2014 (Baseline) 25.06.2014	27.03.2015 (Baseline) 17.06.2015
Plants	Height (average per plot)	Measured from shoot base to the upper growth	Authors	-	-	23.04.2015 05.06.2015 18.05.2015 01.06.2015 08.06.2015
	Weight	8 plants	Authors	-	-	At harvest
	Density (% of plot surface covered by plants) / number of plants per linear meter	Visual scoring	Authors	-	14.05.2014 21.05.2014 11.06.2014	06.05.2015 18.05.2015 18.06.2015 29.06.2015
	Chlorophyll activity	N-tester (YARA)	Authors	-	-	08.06.2015
	Yield (g seeds/plot);	Weighing wheat seeds at dough developmental stage	Authors	-	at harvest	at harvest
	Thousand-seed weight (TSW)	Marvin seed analyzer	Gegas et al. (2010)	-	-	at harvest
	Protein content (%)	Near-infrared spectroscopy	Authors	-	-	at harvest
	Insect pest and pathogen incidence	Visual counts	Authors	Weekly	Weekly	Weekly

[a] -, Measurements were made, but data are considered not representative due to the small size of the plots (COMBINATION assay) and /or the highly heterogeneous growth of the wheat plants within the plots following heavy frit fly damage in the 2014 COMBINATION and PERFORMANCE-1 assays.

Statistical Analysis. All experimental field trials presented a Randomized Complete Block design (**Figure S1**). The data from each group of beneficial organisms were analyzed following standard procedures for their data presentation, transformation, standardization and normalization whenever necessary. In the case of the EPN activity, data from the *G. mellonella* baits were expressed as the percentage of larval mortality per plot, averaged by treatment. The activity was determined with respect to the total mortality caused only by nematodes. For the EPN soil food web analysis, all the organisms (EPN, FLN, NF and bacteria) quantified by using qPCR were expressed per 100 g of dry soil. The parasitism of nematodes by NF was expressed as "infection rate" (IR), which was calculated by dividing the DNA quantity of each species by the total amount of DNA (Campos–Herrera et al., 2012; Duncan et al., 2013). Similarly, to estimate the total FLN and NF, we divided all data within a species by the highest measurement for that species, which allowed the standardization of the units of measurement among species ranging from 0 to 1 (de Rooij van der Goes et al., 1995).

Unless specified, all significant differences between treatments were assessed by one–way ANOVA, using Tukey's HSD test, considering block as co-variable (V 20.0, IBM SPSS Inc., Chicago, IL). In some cases, t-tests were employed to compare pre- and post-augmentation or control *versus* a specific treatment. If necessary, data were transformed to conform the assumptions of normality and equal variances (transformation method is indicated with the respective statistics). The bacterial colonization data were statistically assessed with a non-parametric Kruskal-Wallis test, followed by a post-hoc test (Dunn's test). With the exception of the *Pseudomonas* root colonization data (presented as $\log_{10}$ of the obtained values ± SEM) all data are presented as mean ± SEM of untransformed values.

RESULTS

Survival and Persistence of *Pseudomonas* Inoculants

In the COMBINATION trial, *P. protegens* CHA0-Rif and *P. chlororaphis* PCL1391 reached similar population densities that surpassed the threshold of ~10^5 CFU per gram of roots, which is the level needed for a plant-beneficial effect (Haas and Défago, 2005) (**Figure 1A**; **Table 4**). However, in the PERFORMANCE-1 trial the population density of the *P. chlororaphis* strain on wheat roots was significantly lower ($P < 0.05$) than the density of the *P. protegens* strain at all three monitoring times (i.e., at 67, 89 and 116 days following field inoculation) (**Figure 1B**; **Table 4**). In this trial, in general, strain CHA0-Rif, alone or in the combinations, performed better than strain PCL1391-Rif. If both strains were present in the same treatment, our agar plates almost only reported *P. protegens* CHA0-Rif colonies (data not shown). Moreover, in clear contrast to CHA0-Rif, PCL1391-Rif never approached the population threshold for plant-beneficial effects, neither when applied alone nor when combined with other BeSO, (**Figure 1B**; **Table 4**). In combination with a commercial population of the EPN *H. bacteriophora*, the density of CHA0-Rif was significant reduced for the late June 2014 sample. Late July 2014, i.e. about one month later, CHA0-Rif still maintained its population density in presence of the nematode inoculant while PCL1391-Rif was no longer detectable (**Figure 1B**; **Table 4**).

In the 2015 PERFORMANCE-2 trial, bacterial numbers approached or surpassed the threshold for plant-beneficial effects at all three sampling times (**Figure 1C**; **Table 4**). In

general, no significant differences among treatments were observed, but there was a trend of higher bacterial population densities in the late April and mid-May samplings when bacterial inoculants where combined with the EPN inoculant mixture. In contrast, an opposite trend was observed for late June 2015 samples. These results imply a possible influence of the EPN, but not AMF, on the bacterial inoculants. Finally, as already observed in the PERFORMANCE-1 trial, strain *P. protegens* CHA0-Rif dominated the colonization, while *P. chlororaphis* PCL1391 was hardly detected (data not shown).

In all three field trials, no rifampicin-resistant bacteria were detected in the non-inoculated control treatments, in the buffer zones or in the grassland border zones at the experimental sites (data not shown), hence, the reported CFU data for the augmented bacteria required no baseline correction.

In general, the applied bacteria survived under field conditions until the end of the crop season (**Figure 1**), although the threshold required to provoke beneficial plant effects (~10^5 CFE per g root) was not always attained in all the trials or treatments. Inoculation was successful in all, with good traceability of the different bacterial inocula without cross-contamination. Consistently, the *P. protegens* inoculant showed higher presence on wheat roots, but the effects of the combination with other BeSO were not conclusive and depended on the BeSO species, on the time of exposure to the field conditions and differed between trials.

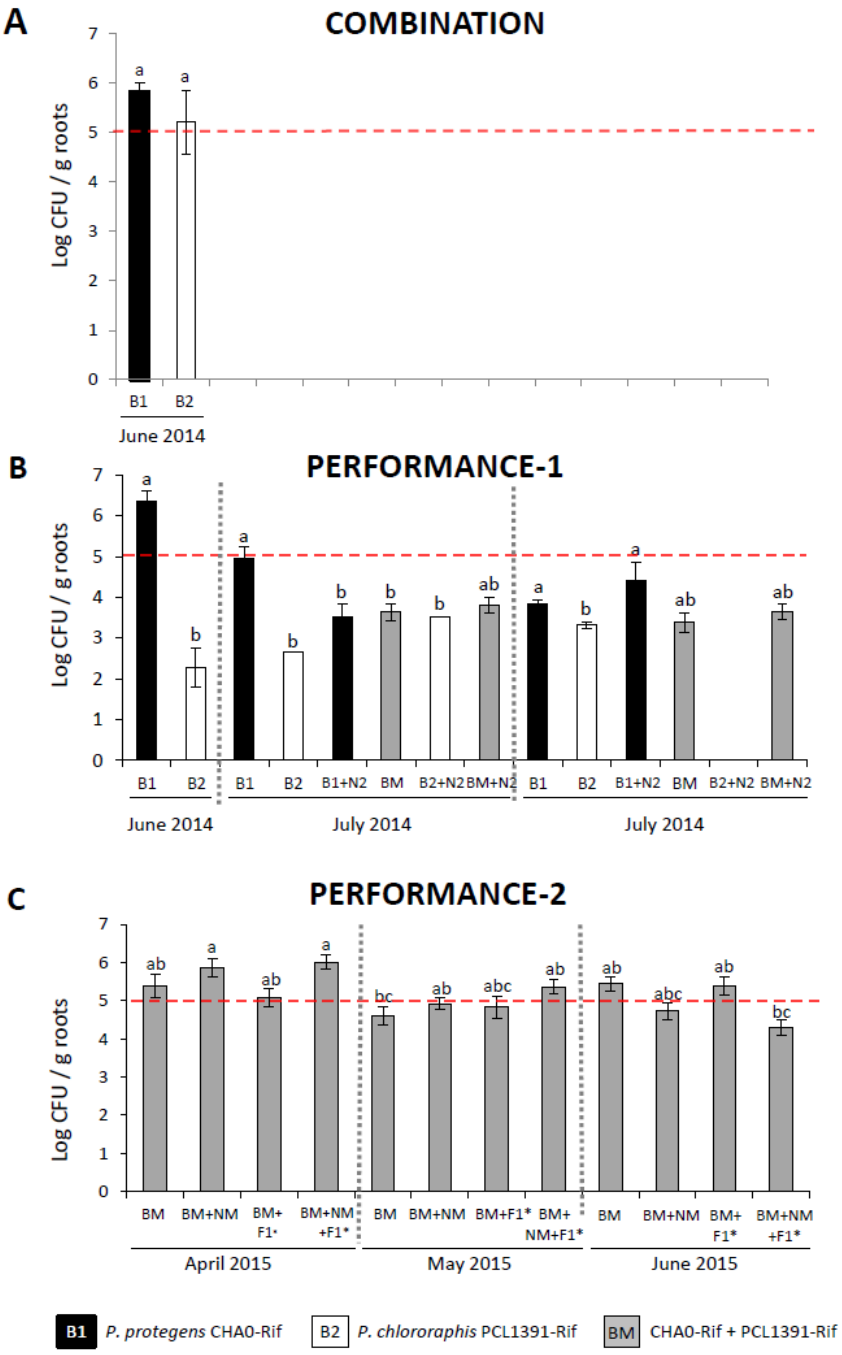

Figure 1 | Survival of *Pseudomonas protegens* strain CHA0-Rif (B1) and *Pseudomonas chlororaphis* strain PCL1391-Rif (B2) on wheat roots in the COMBINATION (A), PERFORMANCE-1 (B), and PERFORMANCE-2 (C) field trials. Bacterial strains were inoculated individually or in combinations with the entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* (N2), an EPN mixture (NM; comprising *Heterorhabditis megidis*, *H. bacteriophora*, and *Steinernema feltiae*) and the arbuscular mycorrhizal fungus *Rhizoglomus irregularis* (F1*). Inoculants were monitored by selective plating on KMB supplemented with rifampicin (100 µg/ml) and cycloheximide (100 µg/ml) at three different time points following seed furrow inoculation. The dashed red line indicates the generally agreed threshold (~10^5 CFU per g root) required to provoke beneficial plant effects with plant growth-promoting pseudomonads (Haas and Défago, 2005). Bar graphs show means of $\log_{10}$ transformed CFU values per gram of dry roots weight (± SEM). Significant differences between treatments were calculated with one-way ANOVA (significance level $P < 0.05$) followed by the Tukey posthoc test, or with a non-parametric Kruskal-Wallis test (significance level $P < 0.05$), followed by Dunn's test for post-hoc comparisons. Inoculants were not detected in the buffer and border zones of the field assays. No Rifampicin-resistant background population was detected at the field sites.

TABLE 4 | Statistical analysis for beneficial soil organisms and plant traits in the three field experiments.

Organisms	Type of measurement	Statistical method	COMBINATION [a]	PERFORMANCE-1 [a]	PERFORMANCE-2 [a]
Bacteria	CFU quantification I	one–way ANOVA (Tukey's HSD test); Kruskal-Wallis (Dunn's test)	$F_{1, 6}$ = 0.1526 , n.s	$F_{1, 2}$ = 2.571 , **	$F_{3, 15}$ = 0.991, n.s.
	CFU quantification II	one–way ANOVA (Tukey's HSD test); Kruskal-Wallis (Dunn's test)	- [b]	$F_{5, 12}$ = 3.675, **	$F_{3, 16}$ = 0.656 , n.s.
	CFU quantification III	one–way ANOVA (Tukey's HSD test); Kruskal-Wallis (Dunn's test)	-	$F_{4, 15}$ = 2.311, n.s.	$F_{3, 16}$ = 1.570, n.s.
AMF	Quantification of INOQ Top [c]	one–way ANOVA	$F_{2, 9}$ = 10.42, **	Not contributed	-
	Quantification of SAF22 [c]	T-tests	T = 10,377 ***	Not contributed	-
	Quantification of INOQ Top in combination samples [c]	one–way ANOVA	$F_{5, 18}$ = 3.712, *	Not contributed	$F_{4, 36}$ = 0.571, n.s.
EPN	EPN presence: pre-inoculation (baseline)	one–way ANOVA (Tukey's HSD test)	$F_{7, 24}$ = 1.273, n.s.	$F_{6, 21}$ = 0.525, n.s.	$F_{4, 40}$ = 0.281, n.s.
	EPN presence: post-augmentation	one–way ANOVA (Tukey's HSD test)	$F_{7, 24}$ = 4.604, **	$F_{6, 21}$ = 2.194, §	$F_{6, 21}$ = 2.888, *
	EPN activity: insect-baits	one–way ANOVA (Tukey's HSD test)	$F_{7, 24}$ = 3.317, *	$F_{6, 21}$ = 1.243, n.s.	$F_{6, 21}$ = 0.722, n.s.
	Free-living nematodes: pre-inoculation	one–way ANOVA (Tukey's HSD test)	$F_{7, 24}$ = 1.051, n.s.	$F_{6, 21}$ = 0.498, n.s.	$F_{6, 21}$ = 0.119, n.s.
	Free-living nematodes: post-augmentation	one–way ANOVA (Tukey's HSD test)	$F_{7, 24}$ = 0.395, n.s.	$F_{6, 21}$ = 1.025, n.s.	$F_{6, 21}$ = 0.341, n.s.
	Nematophagous fungi: pre-inoculation	one–way ANOVA (Tukey's HSD test)	$F_{7, 24}$ = 0.675, n.s.	$F_{6, 21}$ = 0.288, n.s.	$F_{6, 21}$ = 0.618, n.s.

	Nematophagous fungi: post-augmentation	one–way ANOVA (Tukey's HSD test)	$F_{7, 24} = 5.820$, n.s.	$F_{6, 21} = 1.384$, n.s.	$F_{6, 21} = 0.582$, n.s.
Plants	Height (at harvest)	one–way ANOVA (Tukey's HSD test)	-	-	$F_{8, 72} = 1.009$, n.s.
	Density (% plot covered by plants at harvest)	one–way ANOVA (Tukey's HSD test) Kruskal-Wallis (Dunn's test)	-	$F_{7, 88} = 17.219$, ***	$F_{8, 72} = 0.756$, n.s.
	Chlorophyll activity (N-tester)	one–way ANOVA (Tukey's HSD test)	-	-	$F_{8, 72} = 0.161$, n.s.
	Yield (g seeds/plot)	one–way ANOVA (Tukey's HSD test)	-	$F_{7, 23} = 2.069$, **	$F_{8, 72} = 0.026$, n.s.
	Thousand-seed weight (TSW)	one–way ANOVA (Tukey's HSD test)	-	-	$F_{8, 72} = 0.129$, n.s.
	Protein content	one–way ANOVA (Tukey's HSD test)	-	-	$F_{8, 72} = 0.300$, n.s.

[a] Data are presented as the statistical values, degree of freedom and probability levels: § $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., not significant.

[b] For these variables obtained data were not representative because of highly heterogeneous growth of the wheat plants within the plots following frit fly damage and thus were not considered for statistical analysis.

[c] Statistics corresponding to data from two sets of primers, i.e. by Alkan et al. (2006) for INOQ Top, and Bender et al. (under review) for SAF22.

AMF Inoculation Success

For the COMBINATION trial we mainly used the locally well-adapted AMF *Rhizoglomus irregulare* (Schlaeppi et al. 2016) (**Table 1**). We confirmed successful wheat root inoculation for both *R. irregulare* strains that we tested, as well as our custom strain SAF22 and the commercial inoculum INOQ Top (**Table 4**; **Figure 2A**). The higher dosage of the inoculum INOQ Top (80 g per row) corresponded approximately to the amount of SAF22 inoculum and it appeared that both *R. irregulare* strains colonized the wheat roots to a similar extent. The reduced dosage of the commercial inoculum (16 g per row) was reflected in lower levels of root colonization and it only showed a minor tendency of augmentation. We also traced the inoculation of the *Funneliformis mosseae* strain SAF11 and the *Claroideoglomus claroideum* strain SAF12 using specific qPCR primers (Thonar et al. 2012). We did not detect these AMF species at the field site (data not shown) confirming the findings of a previous AMF community profiling (Schlaeppi et al. 2016). Hence, we concluded that these strains failed to establish at the tested field site in the wheat roots. In summary, *R. irregulare* could be augmented in wheat AMF communities using the strains SAF22 or INOQ Top, while this was not successful for *F. mosseae* SAF11 and *C. claroideum* SAF12.

Plots in which soil beneficial organisms showed low AMF colonization levels were not different from control (nothing applied) and mock (application of carrier substrate without AM fungus) plots (**Table 4**; **Figure 2B**). These measured abundances of *R. irregulare* correspond to the native strain in the field and indicated that the application of the carrier substrate on its own did not affect the root colonization by the AM fungus. Although, the level of root colonization by *R. irregulare* showed a slight tendency to increase in the combination treatments of the AM fungus with bacteria, nematodes or both, the AM fungus was not augmented to the same extent as in single application. These first insights on combining soil beneficial organisms suggest possible negative effects on the AMF inoculum if combined with bacteria or nematodes.

In the PERFORMANCE-2 experiment, the commercial *R. irregulare* strain INOQ Top was inoculated using a lower dosage level to larger plots compared to the previous experiments (**Table 2**). Again, there was a tendency of increased colonization of the wheat roots in the combined treatments, however, high inter-plot variation precluded statistic support for this effect (**Table 4**; **Figure 2C**). It remains to be validated, whether the colonization by this *R. irregulare* strain is particularly facilitated if applied in combination with the *Pseudomonas* bacteria.

In summary, *R. irregulare* successfully colonized wheat roots after inoculation, and in the combination experiments, we found varying augmentation efficiencies for *R. irregulare* if combined with pseudomonads, EPN or both, indicating that interactions with these beneficial soil organisms are context dependent.

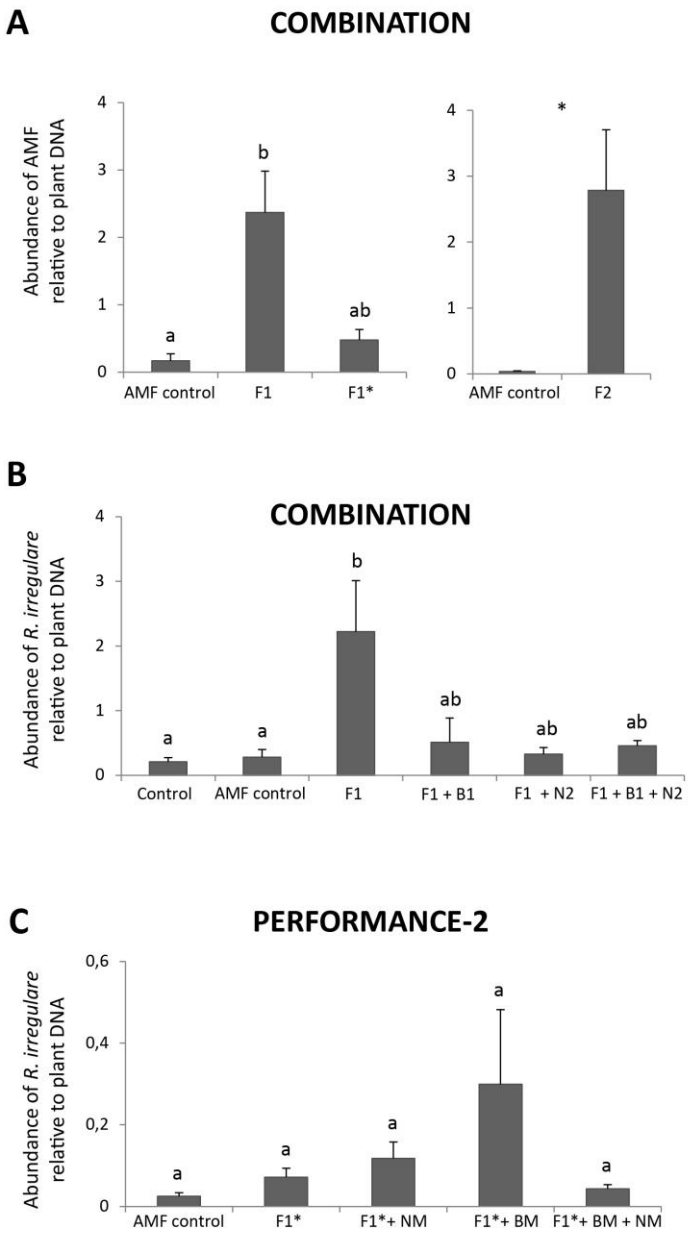

FIGURE 2 | Abundance of *Rhizoglomus irregulare* in wheat roots in the COMBINATION (A, B) and PERFORMANCE-2 (C) field trials. (A) In the COMBINATION experiment, *R. irregulare* strain INOQ TOP was inoculated comparing high (F1) vs. low (F1*) dosages, with one of the treatments including the AMF strain SAF22 (F2). (B) In the same experiment, *R. irregulare* INOQ TOP (F1) was quantified in combination with bacteria, i.e. *Pseudomonas protegens* CHA0-Rif (B1), and nematodes, i.e. *Heterorhabditis bacteriophora* Andermatt (N2). (C) In the PERFORMANCE-2 experiment, *R. irregulare* INOQ TOP at the lower dosage (F1*) was used for the combination treatments with bacterial mixture (BM; i.e. *P. protegens* + *Pseudomonas chlororaphis*) and nematode mixture (NM; i.e. *Heterorhabditis megidis* + *H bacteriophora* + *Steinernema feltiae*; for details see **Figure S1**). Control, non-inoculated control; AMF control, substrate control for AMF inoculation. *R. irregulare* was measured with quantitative PCR employing species-specific primers developed by Alkan et al. (2006) for INOQ TOP or their modified variants with enhanced specificity for SAF22 (Bender et al. under review). Bar graphs report mean normalized (*R. irregulare* relative to plant DNA) abundance (± SEM; COMBINATION, n = 4; PERFORMANCE-2, n = 7-9). Statistical analyses were performed on log-transformed data; asterisks and different letters indicate statistical significance at $P < 0.05$ for t-test and one-way ANOVA followed by the Tukey post-hoc test, respectively.

Nematode Survival, Activity and Interactions with Soil Food Web Members

In all plots, very low numbers of background populations were detected as also found by Campos-Herrera et al. (2015a) and Jaffuel et al. (2017) in the same area. Five to seven species naturally occurred at the experimental field sites, and these species included the taxa that we augmented. Prior to inoculations (baseline; **Table 3**), there were no differences among treatments for any measured variable (EPN, free-living nematodes and nematophagous fungi; data not shown) in any of the three field trials. The evaluation of EPN soil food web members (free-living nematodes and nematophagous fungi) only revealed natural temporal fluctuations between baseline (pre-inoculation) and post EPN augmentation (data not shown), whereas their presence was not significantly affected by the EPN augmentation (alone or in combination) (**Table 4**; **Figure S2**). The nematophagous fungi and free-living nematodes species were in agreement with those already described by Campos-Herrera et al. (2015a, 2015b) and Jaffuel et al. (2017). Finally, the ectophoretic bacterium *P. nematophilus* was not detected in any of plots (control or augmented).

In the COMBINATION trial, the EPN species *H. megidis* and *S. carpocapsae* were recovered in only 25% of the plots four months after augmentation. In contrast, the species *S. feltiae* and *H. bacteriphora*, of which the latter was also combined with other BeSO, were detected in 100% of the plots at the end of the season. The augmentation with *S. feltiae* was the only treatment with a significant increase in total EPN numbers compared with the native populations (**Figure 3A**; **Table 4**). The remarkable persistence of *S. feltiae*, which was the only species detected in the soil in their corresponding plots, was in agreement with the nematode activity measured in the laboratory as % mortality of *G. mellonella* producing progeny. This was the only treatment with significantly higher activity than the control in the whole trial (**Figure 4A**; **Table 4**).

In the PERFORMANCE-1 trial, we only augmented certain plots with *H. bacteriophora*. This EPN was detected in about 50% of the plots when applied alone or in combination with the bacterial inoculant *P. chlororaphis* and in about 75% of the plots when applied with the *P. protegens* in different combinations. No significant difference in EPN populations (qPCR measurements) and their activity (% larval mortality) was observed between plots where the EPN were applied alone or in combination with bacterial inoculants. Nor were they different from control plots (**Figure 3B and 4B**; **Table 4**). In both trials, COMBINATION and PERFORMANCE-1, there was a slight trend to detect more *H. bacteriophora* in the combined treatment with AMF and/or bacterial inoculants (N2+B1+F1 and N2+BM, respectively) (**Figure 3A and 3B**). The same trend was also observed for nematode activity (**Figure 4A and 4B**). In the PERFORMANCE-1 field trials, *Steinernema affine* (**Table S1**) was the dominant native EPN species in the soil of the experimental plots as determined by qPCR (**Figure 3B**).

In the PERFORMANCE-2 trial, the augmented EPN species (a mix of *S. feltiae, H. megidis* and *H. bacteriophora*) could be detected in 100% of the plots inoculated with the three EPN alone or in combination with the *Pseudomonas* inoculants, in 91% of the plots where they were combined with AMF and in only 44.4% of the plots when combined with both bacterial and AMF inoculants. Again, the species *S. affine* was dominant among the native taxa as displayed in the proportional chart, although, contrary to the PERFORMANCE-1 trial, native species were largely displaced in all the treatments where EPN were applied (**Figure 3C**). All plots with EPN application showed significantly higher total numbers of EPN than the control plots (**Figure 3C**; **Table 4**). All the augmented EPN species were detected in each of the plots, but *S. feltiae* and *H. bacteriophora* dominated. The nematode

activity was low and did not significantly vary among treatments (**Table 4**; **Figure 4C**). The progeny from the activity tests belonged mainly to *H. bacteriophora* (62.5%), followed by *S. feltiae* (34.5%), in all the cases we found mixed EPN-free-living nematodes emergence as observed in previous studies in Swiss soils (Jaffuel et al., 2016; 2017).

In general, inoculated EPN persisted during the crop season and remained active until the time for wheat harvest, but with limited pest suppressive potential as measured with a *Galleria* larvae infection assay. We observed that EPN application increased the total numbers of EPN only in specific treatments, displacing at least partially the native populations (**Figure 3**). No long-term effect was observed with respect to soil organisms that can be expected to be modulated by EPN augmentation, such as nematophagous fungi and free-living nematodes. The combined application of EPN with other BeSO indicated compatibility with respect to their persistence, prevalence and activity, when compared with the single EPN application, but some differences depending on EPN species and co-inoculant identity were observed. As for the bacterial and AMF inoculants, the success of EPN inoculants appeared to be context dependent.

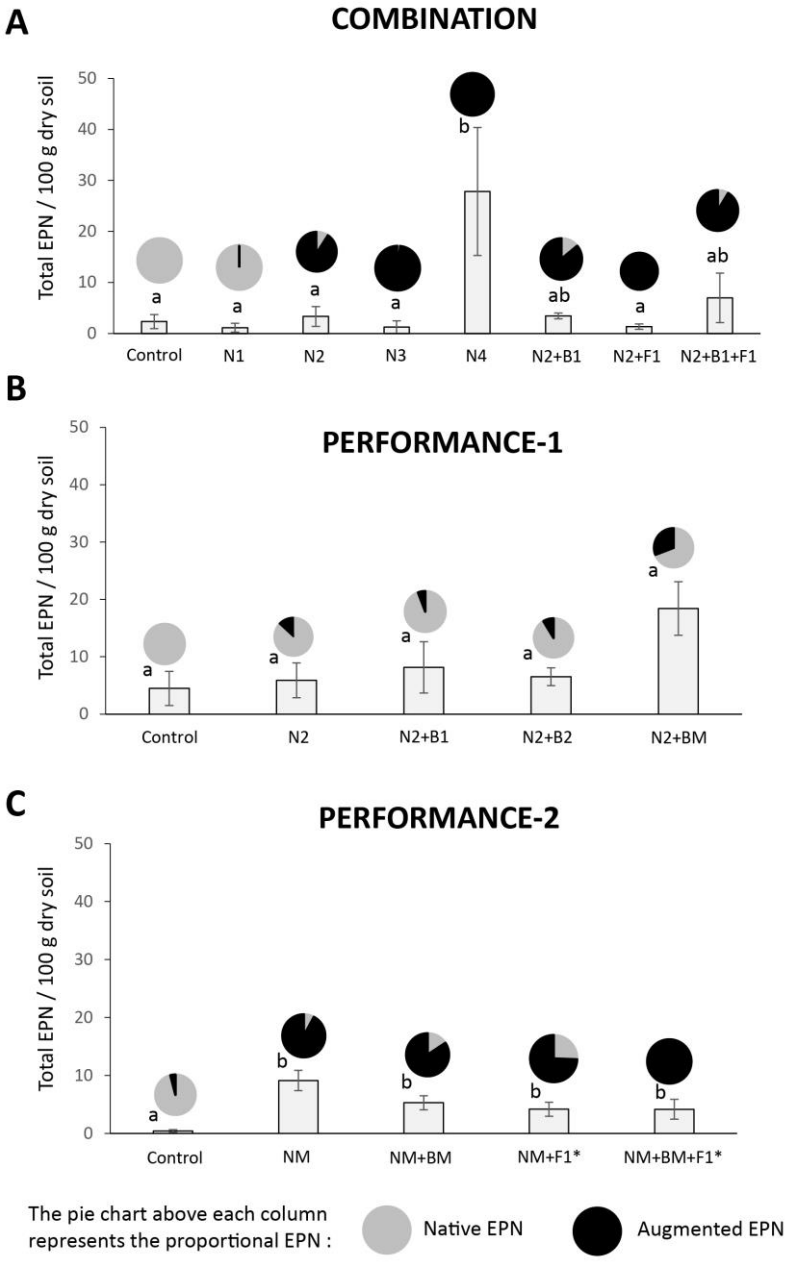

FIGURE 3 | End of the season presence of inoculant and resident entomopathogenic nematodes in the COMBINATION (A), PERFORMANCE-1 (B), and PERFORMANCE-2 (C) field trials. Four different EPN species *Heterorhabditis megidis* (N1), *Heterorhabditis bacteriphora* (N2), *Steinernema carpocapsae* (N3), and *Steinernema feltiae* (N4) were inoculated individually or in combination with *Pseudomonas protegens* (B1), *Pseudomonas chlororaphis* (B2) and *Rhizoglomus irregularis* at two dosages (F1 and F1*). Mixtures of EPN (N1+N2+N4) or of the two bacteria (B1+B2) are indicated with NM and BM, respectively (for details see **Figure S1**). To determine the persistence of the EPN in soil of the different nematode inoculants as well as the impact of each treatment on the resident population of entompathogenic nematodes (EPN), a DNA extraction procedure followed by a qPCR approach was performed. Data are expressed as total EPN 100 g^{-1} of dry soil. Bar graphs report means (± SEM) and pie-charts show the proportion of native EPN *versus* augmented EPN. Significant difference between treatments were calculated with one-way ANOVA (significance level $P < 0.05$) followed by the Tukey post-hoc test.

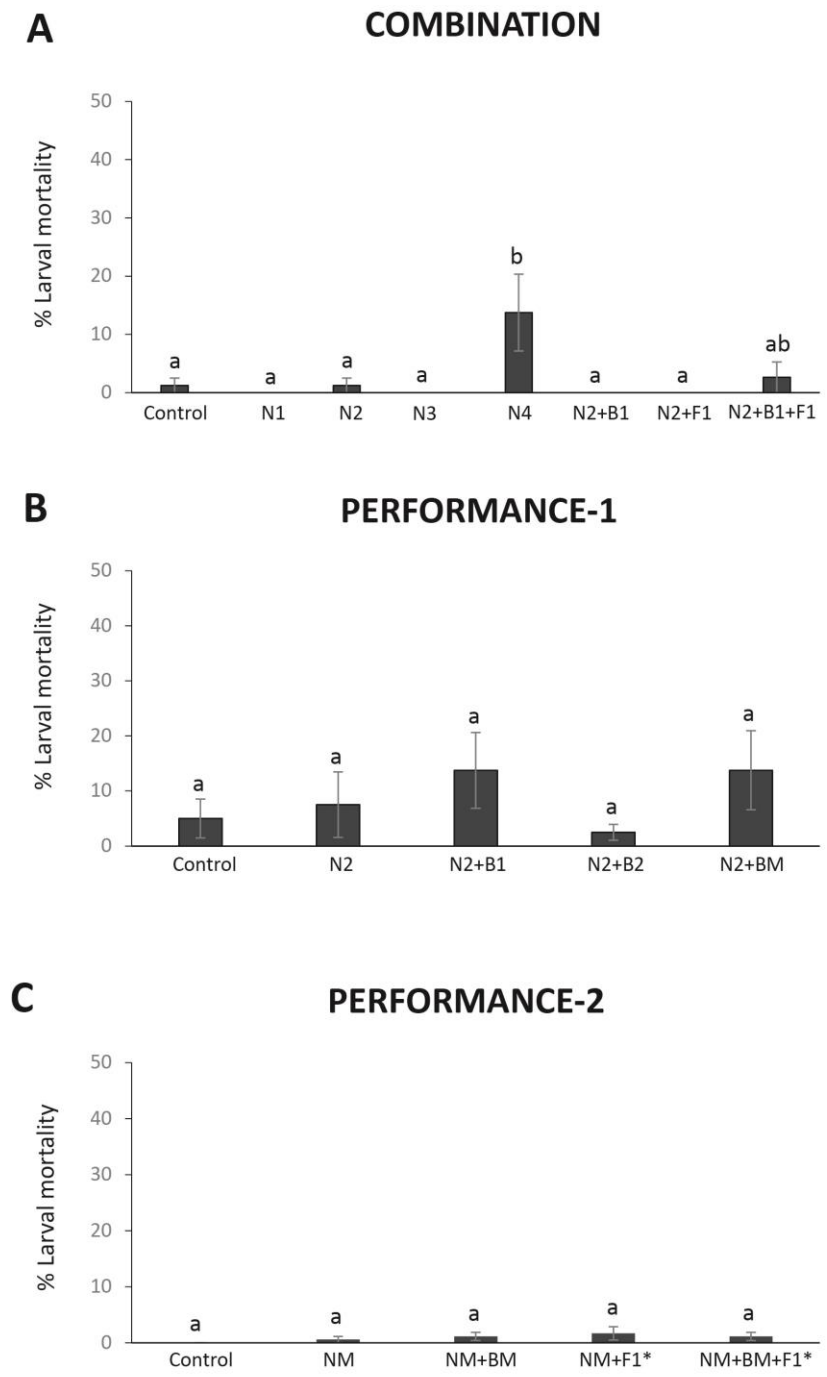

FIGURE 4 | Activity of entomopathogenic nematodes (EPN) post application in three field trials. EPN activity was quantified by a *Galleria mellonella* larvae infection assay in soil samples from the (**A**) COMBINATION, (**B**) PERFORMANCE-1 and (**C**) PERFORMANCE-2 trials. Inoculants were *Heterorhabditis megidis* (N1), *Heterorhabditis bacteriophora* (N2), *Steinernema carpocapsae* (N3), and *Steinernema feltiae* (N4), individually or in combination with *Pseudomonas protegens* (B1), *Pseudomonas chlororaphis* (B2) and *Rhizoglomus irregularis* at two dosages (F1 and F1*). Mixtures of EPN or bacteria are indicated with NM and BM, respectively (for details see Figure S1). Bar graphs report means (± SEM). Significant differences between treatments were calculated with one-way ANOVA (significance level $P < 0.05$) followed by the Tukey post-hoc test.

Agronomic Impact of the Applied Beneficial Soil Organisms

The 2014 trials (COMBINATION and PERFORMANCE-1) were intentionally not subjected to standard pesticide treatments and suffered from heavy attack by frit flies (*Oscinella frit*). For the small scale COMBINATION trial, insect damage was very patchy and therefore not agronomically representative and not included in the plant performance analyses. The larger plot sizes in the PERFORMANCE-1 trial permitted analysis of agronomically relevant plant density and seed yield data (**Table 4**). The % of plot surface covered with plants was significantly higher in augmentation plots than in the control treatment when the two bacterial inoculants, *P. protegens* and *P. chlororaphis*, were applied individually or as a mixture with and without the EPN (**Figure 5A**). Seed yield per plot followed a similar pattern, but only the combined treatment with both bacterial strains and the EPN showed significantly higher values than the control (**Figure 5C**). AMF effects could not be examined in the PERFORMANCE-1 trial due to limited inoculum production. Nevertheless, the neighboring COMBINATION experiment indicated that seedling survival after frit fly attack tended to be higher in plots inoculated with *R. irregulare* (**Figure S3**). In the 2015 PERFORMANCE-2 trial, plots were subjected to pesticide treatment, no pest damage was observed and all plant traits were considered in the analysis. However, none of these measures, including plant density and seed yield per plot (**Figure 5B and 5D**) nor the other plant performance traits (**Table 3 and 4**; **Figure S4**) differed significantly from the control treatment.

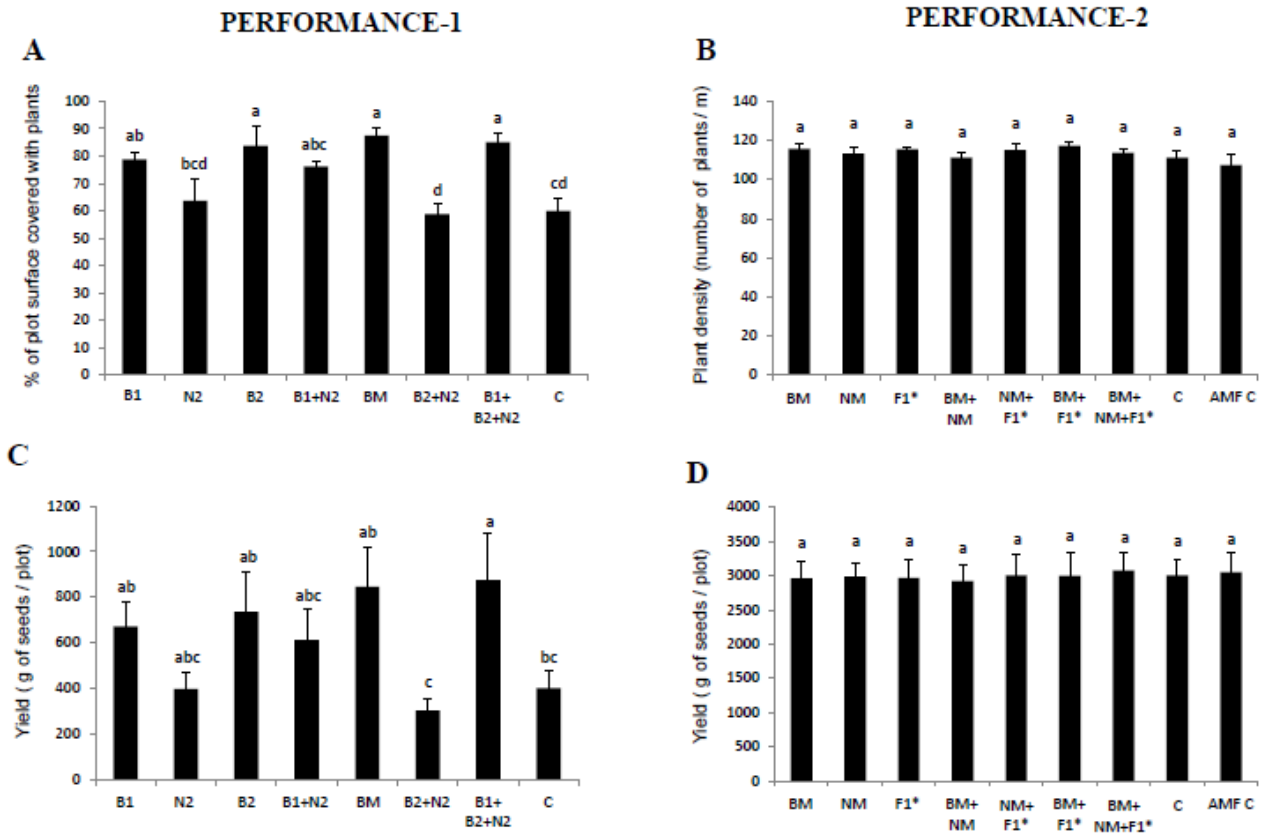

FIGURE 5 | Impact of field inoculations with beneficial organisms on plant performance in the PERFORMANCE-1 (A, C) and PERFORMANCE-2 (B, D) trials. Plant performance was evaluated in terms of plant density (A, B) and yield (weight of wheat seeds) (C, D) for each plot. The PERFORMANCE-1 experiment was exposed to heavy natural infestation with the firt fly (*Oscinella frit*) causing significant plant damage. Plant density in the PERFORMANCE-1 trial was therefore determined by visual scoring the percentage of plot area covered by wheat plants in this experiment why it was determined by counting the number of plants per linear meter in the PERFORMANCE-2 experiment, which had no measurable frit fly damage. Inoculants were *Pseudomonas protegens* (B1), *Pseudomonas chlororaphis* (B2), individually or in combination with *Heterorhabditis bacteriophora* (N2) and *Rhizoglomus irregularis* (F1*). Mixtures of the two bacteria or of the entomopathogenic nematodes (*Heterorhabditis megidis*, *Heterorhabditis bacteriophora*, and *Steinernema feltiae*) are indicated with NM and BM, respectively (for details see **Figure S1**). C, non-inoculated control; AMF-C, substrate control for AMF inoculation. Bar graphs report means (± SEM). Significant differences between treatments were calculated with one-way ANOVA (significance level $P < 0.05$) followed by the Tukey post-hoc test.

In summary, when wheat was exposed to biotic stress (i.e, a heavy insect pest attack in 2014) a significant positive effect of the application of BeSO, notably *Pseudomonas* bacteria, on performance of the crop was observed. The presence of the EPN was only beneficial when combined with both bacteria together. In absence of a biotic stress conditions, as in the PERFORMANCE-2 trial in 2015, there was no measurable plant-beneficial effect of the presence of BeSO, highlighting the context dependence of their protective effect on the crops.

DISCUSSION

Overall, the three field experiments showed consistent results: (1) the inoculated BeSO persisted until the end of the crop season, although their prevalence gradually declined with time; (2) the introduced BeSO in augmented plots were consistently present at higher levels than the native populations, without cross-contamination between plots; (3) the augmented BeSO integrated with or displaced the natural community to varying degrees depending on the strain/population and dosage; and (4) the combined application of *Pseudomonas*, EPN and AMF showed only beneficial effects under conditions with an insect outbreak. In particular and contrary to our expectations, our current tripartite BeSO inoculant system (bacteria + EPN + AMF) did not provide clear additive or synergistic positive effects to allow a better performance of wheat than the application of the individual BeSO. Overall, our results are in agreement with the previous observation that the combination of various BeSO can lead to a beneficial effect under certain conditions (Frey-Klett et al., 2007; Ansari et al., 2010; Walker et al., 2011; Couillerot et al., 2012), but mainly have similar effects as single applications (Tarasco et al., 2011; Glare, Hurst and Narciso, personal communication). We can conclude that there is still a large gap between the promising results from BeSO applications under controlled experiments (laboratory and greenhouse settings) and their performance under field conditions.

Many factors can explain this difference between applications in laboratory/greenhouse and field settings. The characteristics of a particular agroecosystem (i.e., soil type, soil geochemistry, humidity, plant genotype, climate, etc.) play a decisive role in determining the success of augmented BeSO. From a biogeographic point of view, the selection of the BeSO should attend the biology and ecology of the BeSO, so the soil and environmental conditions have to match with the best ecological scenario for expressing the desired activity, within the range of known natural occurrence. The soil is a complex medium, with physicochemical and biological interactions that vary over time and space (Ritz & van der Putten, 2012). In the three trial, the general characteristics of the soil were largely similar (**Table 2**), although unnoticed microhabitat differences might patchily occur and produce internal stochasticity, a factor that is better controlled in any greenhouse experiment where often soils are homogenize first and treatments are confined to smaller experimental units such as pots. In a field experiment, fundamental differences in soil chemistry (acid soils *versus* basic soils, presence of micronutrients, etc.) and soil physical properties (texture, pore size, compaction, available water, etc.), should be considered to select the most appropriate BeSO (Schlaeppi and Bulgarelli 2015). For example, AMF mostly perform better in low nutrient soils (Pellegrini et al., 2012, 2015). Also the effects of AMF on crop productivity are highly dependent on the plant species or genotypes investigated (Lekberg & Koide 2005): plants and crops with fine roots such as wheat (as in this study) are usually less responsive to AMF compared to species

with thicker roots such as red clover (Köhl et al. 2016). Similarly, EPN species have ecological and habitat preferences that are largely determined by texture and moisture of soils (Campos-Herrera et al., 2013, 2016; El-Borai et al., 2016). Soil physico-chemical characteristics can also impact persistence and activity of *Pseudomonas* species (Natsch et al., 1996; Troxler et al., 2012; Mascher et al., 2014; Imperiali et al, 2017). Hence, locally adapted species might have an advantage in persistence over exotic organisms that are not present in the target soil (Schlaeppi et al., 2016).

In addition to the abiotic soil conditions, BeSO inoculants are also subjected to interactions with the resident soil organism community. The diversity of soil organisms can contribute to buffering, masking and silencing beneficial effects of inoculations. This is also a major difference with controlled experiments (growth chamber or greenhouse) where conditions usually limit or simplify the interactions of inoculant BeSO with other soil organisms and the target crop. Often laboratory or greenhouse experiments are conducted with sterilized soils, with entirely or greatly reduced abundance of native soil organisms. Under field conditions, there are also spatial and temporal differences in these effects on the augmented BeSO. This is particular relevant when considering naturally occurring populations of BeSO. In our experiments, we observed that the native populations of AMF and EPN were displaced to varying degrees, depending on the BeSO species/population inoculated in the field plots. In agreement with Schlaeppi et al. (2016) and Jaffuel et al. (2017), we also observed that augmented BeSO species that also occurred naturally in the area performed better than those that were not present or were present in low numbers. Cross-contamination between plots almost did not occur and displacement of native populations was reversed at the end of the crop season, this suggest that stablishment of introduced BeSO do not represent a risk for native populations of soil organism. Yet, more studies are needed to evaluate the potential long-term impacts of implementing inoculation strategies of single or combined BeSO, especially if inoculants are not native or no present in the area of application (Abate et al., 2017; Hart et al., 2017).

Here we introduce a comprehensive toolbox to trace Pseudomonads, AMF and EPN after application. Some of the BeSO did not reach the numbers known to be required to reach beneficial plant effects (Haas and Défago, 2005), did not persist well after application (i.e., the EPN species *H. megidis* and *S. carpocapsae*) or did not establish following field inoculation (i.e., the AMF species *Funneliformis mosseae* and *Claroideoglomus claroideum*). Nevertheless, results for some isolates and combinations were highly promising. Under the experimental field settings, the bacterium *P. protegens* CHA0, the AMF *R. irregularis* and the EPN *S. feltiae* established very well. Under conditions with high biotic stress (frit fly infestation in the PERFORMANCE-1 trial), the combination of bacterial and EPN inoculants produced the highest yields. Because such ecological conditions will change from one season to another, the development of a pre-application diagnosis tool may help the choice of an optimized BeSO (Schlaeppi and Bulgarelli, 2015; Schlaeppi et al., 2016). For example, areas strongly impacted by plant diseases and pests might benefit from the integration of various *Pseudomonas* bacteria. Whereas the presence of insect pests will better support the development and persistence of native and augmented EPN, thereby enhancing their protective effects. Finally, selecting BeOS, in particular AMF, that are compatible with local soil conditions (e.g. low or high nutrient content) is highly advisable (Pellegrini et al., 2015; Schlaeppi et al., 2016).

Advancing our understanding of the soil-plant interface in its broadest sense is critical to achieve sustainable agriculture (Adl, 2016). We evaluated the simultaneous application of three types of BeSO (bacteria, EPN, and AMF) and its impact on wheat productivity under realistic field conditions. While we confirmed the prevalence and persistence of the three

organisms throughout the season, their beneficial effects were variable and differed between inoculant strains. Clear beneficial effects on wheat growth were observed when the plants were exposed to high insect infestation. We learned that there is still a major gap in our understanding of the capacities of BeSO to enhance plant performance under well-controlled conditions and their performance and impacts when applied to the field. We believe that to close this gap and for the successful use of BeSO in agroecosystems there is an urgent need to unravel the context dependency of effective BeSO augmentations. Optimizations should go toward adapting and fine-tuning the selection of inoculant strains that are well adapted to local abiotic and biotic soil conditions. Advancing such an integrative and context-dependent approach is vital before next-generation, sustainable agriculture, in which field crops are protected by applying beneficial soil organisms instead by agrochemicals becomes imaginable.

AUTHOR CONTRIBUTIONS

KS, MvdH, MM, FM, TCJT, CK, and RCH planned the experiments and supervised the study. NI, XC, KS, GJ, SFB, FD, MF, RBP, DV, MvdH, MM, FM, CK and RCH contributed in the field experiments and collected the data. NI, XC, KS, MF, CK, and RCH analyzed the data, discussed the main structure and wrote the manuscript. All authors contributed revisions and commented previous versions of the manuscript.

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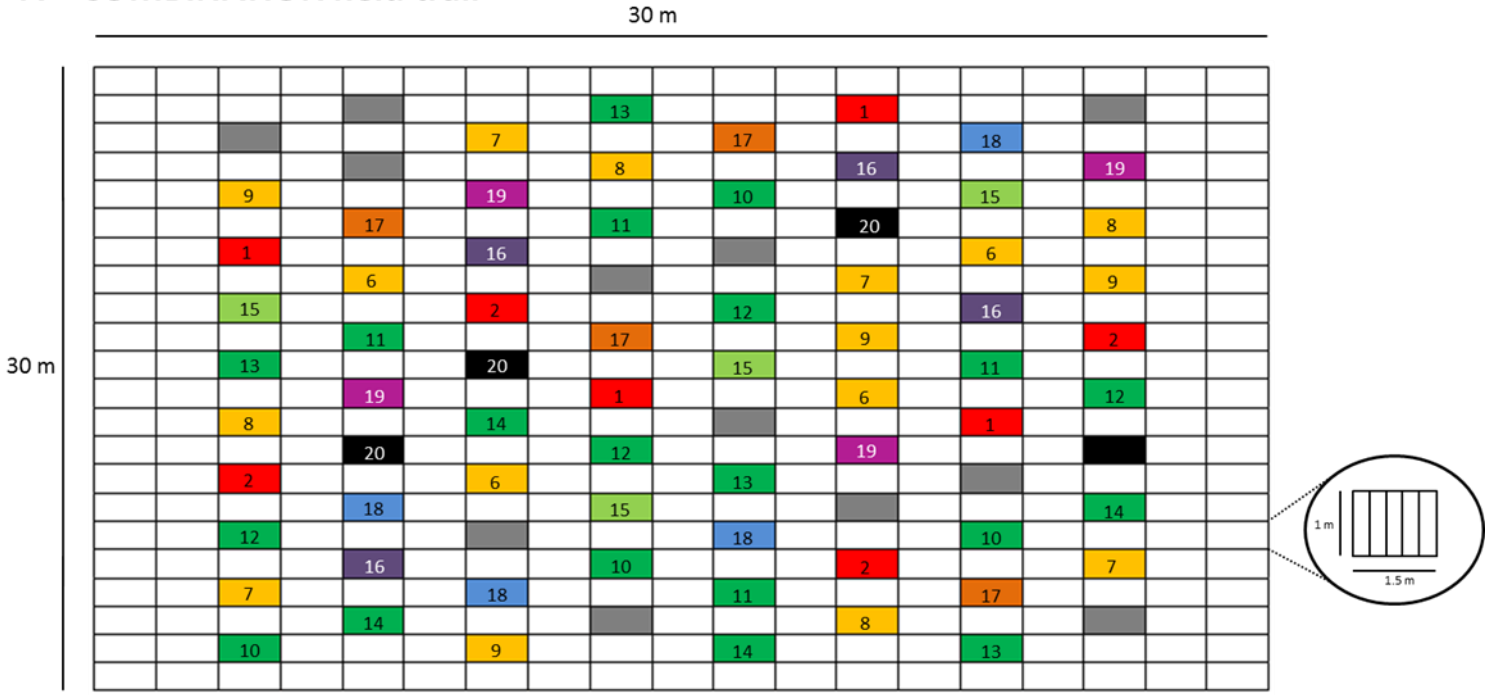
SUPPLEMENTARY MATERIAL

TABLE S1 | Species and sources of nematodes, fungi and bacterium for the entomopathogenic nematode soil food web analysis by real time qPCR.

Type of organism/ species	Population/strain	Material used / Unit of measurements	GenBank accession number[a]
Entomopathogenic nematodes			
Heterorhabditis bacteriophora	Commercial	Infective juvenile (IJs) / no. IJs	KJ938576
Heterorhabditis megidis	Commercial	Infective juvenile (IJs) / no. IJs	KJ938577
Steinernema affine	CH	Infective juvenile (IJs) / no. IJs	KJ938567
Steinernema carpocapsae	DOK-83	Infective juvenile (IJs) / no. IJs	KJ818295
Steinernema feltiae	RS-5	Infective juvenile (IJs) / no. IJs	KJ938569
Steinernema kraussei	OS	Infective juvenile (IJs) / no. IJs	KJ696686
Steinernema poinari	1160	ITS rDNA sequence + pUC57 / pg DNA	KF241754
Free-living and competitor nematodes			
Acrobeloides-group	RT1-R15C	18S rDNA sequence + pUC57 / pg of DNA	JQ237849
Oscheius tipulae	MG68 P29	Nematodes/ ng DNA	KJ938579
Oscheius onirici	MG67 P20	Nematodes/ ng DNA	KJ938578
Oscheius sp. 3	JU75	18S rDNA sequence + pUC57 / pg of DNA	AJ297890
Nematophagous fungi			
Catenaria sp.	1D	ITS rDNA sequence + pUC57 / pg of DNA	JN585805
Arthrobotrys dactyloides	H55	Pure culture / pg of DNA	KJ938574
Arthrobotrys musiformis	11	Pure culture / pg of DNA	KJ938572
Arthrobotrys oligospora	8	Pure culture / pg of DNA	KJ938573
Hirsutella rhossiliensis	2931	Pure culture / pg of DNA	n.a.
Purpureocillium lilacinus	9357	Pure culture / pg of DNA	KJ938575B
Ectophoretic bacteria			
Paenibacillus nematophilus	NEM2	16S rDNA sequence of 490 bp + pUC57 / copy numbers	AF480936

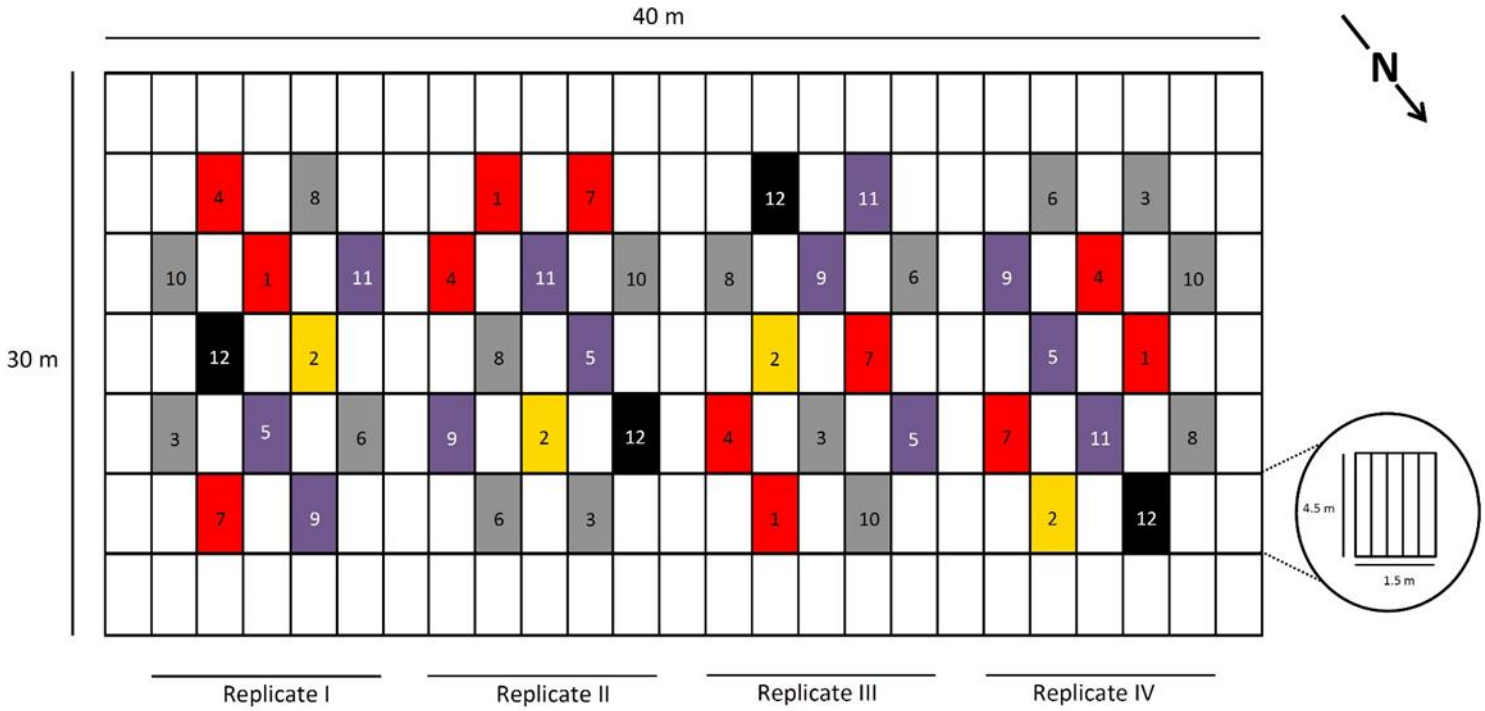
[a] n.a., not available.

A COMBINATION field trail



Bacteria	Nematodes	AMF	Combinations	Controls
1) *P. protegens* CHA0 (B1)	6) *H. megidis* Andermatt (N1)	10) *R. irregulare* INOQ Top (F1-H)	16) B1+ N2	20) Non inoculated plots
2) *P. chlororaphis* PCL1391 (B2)	7) *H. bacteriophora* Andermatt (N2)	11) *R. irregulare* INOQ Top (F1-L)	17) B1+ F1-H	Buffer
	8) *S. carpocapsae* D83 (N3)	12) *R. irregulare* SAF22 (F2)	18) N2+ F1-H	3,4,5) Not considered treatments
	9) *S. feltiae* RS5 (N4)	13) *F. mosseae* SAF11 (F3)	19) B1+ N2+ F1-H	
		14) *C. claroideum* SAF12 (F4)		
		15) AMF control		

B PERFORMANCE-1 field trail



Bacteria	Nematodes	Combinations	Controls
1) *P. protegens* CHA0 (B1)	2) *H. bacteriophora* Andermatt (N2)	5) B1 + N2	12) Non inoculated plots
4) *P. chlororaphis* PCL1391 (B2)		9) B2 + N2	Buffer
7) *P. protegens* CHA0 + *P. chlororaphis* PCL1391 (BM)		11) BM + N2	3,6,8,10) Not considered treatments

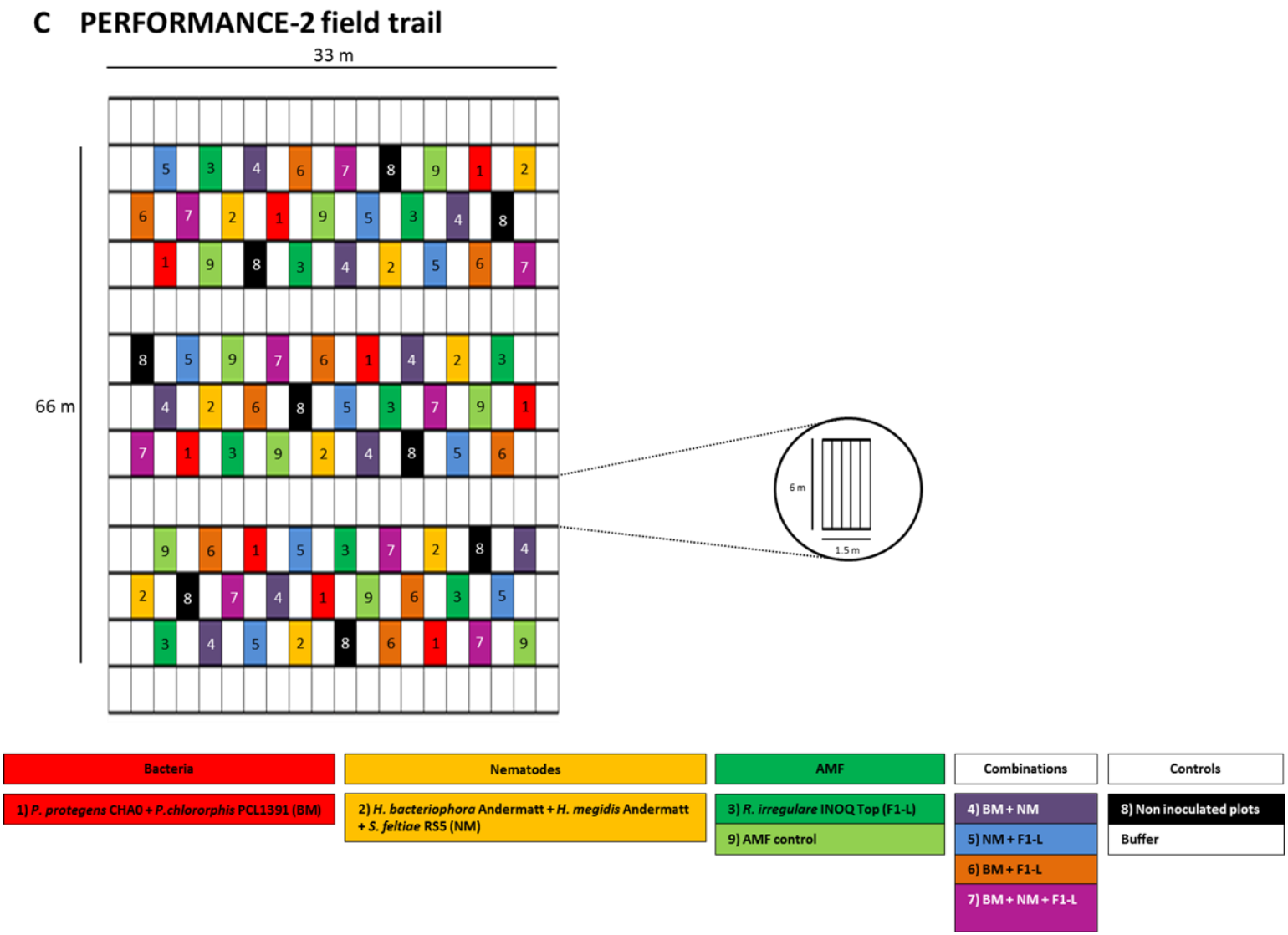


FIGURE S1 | Experimental designs of the three field experiments performed in 2014 and 2015 at the agricultural research station Agroscope near Prangins, Switzerland. Experiments were carried out using the spring wheat variety Rubli and individual or combined

inoculations with rifampicin-resistant pseudomonads, entomopathogenic nematodes and arbuscular mycorrhizal fungi (AMF). Plots treated with different inoculants are indicated with different colours. Each plot consisted of five plant rows (row spacing of 50 cm). Inoculants were applied to the seed furrow immediately following sowing of wheat seeds. Non-coloured portions indicate buffer zones in which the triticale variety Trado was cultivated. Negative controls were untreated plots and, in addition for AMF, plots treated with a "mock inoculum" consisting of *Plantago* roots and substrate free of AMF propagules (AMF control). In each experiment, all replicates were disposed in Randomized Complete Block design. The COMBINATION field trial **(A)** conducted in spring 2014 included twenty treatments with four replicates per treatment. *Pseudomonas protegens* CHA0-Rif (B1) and *Pseudomonas chlororaphis* PCL1391-Rif (B2) were inoculated into the seed furrows of the 1.5 m^2 plots, individually or in combination with the entomopathogenic nematodes *Heterorhabditis megidis* Andermatt (N1), *Heterorhabditis bacteriophora* Andermatt (N2), *Steinernema carpocapsae* D83 (N3), *Steinernema feltiae* RS5 (N4) and the AMF strains *Rhizoglomus irregularis* INOQ Top, inoculated at high (i.e. 250 ml/row; (F1-H) and low concentration (i.e., 50 ml/row; (F1-L), *Rhizoglomus irregularis* SAF22 (F2), *Funneliformis mossae* SAF11 (F3) and *Glomus claroideum* SAF12 (F4). Grey-colored plots indicate three treatments (3, 4 and 5) with bacterial strains that were not considered for the present study. The PERFORMANCE-1 field experiment **(B)** was performed in spring 2014 and included four replicates of twelve treatments. *P. protegens* CHA0-Rif (B1) and *P. chlororaphis* PCL1391-Rif (B2) were inoculated into seed furrows of 6.75 m^2 plots, either individually, mixed (BM) or in combination with the entomopathogenic nematode *H. bacteriophora* Andermatt (N2). Grey-colored plots indicate four treatments (3, 6, 8 and 10) with application of chitosan that were not considered for the present study. The PERFORMANCE-2 field experiment **(C)** was conducted in spring 2015 and included nine replicates of nine treatments. For the different treatments, a mixture of the bacteria *P. protegens* CHA0-Rif and *P. chlororaphis* PCL1391-Rif (BM), a mixture of the entomopathogenic nematodes *H. megidis* Andermatt, *H. bacteriophora* Andermatt, and *S. feltiae* RS5 (NM), the AMF *R. irregularis* INOQ Top at 50 ml/row (F1-L) or combinations of the bacteria, nematodes, and AMF were inoculated into seed furrows of 9 m^2 plots.

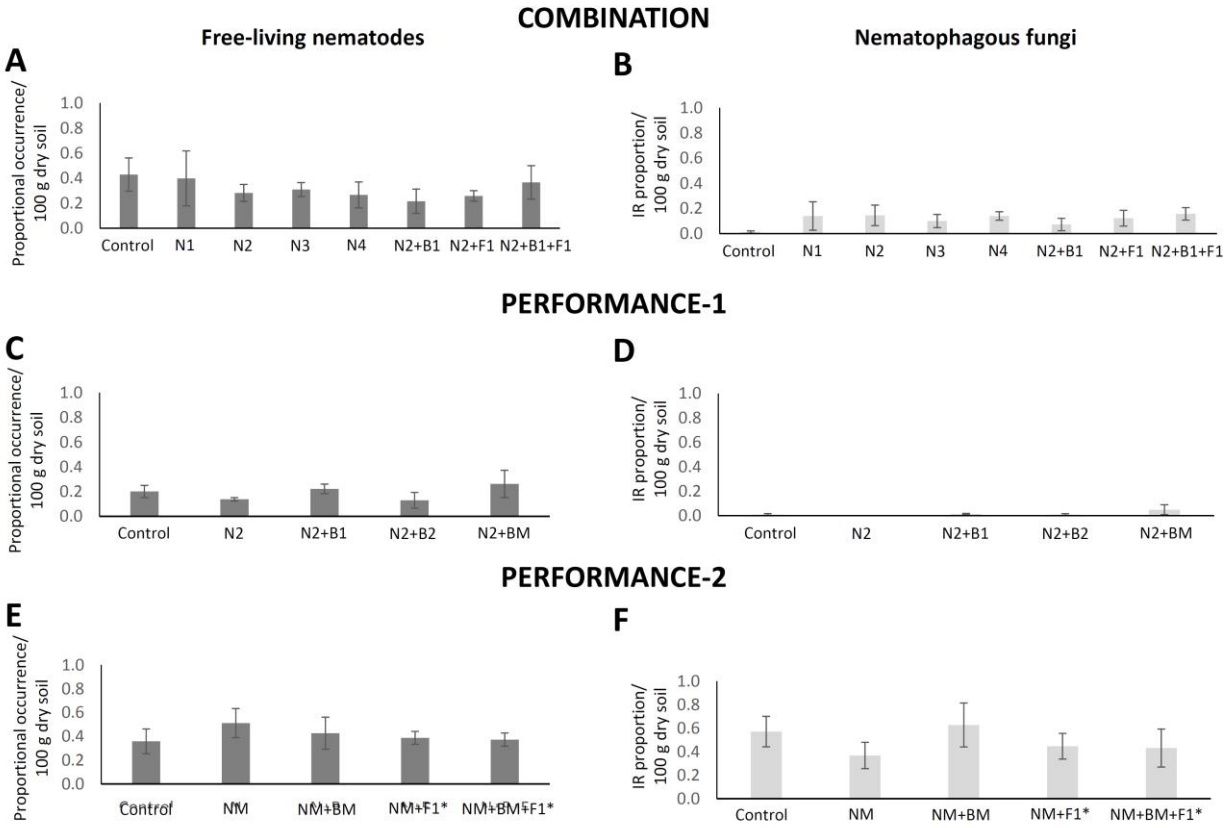


FIGURE S2 | Natural occurrence of free living nematodes (A, C and E) and nematophagous fungi (B, D, and F) in the three field experiments following application of entomopathogenic nematodes. Treatments: No inoculants (control), *H. megidis* Andermatt (N1), *H. bacteriophora* Andermatt (N2), *S. carpocapsae* D83 (N3), *S. feltiae* RS5 (N4), *P. protegens* CHA0 (B1), *P. chloraphis* PCL1391 (B2), AMF *R. irregularis* strain INOQ Top (high dosage, F1-H), AMF *R. irregularis* strain INOQ TOP (low dosage, F1-L), *P. protegens* CHA0 + *P. chloraphis* PCL1391 (BM), *H. megidis* Andermatt + *H. bacteriophora* Andermatt + *S. feltiae* RS5 (NM). The parasitism of nematodes by nematophagous fungi was determined by dividing the DNA quantity of each species by the total amount of DNA and expressed as "infection rate" (Campos–Herrera et al., 2012; Duncan et al., 2013). To estimate the total free-living nematodes and nematophagous fungi, we standardized the units of measurement among species to be on a scale of 0 to 1, by dividing all data within a species by the highest measurement for that species (de Rooij van der Goes et al., 1995). Both free-living nematodes and nematophagous fungi were expressed per 100 g of soil ± SEM. For statistical analysis, see details in Table 4.

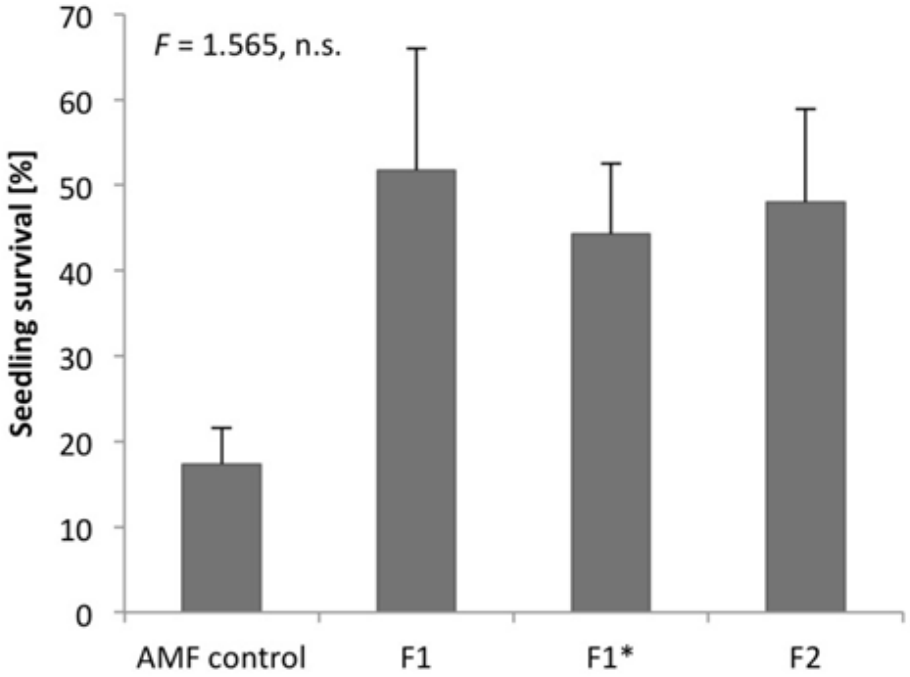


FIGURE S3 | Seedling survival after frit fly attack in the small AMF plots of the COMBINATION experiment. Treatments: AMF-free carrier (AMF control), AMF *Rhizoglomus irregularis* strain INOQ Top (high dosage, F1-H), AMF *Rhizoglomus irregularis* strain INOQ TOP (low dosage, F1-L) and AMF *Rhizoglomus irregularis* strain SAF22 (F2). Values presenting the mean (± SEM) plant density in the plots in percentages were analysed with one-way ANOVA (significance $P < 0.05$, n.s. not significant).

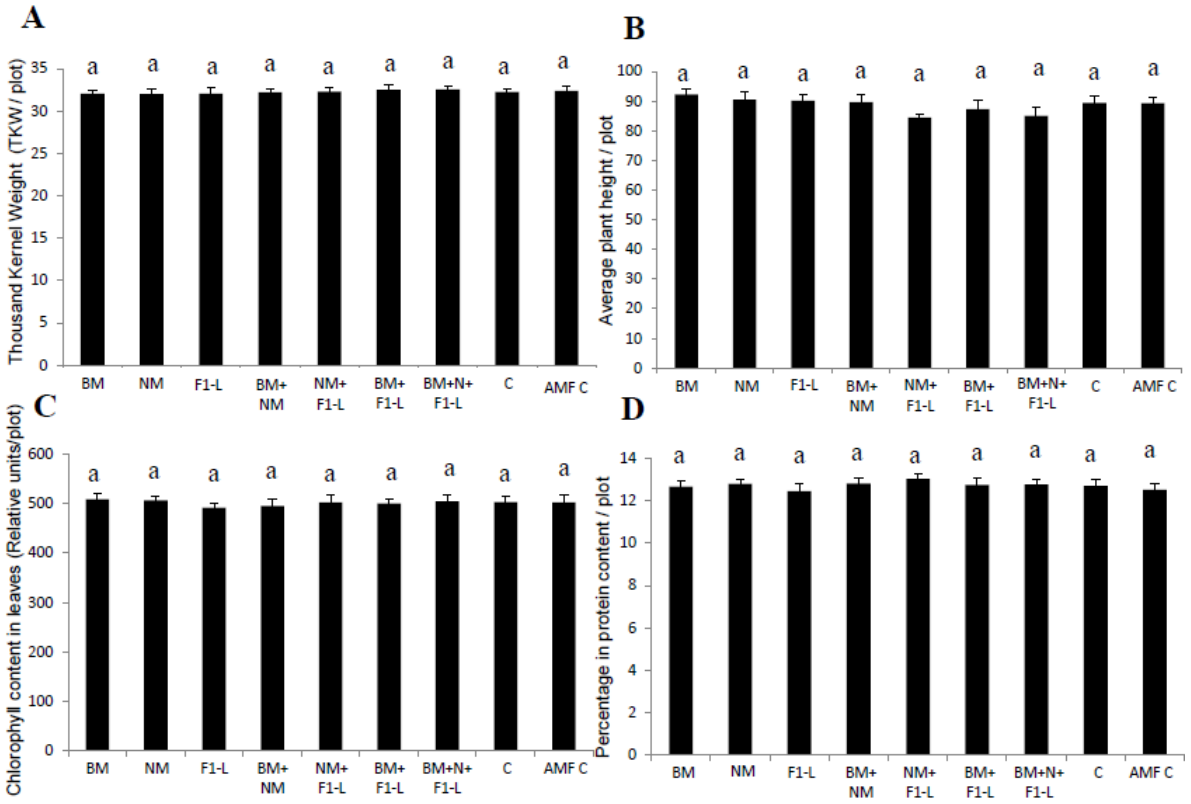


FIGURE S4 | Additional plant traits measured in the PERFORMANCE-2 experiment. Thousand kernel weight (TKW, expressed in g^{-1} per plot) values were determined by measuring the weight of wheat seeds in each plot (**A**). Plant height was measured from soil surface to the top of wheat plants, using a metre. Data are expressed as average plant height per plot (**B**). The chlorophyll content in wheat leaves was measured using a N-tester. Such data are strongly correlated with the state of nitrogen nutrition if the plant. Data are given in relative units per plot (**C**). Protein content in wheat plants were determined by infrared spectroscopy, and results are expressed in percentages per plot (**D**).

-APENDIX-

Biocontrol of larvae of the banded cucumber beetle *Diabrotica balteata* (Coleoptera: Chrysomelidae) with combinations of entomopathogenic nematodes and pseudomonads root-colonizing bacteria

Xavier Chiriboga M.[1], *Raquel Campos-Herrera*[2], *Caroline Pons*[3] and *Marie-Jeanne Tschudi*[1]

University of Neuchatel, Switzerland[1]
University of Algarve, MeditBio, Portugal[2]
University of Bielefeld, Germany[3]

ABSTRACT

Insect root feeders are difficult to control due to their cryptic habits, which limit that control methods can reach them in the soil. Certain species of root-associated bacteria have been shown to possess oral insecticidal activity against lepidopteran insect larvae in experimental infections at laboratory level. Here I present a first experiment that aimed to demonstrate the pathogenic capacity of pseudomonads root-colonizing bacteria in naturalized conditions against an insect root feeder. In addition, because entomopathogenic nematodes (EPN) are a successful biological alternative to control belowground feeders, we also tested if the combination of entomopathogenic nematodes and root-associated bacteria enhance the control of the larvae of the root feeder *Diabrotica balteta*. Overall, the EPN *Heterorhabditis bacteriophora* were found to be more virulent than the bacteria *Pseudomonas protegens* CHA0 against *D. balteata*. When both were combined, bacteria may display antagonism against the EPN and/or its enteric bacteria. In contrast, the combination of *Pseudomonas chlororaphis* plus *Steinernema feltiae* enhanced larvae mortality compared to bacteria treatment alone. This opens questions about potential mechanisms of this effect on larvae mortality, to improvement strategies of biological control of belowground insect feeders. Future research can establish the best combination of certain soil beneficial organisms such as pseudomonads bacteria and EPN species.

INTRODUCTION

Soil harbors a great variety of organisms from macro and mesofauna such as earthworms and nematodes to microfauna such as bacteria and fungi (Hol et al., 2013). Microbial communities associated with roots are composed of tens of thousands of species and constitute a key determinant of plant health and productivity (Berendsen et al. 2012). Such is their importance that the plant microbiome has been considered as the second genome of the plant (Berendsen et al., 2012). Furthermore, increasing evidence has acknowledged the fact that rhizosphere microbial communities affect the plant and vice versa (Berendsen et al., 2012; Turner et al., 2013). In addition to direct effects on deleterious microbes on the rhizosphere (e.g.: competition for micronutrients, production of antibiotic compounds or enzymes, consumption of pathogen stimulatory compounds), many beneficial soil borne microorganism (e.g.: *Pseudomonas* spp.) have been found to possess insecticidal activity against several insect herbivore species (Ruffner et al., 2013). The species *Pseudomonas protegens* CHA0 is a root-associated bacteria that not only produce antifungal metabolites but also is able to produce a protein that is very similar to the potent insect toxin Mcf1of the entomopathogen *Photorhabdus luminescens* (γ-Proteobacteria: Enterorbacteriaceae) (Péchy-Tarr et al., 2008).

The entomopathogenic nematodes (EPNs) that are applied in pest control comprise the families Steinernematidae (genus *Steinernema* and *Neosteinernema*) and Heterorhabditidae (genus *Heterorhabditis*). They have lethal effects on insect pests, resulting from their association with a mutualistic enteric γ–Proteobacteria in the genera *Xenorhabdus* and *Photorhabdus*, respectively (Boemare, 2002). The third stage infective juvenile (IJ) is the free-living stage naturally occurring in the soil, which is in charge of searching and penetrating potential hosts. Once inside the host, they release the symbiotic bacteria, which contribute together with the nematode to kill the host (Boemare, 2002). The bacteria proliferate rapidly, nematodes feed on the degraded host tissues and bacteria, develop and reproduce, until food is depleted and the excretion products are a limiting factor (San-Blas et al. 2008). Due to their shared characteristics with predators/parasitoids and microbial pathogens, and the facility of large-scale culturing, EPNs are suitable organisms for biological control of several insect pests, especially root feeders (Lacey et al., 2015).

Several studies on the behavior and ecology of EPN have been carried out during the past decades to optimize their use as efficient biocontrol agents (Lewis et al., 2006, 2015; Griffin, 2015). In this study, I tested the efficiency of EPN and pseudomonads root bacteria in individual treatments and combinations for biocontrol *Diabrotica balteata* (Coleoptera: Chrysomelidae) larvae in controlled naturalized conditions (soil + plant setting). To our knowledge this is the first time that virulence of *Pseudomonas* spp. is tested against a belowground insect feeder. I hypothesized that combinations of EPN and root colonizing bacteria species can act synergistically to produce mortality in the insect feeder *D. balteta*.

MATERIALS AND METHODS

Plants. Squash plants (*Cucurbita pepo* L.) Bio Zucchetti "Fruhbusch" (SELECT, Switzerland) were sown in a mixture (1:1) of sterilized potting soil (Terreau semis Capito, LANDI-Switzerland) and sand (MIGROS, Switzerland). Seedlings were watered with sterile water as required and kept at greenhouse conditions (22 ºC, 50% Relative humidity). After 3 weeks each seedling was transplanted in one clean plastic pot (12.5 cm x 16.5 cm, diam.) with additional sterilized potting mixture, that served as experimental units.

Insects. Second instar *D. balteata* larvae were reared from eggs provided by Syngenta (Stein, Switzerland) and they were fed with maize seedlings in quarantine rearing facilities.

Entomopathogenic nematodes. Infective juveniles (IJs) of *Heterorhabditis bacteriophora* (Andermatt) and *Steinernema feltiae* (Sf-5) were cultured in *Galleria melonella* (Lepidoptera: Pyralidae) (Au Pecheur, Switzerland) larvae in laboratory conditions. Suspensions of IJs, no older than 2 weeks from emergence, were adjusted to inoculate each plant at a rate of 50 IJs / cm^2 in 200 ml of sterile water. Suspensions were maintained at 11°C until inoculation.

Bacteria. *P. protegens* CHA0-Rif and and *P. chloraphis* PCL1391-Rif (selected rifampicin resistant strains) were cultured in LB agar (Luria-Bertani broth, Miller, Sigma-Aldrich) containing 100 µg/ml of rifampicin (≥ 97% powder, Sigma-Aldrich), for 48 hours in 9 cm. diam. Petri dishes at 30 ºC. Bacteria were scratched from the plates under sterile conditions and transferred to 100 mL of sterile rifampicin supplemented-LB broth. Both species were cultivated independently in an orbital agitator (IKA- KS 4000) at 30 ºC and 190 rpm for 16 hours. Bacteria liquid cultures were centrifuged at 6846 x g for 10 minutes to separate bacteria pellet from the liquid culture media, which were diluted again in sterile distilled water. Standard bacteria concentrations (1 x 10^6 CFU) were obtained, calibrating the inoculum with a spectrophotometer at an optical density of 0.2A at 600 nm. Suspensions were maintained at 11°C until inoculation.

Inoculum. Two EPN species and two pseudomonads species were selected for the treatments. Combinations of EPN and *Pseudomonas* species were selected based on previous experiments in field conditions (Imperiali, Chiriboga et al., under review). Not all possible combinations were done due to limited inoculum production and space. Inoculation was done after transplanting with the following treatments: a) *P. protegens* CHA0-Rif, b) *P. protegens* CHA0-Rif + *H. bacteriophora*, c) *H. bacteriophora*, d) *P. chloraphis* PCL1391-Rif, e) *P. chloraphis* PCL1391-Rif + *S. feltiae*, f) *S. feltiae* and g) Control. Each treatment had 6 replicates (n = 6) and the experiment was done twice.

Experimental procedures. Combined inoculations treatments contained 200 ml of EPN suspension and 50 ml of bacterial inoculum, single EPN treatments contained 200 ml of EPN suspension and 50 ml of sterile water, individual *Pseudomonas* spp. treatments contained 50 ml of bacterial inoculum and 200 of sterile water. Control plants received 250 ml of sterile water.

Ten larvae were used to infest each squash plant (5 weeks old, after a period of 2 weeks of EPN inoculation and bacteria colonization) by burying them in the soil. Dead and living larvae were recovered after 3 days of insect infestation and kept 3 additional days in humid chambers to verify infection.

Colonization of squash roots with *P. protegens* CHA0 or *P. chloraphis* PCL1391 was verified with same plants used in the experiment after *D. balteata* larvae mortality evaluation. Roots of inoculated plants were harvested, soil was gently removed and roots were weighed. Roots were suspended in flasks with 40 ml of sterile water. Flasks were shaken vigorously for 10 minutes to wash off all bacteria from the roots, serial dilutions of the washed roots were prepared and plated on rifampicin-LB agar Petri dishes. Plates were incubated at 30 ºC and after 24 h the numbers of colony-forming units (CFU) were counted and CFU / g of root calculated.

Activity of EPNs was verified by doing a baiting assay with 250 g of soil from each treatment. Five *G. melonella* larvae were put within plastic containers with soil from each treatment. Dead larvae from each container were recovered after 4 days, rinsed with tap water and transferred to White traps (White, 1927). Nematode emergence was checked after 5-7 days.

Statistical analysis. Mortality data were corrected with the Abbot method (Abbot, 1925). A statistical model showed that the variable "trial" was not significant, therefore we pooled data from the two experiments. Thereafter, differences among treatments were assessed by One-Way ANOVA in R 3.3.2. (2016) and differences between means were calculated by Tukey HSD method.

RESULTS

Larvae mortality. Overall, we found a marginally significant difference ($F_{5,66} = 3.4$, $P = 0.06$) in the percentage of mortality between treatments. Percentage of mortality of the EPN *H. bacteriophora* was significant higher comparing with efficiency of *P. protegens* CHAO. Moreover, percentage of mortality in the combination of *P. protegens* plus *H. bacteriophora* was slightly lower than mortality casued by the EPN alone. Similarly, mortality produced by *S.feltiae* was slightly lower than the mortality caused by *H. bacteriophora*. However, the combination of *P. chloraphis* PCL with *S. feltiae* produced a higher rate of mortality on larvae comparing with the effect of the single treatment with *P. chloraphis* PCL (Fig. 1).

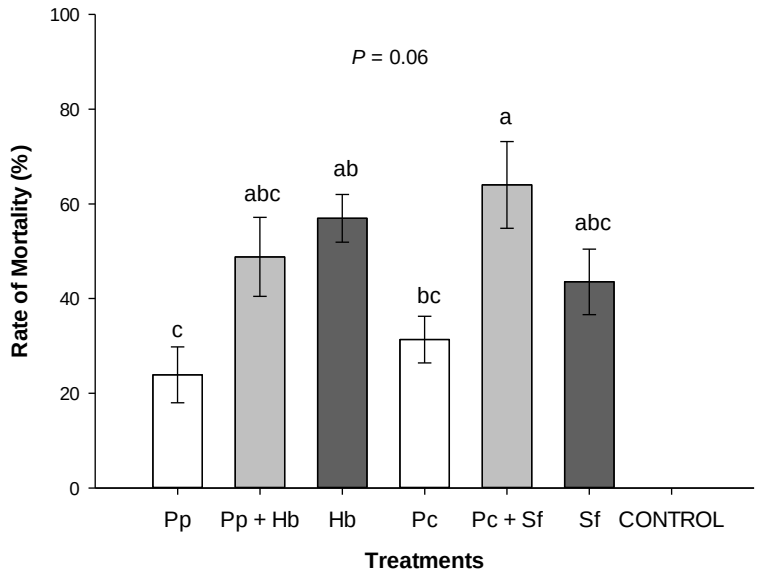


Figure 1. Rate of mortality (±SEM) of *Diabrotica balteata* larvae, values subjected to Abbot correction. One-Way ANOVA. Tukey-HSD Test 5%. Different letters show significant differences.

Root colonization. Roots were colonized by the bacterial inoculants and we did not find differences in root colonization by *P. protegens* CHA0-Rif and *P. chloraphis* PCL1391-Rif in squash plants between treatments (Table 1).

Table 1. Quantification of root colonization by *P. protegens* CHA0-Rif and *P. chloraphis* PCL1391-Rif. One-Way ANOVA ($F_{3, 12}$=3.75, P > 0.1)

Treatment	CFU / g root (MEAN ± SEM)
***P. protegens* CHA0-Rif**	$1.8 \times 10^6 \pm 0.4$ a
P. protegens* CHA0-Rif + *H. bacteriophora	$5.7 \times 10^5 \pm 0.3$ a
***P. chloraphis* PCL1391-Rif**	$1.4 \times 10^6 \pm 0.2$ a
P. chloraphis* PCL1391-Rif + *S. feltiae	$7.5 \times 10^6 \pm 0.6$ a

Nematode activity. Baiting with *G. melonella* larvae (at the end of the experiment) revealed 100% of nematode activity in the soils of all treatments in which we applied nematodes.

DISCUSSION AND CONCLUSIONS

Our experiment evaluated the biocontrol efficiency of *D. balteta* larvae with individual treatments of two species of EPNs, two species of pseudomonads bacteria and selected combinations of EPNs and bacteria species in potted plants. Control efficiency of *D. balteata* larvae by *S. feltiae* and *H. bacteriophora* ranged between 45% and 55%, respectively. Generally, *H. bacteriophora* display a

low penetration and infection rates in several insect species (Caroli et al., 1996); however, these characteristics are not necessarily related with low nematode efficacy (Ricci et al., 1996). Indeed, other studies have shown that *H. bacteriophora* is the most effective EPN against white grubs even in field conditions (Georgis and Gaugler, 1991). Despite the fact that *S. feltiae* has been shown to have a high penetration rate and produce fast invasions of insects (Ricci et al., 1996), control efficiency of *S. feltiae* was relatively low for the type of experiment we performed (closed environment and high EPN dosage, EPN do not have to disperse to find a host). We also cannot acknowledge an effect of EPN size and host size having impact on *D. balteata* infection and mortality. Indeed, *S. feltiae* is an EPN of medium size and *D. balteata* second instar larvae is not a micro-host (< 5 mm) (Bastidas et al. 2014). In our experiment, it is difficult to explain why *S. feltiae* tended to produce lower mortality than *H. megidis* in *D. balteata* in terms of foraging behavior. There is a lot of questioning around "ambusher" and "cruiser" theories. For example, some authors recognize that *H. megidis* tend to adopt a cruiser foraging strategy and *S. feltiae* behavior varies from cruising to ambushing. However, it has been acknowledged that the foraging behavior of EPN can change depending on the substrate in which they forage (Griffin, 2015). Our results may be explained by the poor quality inoculum of *S. feltiae* and/or the sensivity of IJs to detrimental environmental factors (i.e. dessication and UV radiation) (Gaugler and Boush, 1978). The fact that EPN produced in *G. melonella* larvae, were transferred to water suspensions before inoculum preparation and application may also have affected EPN performance and/or behavior. Indeed, EPN emerging directly from cadavers disperse more and are more infective than those that are applied in aqueous suspensions (Griffin, 2015).

The combination of the bacteria *P. protegens* CHA0-Rif with *H. bacteriophora* did not improve control efficiency of the EPN treatment. This result suggests that root colonization by *P. protegens* CHA0-Rif did not contribute to infect and/or kill *D. balteata* larvae during the 3 days of exposure to the pathogens. We found a slightly lower mortality of larvae in the combination *P. protegens* CHA0-Rif plus *H. bacteriophora*, this may suggest a potential antagonism of the bacteria on the EPN, probably mediated by the production of antibiotics. Thus, we can suggest that antibiotics produced by *P. protegens* CHA0 may have affected virulence of *H. bacteriophora* enteric bacteria *Photorhabdus*. We should also acknowledge the fact that infection process by *P. protegens* CHA0-Rif bacteria may be longer than the parasitization by the EPN, bacteria need to be ingested, the toxin released and bound, the gut wall broken to provoke the death of the insect in this experiment. In contrast, *Pieris brassicae* (Lepidoptera: Pieridae) feeding on Chinese cabbage leaves containing drops of a suspension of *P. protegens* CHA0, produced invasion of the insect blood system within less than one day after oral uptake (Ruffner et al., 2013). This suggest that pseudomonads bacteria may not display their pathogenicity potential when is colonizing roots. Indeed, there is evidence that their toxin is expressed only when the bacteria enter the insect gut, but not when growing on plant roots or common laboratory media. Thus, expression of their insect toxin is activated in a host-dependent manner (Pechy-Tarr et al., 2013).

The treatment with the EPN *S. feltiae* plus *P. chloraphis* PCL1391 resulted in increased mortality of *D. balteata* larvae, comparing with the single bacteria treatment, suggesting that these two entomopathogenic organisms tend to act in an additive manner. The mechanisms of this effect remain to be investigated. It is not known yet if this enhancing effect occurs in other root feeder-insect species. It is also necessary to test biocontrol potential of other EPN-pseudomonads bacteria combinations against root feeders. Consistently, for single bacteria treatments, in a different experiment with maize plants in smaller pots (Chapter 3, this thesis) *P. chloraphis* PCL1391-Rif produced 30% of mortality and *P. protegens* CHA0-Rif 20% after 72 hours of insect feeding.

In conclusion, we found that in the combination of *P. protegens* plus *H. bacteriophora* the bacteria may display an antagonism against the EPN and/or its enteric bacteria. The combination *P.*

chloraphis plus *S. feltiae* caused enhanced larvae mortality compared to bacteria treatment alone, which is worth to be studied in more detail. Unraveling the mechanisms of this effect would potentially improve biological treatments to control belowground insect feeders with certain EPN species and pseudomonads bacteria combinations.

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-CHAPTER 3-

Root-colonizing bacteria enhance the levels of (*E*)-β-caryophyllene produced by maize roots in response to rootworm feeding

Xavier Chiriboga M.[1], Huijuan Guo[1,2], Raquel Campos-Herrera[1,3], Gregory Röder[1], Nicola Imperiali[4], Christoph Keel[4], Monika Maurhofer[5], Ted C.J. Turlings[1]

[1] *Fundamental and Applied Research in Chemical Ecology Laboratory, University of Neuchâtel, Emile-Argand 11, Neuchâtel CH 2000 (Switzerland)*
[2] *State Key Laboratory of Integrated Management of Insect Pests and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijin 100101 (China)*
[3] Current address: *Centro para os Recursos Biológicos e Alimentos Mediterrânicos (MeditBio), Campus Gambelas, Edf. 8, FCT, Universidade do Algarve, 8005-139, Faro (Portugal)*
[4] *Department of Fundamental Microbiology, University of Lausanne (Switzerland)*
[5]*Plant Pathology, Institute of Integrative Biology, Swiss Federal Institute of Technology, Zurich (Switzerland)*

accepted by *Oecologia*

ABSTRACT

When larvae of rootworms feed on maize roots they induce the emission of the sesquiterpene (*E*)-β-caryophyllene (*E*βC). *E*βC is attractive to entomopathogenic nematodes, which parasitize and rapidly kill the larvae, thereby protecting the roots from further damage. Certain root-colonizing bacteria of the genus *Pseudomonas* also benefit plants by promoting growth, suppressing pathogens or inducing systemic resistance (ISR), and some strains also have insecticidal activity. It remains unknown how these bacteria influence the emissions of root volatiles. In this study, we evaluated how colonization by the growth-promoting and insecticidal bacteria *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391 affects the production of *E*βC upon feeding by larvae of the banded cucumber beetle, *Diabrotica balteata* Le Conte (Coleoptera: Chrysomelidae). Using chemical analysis and gene expression measurements, we found that *E*βC production and the expression of the *E*βC synthase gene (*tps*23) was enhanced in *Pseudomonas protegens* CHA0-colonized roots after 72 h of *D. balteata* feeding. Undamaged roots colonized by *Pseudomonas* spp. showed no measurable increase in *E*βC production, but a slight increase in *tps*23 expression. *Pseudomonas* colonization did not affect root biomass, but larvae that fed on roots colonized by *P. protegens* CHA0 tended to gain more weight than larvae that fed on roots colonized by *P. chlororaphis* PCL1391. Larvae mortality on *Pseudomonas* spp. colonized roots was slightly, but not significantly higher than non-colonized control roots. The observed enhanced production of *E*βC upon *Pseudomonas protegens* CHA0 colonization may be beneficial for entomopathogenic nematodes attractiveness but this still remains to be tested.

Key words: Root-colonizing bacteria, *Diabrotica balteata*, (*E*)-β-caryophyllene, terpene synthase, maize

INTRODUCTION

Insect-damaged roots emit volatile compounds that serve as attractants for the natural enemies of the damaging insects (Rasmann et al. 2005; Ali et al. 2010; Tonelli et al. 2016). The first such attractant was identified for maize roots, which respond to feeding by larvae of *Diabrotica virgifera virgifera* Le Conte (Coleoptera: Chrysomelidae) with the release of the sesquiterpene (*E*)-β-caryophyllene (*E*βC). This herbivore-induced volatile (HIPV) attracts entomopathogenic nematodes (EPN) and, thereby, helps to protect maize roots against further herbivore damage (Rasmann et al. 2005; Degenhardt et al. 2009). Although similar root signals have been studied for several other plants (Boff et al. 2001; Ali et al. 2011), it is still poorly understood how soil microorganisms affect their production or may respond to them.

Besides root herbivores, numerous other organisms that live in the rhizosphere form associations with plants. Their effects may be beneficial (e.g. mycorrhizal fungi, N-fixing bacteria) or detrimental (e.g. pathogenic fungi or bacteria) to plant performance (Brussaard 1998; Rasmann and Turlings, 2016). There is increasing interest in some strains of root-associated bacteria of the genus *Pseudomonas* that benefit plants by promoting plant growth, suppressing pathogens and/or inducing systemic plant defenses (van Oosten et al. 2008; Lugtenberg and Kamilova 2009; Kupferschmied et al. 2013). Recent studies have also revealed that specific *Pseudomonas* strains possess insecticidal activity against several insect herbivore species (Ruffner et al. 2013). It has been suggested that natural isolates of *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* (γ-Proteobacteria: Pseudomonaceae) have a high potential to be applied as plant protection products. Since they are adapted to live on plant roots, show environmental persistence and are competitive root colonizers, they may be ideal not only to enhance plant growth, but also to control insects pests (Lugtenberg and Kamilova 2009; Kupferschmied et al. 2013). The current study is an interdisciplinary effort to explore potential synergies in applying combinations of plant beneficial soil organisms (http://www.nrp68.ch/en).

Studies measuring the effects of root-associated bacteria on volatile organic compounds have been limited to aboveground volatiles (Ballhorn et al. 2013; Pineda et al., 2013; Pangesti et al., 2015a) and the reported effects are greatly contrasting, depending on the insect herbivore attacking the plants. We are aware of only one study that looked at the effects of root-colonizing bacteria on root-produced HIPVs. Santos et al. (2014) found that maize root colonization by *Azospirillum brasilense* (α-Proteobacteria: Rhodospirillaceae) produced higher amounts of *E*βC compared to non-colonized maize roots, in this case without insect damage. They further found that larvae of the generalist root feeder *Diabrotica speciosa* (Coleoptera: Chrysomelidae) oriented preferentially towards non-inoculated maize roots *versus* inoculated roots and gained less weight when feeding on inoculated roots.

It remains unknown how root-associated bacteria affect the induction of belowground volatiles in response to root herbivory. This prompted this study in which we investigated these effects in maize roots damaged by larvae of another generalist, the banded cucumber beetle *Diabrotica balteata* Le Conte (Coleoptera: Chrysomelidae). *D. balteata* larvae induce lesser amounts of *E*βC in maize roots than *D. virgifera* larvae, but this still results in some attraction of EPN (Rasmann and Turlings 2008). *D. balteata* is an important agricultural pest in Central and North America (Capinera 2011), attacking a broad spectrum of crops, including maize (Saba, 1970; Chittenden, 1992; Capinera, 2011). It may damage all parts of a plant, but the most serious injury caused by *D. balteata* is to the roots (Capinera 2011). Enhancing *E*βC emissions in maize roots damaged by *D. balteata* might render EPN more effective in finding and killing the larvae of this

important generalist root pest. This is therefore a good model to test the possible effects of root-colonizing bacteria on *E*βC emissions.

We used a chemical and a molecular approach to evaluate the effects of maize root colonization by the bacterium *P. protegens* CHA0 and *P. chlororaphis* PCL1391 on the production of *E*βC. Roots were inoculated (or not) by one of the bacteria and infested or not by *D. balteata* larvae, we then collected and analyzed volatiles produced by the roots and we measured the expression of the maize terpene synthase gene (*tps*23) (Köllner et al. 2008).

The species *P. protegens* CHA0 is a root-associated bacterium that not only produces antifungal metabolites, but also an insecticidal protein that is very similar to the insect toxin Mcf1 of the entomopathogen *Photorhabdus luminescens* (Péchy-Tarr et al., 2008). *P. protegens* CHA0 causes insect toxicity in experimental infections of aboveground feeding insect larvae (Péchy-Tarr et al., 2008) and also in feeding assays with artificial diets or leaves treated with the bacterium (Ruffner et al., 2013). It is unknown how these root-associated bacteria affect root feeding insect larvae, we therefore studied the effect of *Pseudomonas* spp. on the performance and mortality of *D. balteata* larvae.

We tested if colonization by *P. protegens* CHA0 or *P. chlororaphis* PCL1391: i) induces a change in the production of *E*βC after *D. balteata* attack in maize roots, ii) changes the expression of the gene *tps*23, iii) affects root growth in maize plants, and iv) affects the weight gain and mortality of *D. balteata* larvae. We discuss our results in terms of the physiological changes that may occur in plants upon *Pseudomonas* colonization and how these changes may influence HIPVs. We suggest possibility of applying bacteria in combination with EPN for the effective control of diabroticine beetle larvae in maize and other crops.

MATERIALS AND METHODS

Soil, plants and insect larvae

A substrate containing potting soil (Terreau semis Capito, Landi-Switzerland, pH = 5.8-6.8) and white sand (Migros, Switzerland) in proportion 1:1 (v/v) was used to grow the plants. The substrate was autoclaved twice at 120 ºC for 120 min. Plastic pots (11 cm, height x 4 cm, diameter) were autoclaved once at 120 ºC for 120 min before each sowing.

Maize seeds (var. Delprim and var. F268) were surface sterilized by washing them with ethanol 70% for 2 min and sodium hypochlorite 3% for 2 min and rinsing them with sterile water. Plants were watered with 20 mL of sterile distilled water every 2-3 days. Plants were grown either in a greenhouse (30±5 ºC, 8:16 h dark:light photoperiod) in summer or in a phytotron (30±2 ºC, 8:16 h dark:light photoperiod, 300 µmol m^{-2} s^{-1}, CLF Plant Climatics, Germany) in winter.

Second instar larvae of *D. balteata* were reared from eggs provided by Syngenta (Stein, Switzerland) and they were fed with maize germinate. Larvae were used to infest 11 days old maize plants (after a period of 6 days of roots colonization by bacteria), by burying them (at 1-1.5 cm) in small holes in the soil. Each plant was infested with six *D. balteata* larvae.

Bacteria cultures and inoculation

The bacteria *P. protegens* CHA0 and *P. chlororaphis* PCL1391 (Department of Fundamental Microbiology, University of Lausanne) were cultured in LB agar (Miller, Sigma-Aldrich) supplemented with 100 µg/mL of rifampicin (≥ 97% powder, Sigma-Aldrich) for 48 h in 9 cm diam. Petri dishes at 30 ºC. Bacteria were scratched from the plates under sterile conditions and transferred to 100 mL of sterile rifampicin supplemented-LB broth. Both species were cultivated independently in an orbital agitator (IKA-KS 4000) at 30 ºC and 190 rpm for 16 h. Bacterial cultures were then centrifuged at 6846 x g for 10 mi to separate bacterial cells from the liquid culture media. Resulting bacterial cell pellets were diluted again in sterile distilled water. Standard bacteria concentrations (1 x 10^6 CFU ml^{-1}) were obtained, calibrating the inoculum with a spectrophotometer at an optical density of 0.2A at 600 nm.

At the shoot emergence stage (4-5 days after sowing) , plants were selected for the application of different treatments: a) inoculated with *P. protegens* CHA0, and infested with *D. balteata* (CHA0+Db), b) inoculated with *P. chlororaphis* PCL1391, and infested with *D. balteata* (PCL+Db), c) not inoculated with bacteria, infested with *D. balteata* (Db), d) control healthy plants (Healthy), e) only inoculated with *P. protegens* CHA0 (CHA0), and f) only inoculated with *P. chlororaphis* PCL1391 (PCL). Plants treated with root-colonizing bacteria were inoculated with 20 mL of *P. protegens* CHA0 or *P. chlororaphis* PCL1391 inoculum prepared as described above. Plants infested only with *D. balteata* and control-healthy were watered with 20 mL of sterile distilled water. Preliminary experiments were performed before, measuring production of *E*βC after 72 h of insect feeding in the maize inbreed line F268, with six replicates per treatment (n = 6). Nine replicates (n = 9) per treatment were done in a final time-course experiment. Plants of different treatments were kept separated in different plastic trays to avoid cross-contamination and kept either in a greenhouse or a phytotron for 6 days during the root colonization period.

Colonization of maize roots with *P. protegens* CHA0 or *P. chlororaphis* PCL1391 was verified for a subset of plants of the same batch used for the volatiles and gene expression analysis. For this, roots of inoculated plants were harvested and the soil was gently removed with the palm of the hand and roots were weighed. Then the roots were suspended in flasks with 40 mL of sterile water and the flasks were shaken vigorously for 10 min to wash off the bacteria from the roots. Serial dilutions of the washed roots were prepared and plated on rifampicin-LB agar Petri dishes. Plates were incubated at 30 ºC and after 24 h the numbers of colony-forming units (CFU) were counted and CFU per gram of root calculated.

Volatile extraction and analyses

In preliminary experiments, we analyzed volatiles produced by the whole root system after 72 h of *D. balteata* infestation, whereas in the final time-course experiment, we standardized the amount of ground root sample per vial for volatile analysis. We quantified the amount of *E*βC produced by roots of maize plants var. Delprim after 6 and 72 h of insect infestation.

Roots were harvested and washed gently with tap water 6 and 72 h after insect infestation and immediately frozen in liquid nitrogen for grinding. Roots were ground in a frozen mortar with liquid nitrogen. Root volatiles were extracted following the standard procedure by Rasmann et al (2005): 500 mg of ground root material were transferred to 10-mL glass vials sealed with a Teflon-

coated septum and stored at -80 ºC for analysis. A 100 µm polydimethylsiloxane SPME fiber (Supelco, Sigma-Aldrich Chemie SA, Buchs, Switzerland) was inserted through the septum and exposed in the headspace for 60 min at 40 ºC. The compounds adsorbed onto the fiber were analyzed with an Agilent 7890a Series GC system coupled to mass-selective detector (Agilent 5975c, transfer line 280 ºC, source 230 ºC, quadrupole 150 ºC, ionization potential 70 eV) (Palo Alto CA, USA). The fiber was inserted into the injector port (250 ºC), desorbed and the volatile compounds were separated on a non-polar column (HP1-MS; 30 m, 0.25 mm internal diameter, 0.25 mm film thickness; J & W Scientific, Agilent Technologies SA, Basel, Switzerland). Helium at a constant flow mode of 0.9 mL min^{-1} was used as a carrier gas. After fiber insertion, the column temperature was maintained at 50 ºC for 3 min, then increased to 180 ºC at 5 ºC min^{-1}, before a final ramp at 8 ºC min^{-1} to reach 250 ºC (hold 3 min). Chromatograms processing were carried out with ChemStation software (Agilent Technologies SA, Basel, Switzerland). Relative abundance of the root volatiles was calculated by integrating peaks and values were corrected for sample weight to calculate relative abundance of the volatile per gram of root.

cDNA synthesis and gene expression analysis

Approximately 60 mg of ground root material was used for the analysis of Zm-*tps*23 gene expression. RNA from roots was extracted using the Isolate II RNA Plant Kit (Bioline, Germany), and RNA concentration was determined using a Nanodrop (Control Program ND-1000 v.3.3.0., ThermoScientific, Wilmington, DE). cDNA was synthetized using Sunscript RT RNAse H+ (Bioline, Germany). Real-time qPCR was performed in 100-well gene discs reaction plates (Biolabo, Scientific Instruments, Switzerland) in the Corbett Research real-time qPCR using Zm-*tps*23 specific primers (F: GTGGGCCTCTACCTATCCA, R: CTGTGGTGGTGCCGTATTT) and Zm-*actin* specific primers (F: CAGTGGTCGAACAACGGGTA, R: GGTAAGGTCACGACCAGCAA) as a reference gene (Köllner et al. 2008). The qPCR mix was adjusted to a final volume of 10 µL, using RNA-free water, specific primers (either for *tps*23 or for *actin* detection) both forward and reverse (0.05 µM) and SYBR Green (Bioline, Germany) and 1 µL of DNA template. Negative control contained free RNAase water instead of DNA template, to verify there is not contamination in the reactions. A qPCR analysis was carried out using the following thermal cycling conditions: a hold at 95 ºC for 10 min and 40 cycles, at 95 ºC for 10 s and at 60 ºC for 45 s. Relative expressions of the genes *tps*23 and *actin* for different treatments were obtained using the correction method $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

Assessment of larvae weight gain and mortality

For this evaluation, we used the same set of plants that we used for volatile extraction in the time-course experiment. We weighed *D. balteata* larvae (Mettler Toledo MX5 microbalance) before placing them on the plants and we recorded weight gain of the larvae after 6, 48 and 72 h of feeding. We also recorded the number of dead larvae per treated plant.

Statistical analysis

Relative abundance of volatiles per gram of root values (*E*βC) were normalized prior statistical analysis with square root transformation. Transformed data from each time-point was analyzed separately with One-way ANOVA model. Data of the relative expression of the terpene synthase gene were subject of the same transformation and analyzed with One-way ANOVA.

Tukey method ($P < 0.05$) was used to compare Least Square Means of treatments in both cases and t-test was used to compare differences between time-points. Root-biomass data was analyzed with One-way ANOVA. Percentage of larvae weight gain were analyzed with Two-Way ANOVA, with treatment and time of feeding as variables. Mortality data were subjected to arcsin transformation and analyzed with Two-way ANOVA, with treatment and time of feeding as variables. All data were analyzed using R 3.3.2. (2016). Data is presented as mean ± SEM of untransformed values.

RESULTS

Maize root colonization by *Pseudomonas* spp. and production of (*E*)-β-caryophyllene after *Diabrotica balteata* damage

The root colonization by *Pseudomonas* spp. was similar for all bacterial treatments ($F_{3,12} = 1.43$, $P = 0.35$) (Table 1).

Table 1. Quantification of root colonization by *P. protegens* CHA0 and *P. chloraphis* PCL1391 in different treatments

Treatment	C.F.U. / g of root (±SEM)
P. protegens* CHA0 + *D.balteata	$(5.7 \times \pm 0.20) \times 10^7$ a
P. chloraphis* PCL + *D.balteata	$(1.3 \times \pm 0.07) \times 10^8$ a
***P. protegens* CHA0**	$(2.4 \times \pm 1.70) \times 10^8$ a
***P. chloraphis* PCL**	$(3.5 \times \pm 0.65) \times 10^7$ a
Control healthy	0

Our preliminary experiments, in which we analyzed the roots from two maize genotypes (var. Delprim and inbred line F268), showed a trend of higher production of *E*βC in response to *D. balteata* feeding on *Pseudomonas*-colonized roots as compared to non-colonized roots (72 h post-attack) (Supplementary Fig.1a and 1b). However, variability within the treatments was high and no significant differences were detected.

The subsequent experiments showed that the production of *E*βC in maize roots was affected by treatment after 6 h ($F_{5,42} = 9.12$, $P < 0.001$) and 72 h ($F_{5,42} = 10.8$, $P < 0.001$) of insect feeding (Fig. 1). After 6 h, there was no difference between *D. balteata*-damaged roots colonized by any of the bacteria species ($P = 0.32$ and $P = 0.38$) and non-colonized roots attacked by the insects. There was a difference in *E*βC quantities between non-colonized roots attacked by the insects and undamaged roots colonized by any of the bacteria species ($P < 0.001$) (Fig. 1).

Seventy two hours after *D. balteata* attack, roots colonized by *P. protegens* CHA0 produced significantly larger amounts of *E*βC ($P = 0.001$) than non-colonized roots attacked by *D. balteata*, whereas roots colonized by *P. chlororaphis* PCL1391 produced similar ($P = 0.22$) amounts of *E*βC than non-colonized roots attacked by the insects. Non-colonized roots attacked by *D. balteata* produced slightly higher amounts of *E*βC than undamaged roots colonized by either bacterium (Fig. 1). We found a significant higher production of *E*βC ($P < 0.001$) after 72 h than after 6 h of insect

damage in roots colonized by *P. protegens* CHA0. For the other five treatments, there were no differences between the two time points (Fig. 1).

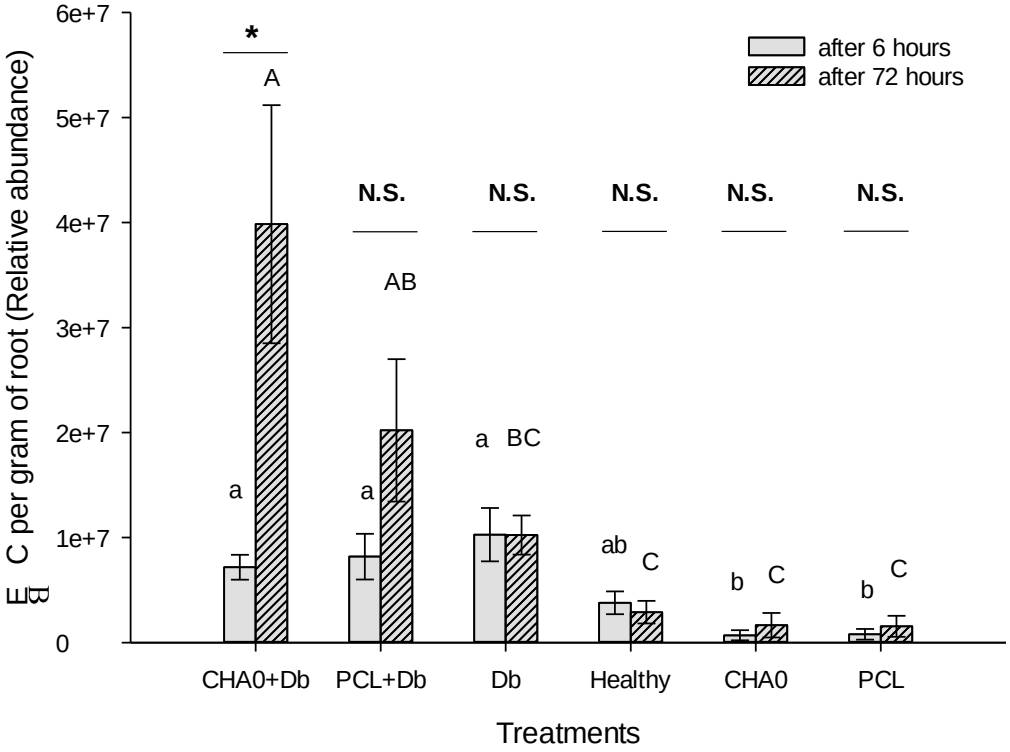


Fig. 1 Relative abundance of *E*βC (mean ± SE) released by maize roots *var.* Delprim after different treatments: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0+Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D.balteata* (PCL+Db), non-inoculated plants infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), (n = 9). Abundance of *E*βC was analyzed separately for each time-point with One-way ANOVA. Differences between means of the treatments were compared with Tukey tests ($P < 0.05$) and differences between time-points with t-test. Lower case letters indicate significant differences between treatments after 6 hours of feeding. Capital letters indicate significant differences between treatments after 72 hours of feeding. Stars indicate significant differences between times. * $P < 0.05$ N.S. indicate no significance

Expression of the terpene synthase gene *tps*23 after *Diabrotica balteata* damage in maize roots colonized by *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391

The treatments also affected the expression of *tps*23 (after 6 h: $F_{5,32} = 4.41$, $P = 0.003$; after 72 h: $F_{5,23} = 18.32$, $P < 0.001$). After 6 h of insect feeding, the expression of the gene *tps*23 in roots colonized either by *P. protegens* CHA0 or *P. chlororaphis* PCL1391 and attacked by *D. balteata* was not different from non-colonized roots attacked by the insect ($P = 0.83$ and $P = 0.75$) (Fig. 2).

We also found a significant higher expression of the gene in non-colonized roots attacked by the insect than ($P = 0.001$) than in healthy control roots (Fig. 2).

After 72 h of *D. balteata* attack, gene expression in insect-damaged roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 was significantly higher ($P < 0.001$ and $P = 0.001$) than in insect-damaged non-colonized roots (Fig. 2). The expression in the latter roots was not different from the expression in undamaged roots colonized by either one of the bacteria species ($P = 0.99$ and $P = 0.33$). Similarly to what found for the release of *E*βC (Fig. 1), *tps*23 expression was significantly higher ($P < 0.001$) after 72 h of insect attack than after 6 h in insect-damaged roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 (Fig. 2). In all of the other four treatments, gene expression was not statistically different between the two time-points.

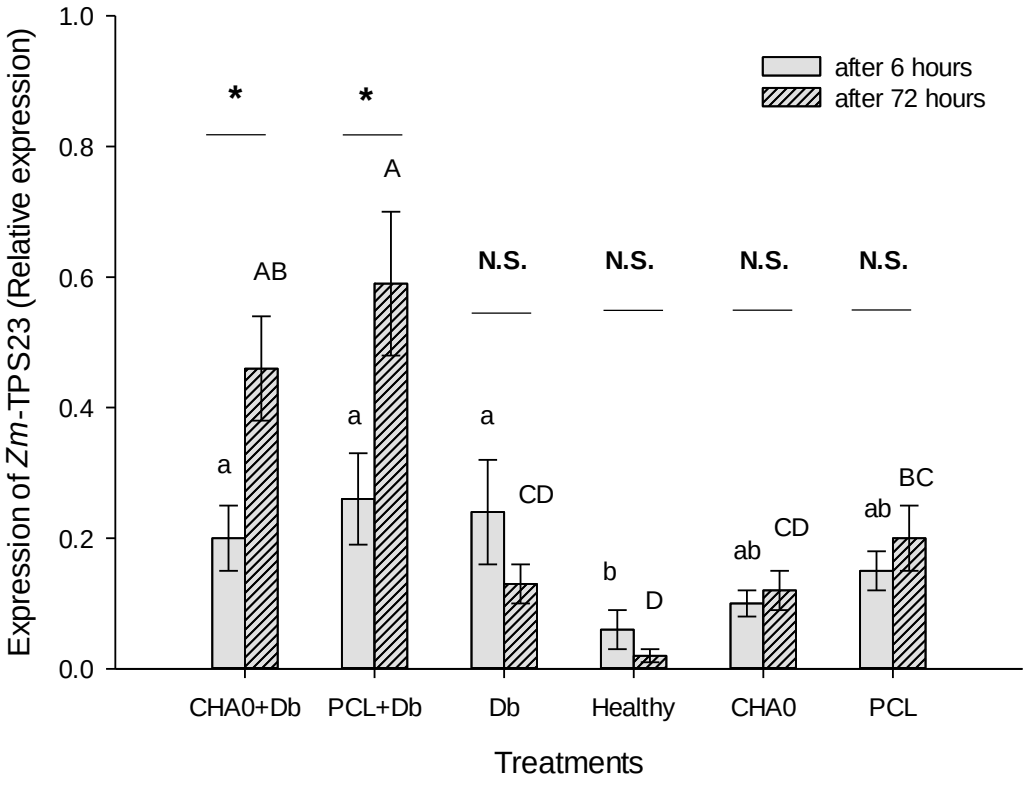


Fig. 2 Relative expression (calculated in relation to actin relative expression) of the terpene synthase gene *Zm*-tps23 (mean ± SE) in maize roots *var.* Delprim after treatments: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0+Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D. balteata* (PCL+Db), non-inoculated plants infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), ($n = 9$). Expression of the *tps*23 gene was analyzed separately for each time-point with One-way ANOVA. Differences between means of the treatments were compared with Tukey tests ($P < 0.05$) and differences between time-points with t-test. Lower case letters indicate significant differences between treatments after 6 hours of feeding. Capital letters indicate significant differences between treatments after 72 hours of feeding. Stars indicate significant differences between times. * $P < 0.05$ N.S. indicate no significance

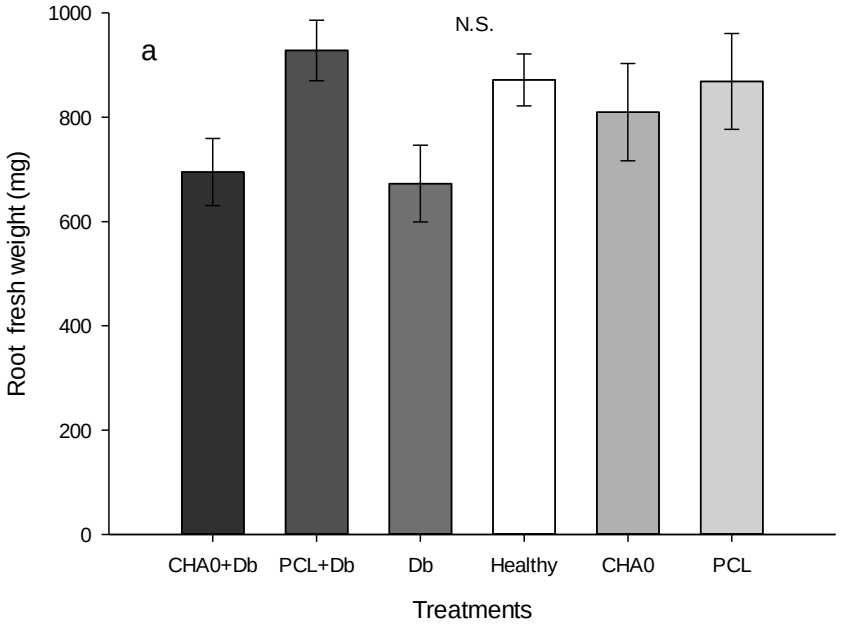
Root colonization does not change root biomass

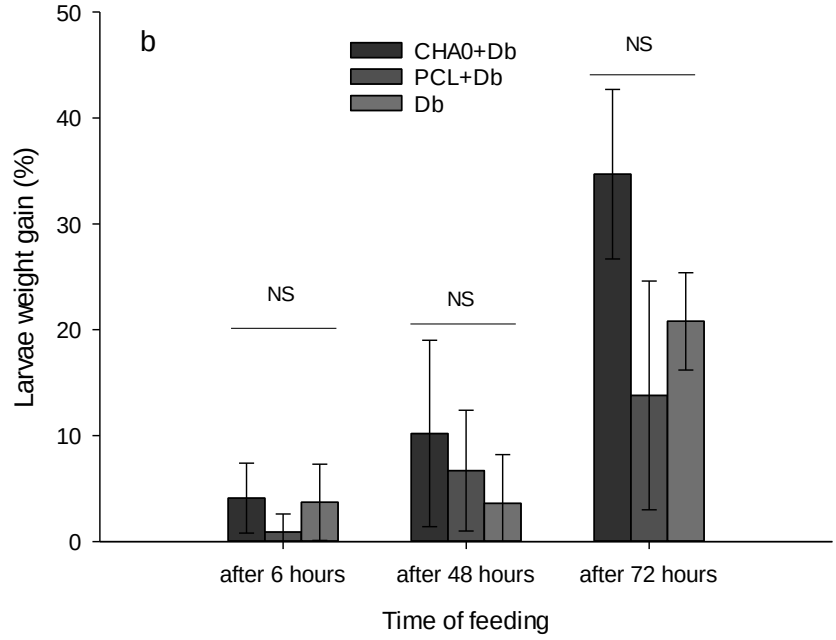
We did not find an effect of any of the treatments on root fresh weight ($F_{5,72}$ = 1.95, P = 0.09), measured after the 72 h of *D. balteata* feeding (Fig. 3a). However, there was a trend that biomass of insect-damaged roots was higher for plants colonized by *P. chlororaphis* PCL1391 as compared to the insect-damaged roots grown in presence of *P. protegens* CHA0 or in absence of bacterial inoculants.

Effects of bacterial colonization on the weight gain and mortality of *Diabrotica balteata* larvae

Although none effect of treatment was found for larval weight gain ($F_{2,72}$ = 1.72, P = 0.18), there was a trend towards increased weight gain when larvae were feeding on *P. protegens* CHA0 colonized roots than when feeding on *P. chlororaphis* PCL1391-colonized roots (Fig. 3b), and this correlates with differences in root biomass (Fig. 3a). We found an overall increase in weight over time ($F_{2,72}$ = 8.59, P < 0.001) but no a significant interaction between time and treatment ($F_{4,72}$ = 0.72, P = 0.57). In a preliminary experiment with maize plants inbreed F268, we found a similar pattern of weight gain for *D. balteata* feeding on roots colonized by *P. protegens* CHA0, *P. chlororaphis* PCL1391 and non-colonized roots (Supplementary Fig. 2). In this experiment, we detected a significant effect of time ($F_{2,95}$ = 7.09, P = 0.001), but no obvious effect of the treatment ($F_{2,95}$ = 0.8, P = 0.44), nor an interaction between time and treatment ($F_{4,95}$ = 0.23, P = 0.92).

For the time-course experiment, we also found an effect of time on the mortality of *D. balteata* larvae ($F_{2,72}$ = 21.76, P < 0.001), but no effect of the treatment ($F_{2,72}$ = 2.03, P = 0.13), nor an interaction between time and treatment ($F_{4,72}$ = 0.98, P = 0.41) (Fig. 3c).





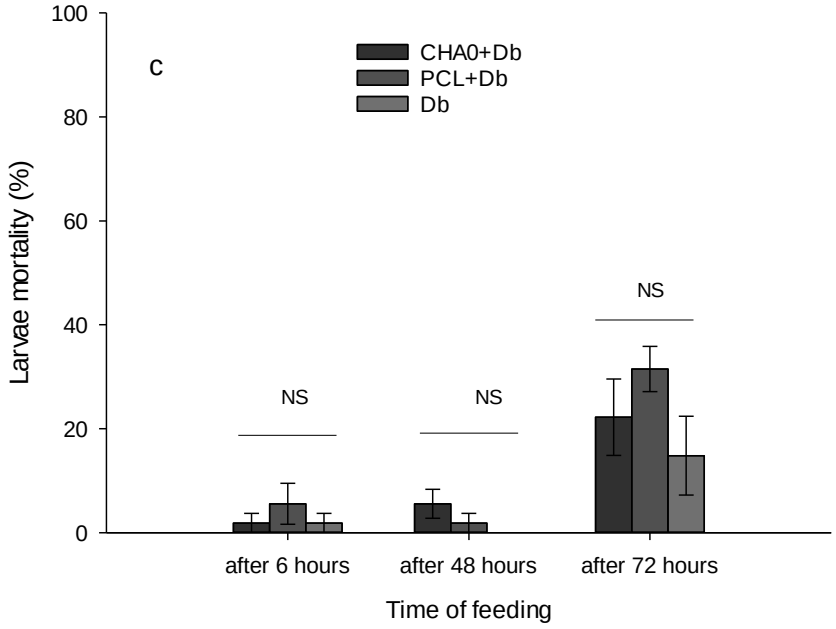


Fig.3a Root fresh weight (mean ± SE) of 14-days-old maize plants *var.* Delprim: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0+Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D. balteata* (PCL+Db), non-inoculated plants infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), (*n* = 12). Root weight was analyzed with One-way ANOVA. **b** Weight gain (percentage, mean ± SE) of *D. balteata* larvae after 6, 48 and 72 hours of feeding on maize roots *var*. Delprim with different treatments, (*n* = 9). Weight gain of larvae was analyzed with Two-way ANOVA. **c** Percentage of mortality of *D. balteata* larvae after 6, 48 and 72 hours of feeding on roots with different treatments, (*n*=9). Mortality of larvae was analyzed with Two-way ANOVA. Different letters show significant differences between treatments. N.S. no significance

DISCUSSION

We found differences in quantities of the root volatile *E*βC produced by different treatments. Although, our method does not measure the real emissions of *E*βC in the rhizosfere, it reflects the production of the sesquiterpene by maize roots. Other volatiles different than *E*βC were also detected in ground maize roots; however, the amounts of these volatiles were not contrasting between treatments.

Colonization by *P. protegens* CHA0 significantly enhanced the production of the sesquiterpene in maize after 72 h of *D. balteata* feeding (Fig. 1). Moreover, undamaged maize roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 bacteria produced only minor quantities of the root volatile *E*βC (Fig.1 and Supplementary Fig.1a and b). To our knowledge, ours is the first study that evaluates how root-associated bacteria affect the production of a belowground HIPV upon root herbivory. Yet, Santos et al. (2014), using the same maize variety (Delprim), showed that the plant-beneficial bacterium *Azospirillum brasilense* affects *E*βC emissions in plants but without insect damage. They found also that colonized roots released more *E*βC and repelled larvae of *Diabrotica speciosa*.

Other studies on how root-associated bacteria affect volatile emissions have focused on volatiles released from aboveground plant parts, and show contrasting results. Root colonization by pseudomonads can decrease (Pangesti et al. 2015a) or increase (Pineda et al., 2013) aboveground HIPVs. For example, *Arabidopsis thaliana* plants colonized by *Pseudomonas fluorescens* WCS417r and subsequently attacked by *Mamestra brassicae* caterpillars, produced lower amounts of methyl salicylate, lilial and the terpene (*E*)-α-bergamotene in comparison with non-colonized plants infested with caterpillars (Pangesti et al., 2015a). In contrast, Pineda et al. (2013) showed with the same plant-bacteria system, but using the aphid *Myzus persicae* as herbivore, that the aphid-induced production of eight leaf volatiles (2-nonenal, isovaleric acid, dimethyl sulfoxide, 2-cyclopente-1-one, (*R*)-verbenone, (*E*)-2-heptanal, 1-pentanol and 5,5 dimethyl-2(5H)-furanone) was enhanced in soil bacteria-colonized plants compared with non-colonized plants. Hence, effects of root colonizing bacteria on inducible volatiles appear to vary strongly, depending on the plants species, root-associated bacteria and on the insect herbivores.

These differences can be explained by the different hormonal pathways that are activated by different plant antagonists. Chewing insects and necrotrophic pathogens typically induced the jasmonic acid pathway, whereas phloem-feeding insects and biotrophic pathogens usually upregulate the salicylic acid pathway (Zarate et al. 2006; Thaler et al., 2012; Jacobs et al. 2011; Pieterse et al. 2012). This is also a possible explanation for the results found by Ballhorn et al. (2013), who compared volatile emissions by rhizobia-colonized lime bean plants after experimental induction with jasmonic acid. Colonized plants produced higher amounts of shikimic acid-derived compounds than non-colonized plants, whereas the emission of compounds produced via the octadecanoid, mevalonate and non-mevalonate pathways was reduced.

Our findings on *E*βC emissions correlate nicely with the results for the expression of the terpene synthase gene *tps*23. In roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391, the expression was enhanced after 72 h of *D. balteata* infestation in comparison with non-colonized roots attacked by the insect (Fig. 2). Interestingly, we also found a higher expression of the gene *tps*23 in undamaged roots colonized by *P. chlororaphis* PCL1391 than in control healthy roots at the second time-point (72 h). This is again different from Pangesti et al. (2015a), who reported a negative effect of *P. fluorescens* colonization on the expression of the terpene

synthase genes *tps*03 and *tps*04 in *Arabidopsis* upon insect leaf herbivory. These contrasting results confirm, as mentioned above, that the effects of root-associated bacteria on volatile emissions may vary depending on the system under study.

Inducible plant defenses, including volatile emissions, are mediated by wound-induced jasmonic acid (JA), which is derived from the lipoxygenase (*LOX*) pathway (Turner et al. 2002; Schmelz et al. 2003; Maffei et al. 2011; Dudareva et al, 2013). Previous studies found that *Pseudomonas* colonization of *A. thaliana* plants promotes the expression of the gene *lox*2 (Pineda et al. 2012) and JA-responsive genes (van Oosten et al. 2008), and results in stronger JA-signaling after insect attack (Pangesti et al., 2015b). We also know that the gene Zm-*tps*23 is locally and systemically induced in maize roots in response to feeding by *D. virgifera*. This appears to be triggered by local induction of jasmonic acid (JA) and its isoleucine conjugate (JA-Ile) after 30 min, resulting in an exponentially increasing production of *E*βC over 48 h of feeding (Erb 2009; Hiltpold et al. 2011). Taking all together, we can hypothesize that belowground enhanced production of *E*βC in maize roots colonized by *P. protegens* CHA0 might be mediated by increased JA-signaling (Erb 2009; Hiltpold et al. 2011)

Pangesti et al. (2015b) point out that differences in soil composition may explain some of the variable outcomes of plant-mediated effects of root-associated microbes on volatile signals and insect performance. It remains to be investigated if the effects of *P. protegens* CHA0 and *P. chlororaphis* PCL1391 on the enhanced production of the root sesquiterne *E*βC and/or expression of the gene *tps*23 are consistent in different types of soils. We previously showed the importance of studying the dynamics of *E*βC production and diffusion under different soil conditions (Chiriboga M. et al. 2017).

It has also been proposed that the effect of root-associated microbes on insect herbivores is different for specialist and generalist herbivores and for insects with different modes of feeding (Pineda et al., 2010). The latter authors expect a negative effect on generalist chewing insects and mesophyll feeders, and positive or neutral on specialist chewing insects and phloem feeders. The effects on herbivore performance are directly related to the activation of defensive responses in the plant, including the production of HIPVs.

In the study by Pineda et al. (2013), different volatiles were also produced in high quantities in plants colonized by *P. fluorescens* even without insect damage. It is also pertinent to focus on additional volatiles and/or non-volatile secondary metabolites (Walker et al., 2012) produced upon bacteria root-colonization by maize plants and by bacteria themselves (D'Alessandro et al., 2014), and on the effects of these compounds on the interactions with other soil organisms. Non-target effects on soils organisms need also to be evaluated (Ali et al., 2013). Indeed, numerous studies have shown that microbes trigger significant responses in terms of volatiles production in plants (Ballhorn et al. 2013; Pineda et al., 2013; Pangesti et al., 2015a; Fontana et al., 2009).

We did not observe a clear effect of any treatment on root biomass (Fig. 3a), but there was a trend of lower biomass for insect-damaged roots that were colonized by *P. protegens* CHA0 compared to insect-damaged roots colonized by *P. chlororaphis* PCL1391 (Fig. 3a). The relatively poorer performance of the larvae on PLC-colonized plants may have contributed to this trend (Fig. 3b). Indeed, *D. balteata* larvae feeding on maize roots colonized by *P. protegens* CHA0 tended to gain relatively more weight than larvae feeding on roots colonized by *P. chlororaphis* PCL1391 after 72 h of feeding. Possibly, the increased emissions of *E*βC on roots colonized by *P. protegens* CHA0 stimulated feeding and/or benefitted *D. balteata* weight gain. This has been shown for larvae of the maize specialist *D. virgifera*, which are attracted to *E*βC (Robert et al. 2012a) and perform better on already infested roots (Robert et al. 2012b). In sharp contrast, in a different study, larvae

of the generalist *D. speciosa* larvae gained less weight on and were less attracted to roots that produce increased amounts of *E*βC (Santos et al. 2014).

It is further possible that the differences in weight gain by feeding on roots with different treatments were due to differences in nutritional quality and/or biomass of the roots. Mutualistic microorganisms are known to influence plant tolerance to herbivory (Strauss and Agrawal 1999). *Diabrotica* feeding also triggers tolerance responses, including regrowth of roots and resource reallocation in maize (Erb, 2009). Thus, it would be worthwhile to determine if PCL1391-colonization has an effect on these responses.

There were no significant differences in mortality among treatments (Fig. 3c), but there was a trend for higher mortality in larvae feeding 72 h on *P. chlororaphis* PCL-treated plants. If we had let the larvae feed longer this might have resulted in clearer effects, as pathogenicity of *Pseudomonas* bacteria can be rather a long process that involves several steps: bacteria ingestion, release of the toxin, toxin binding, breaking of the gut wall and insect death (Kupferschmied et al. 2013, Keel 2016). The observed enhanced signaling ability and relatively higher larval mortality on *Pseudomonas*-colonized roots imply that the application of the bacteria in combination with EPNs may be an effective strategy for the control of root herbivores in maize crop. The compatibility of soil bacteria with a EPN was confirmed in a field study, in which two species of *Pseudomonas* in combination with the EPN *Heterorhabitis bacteriophora* were found to be best in enhancing wheat plant performance (Imperiali-Chiriboga et al, 2017). How the application of such combinations plays out against *Diabrotica* pests under realistic field condition remains to be determined.

CONCLUSIONS

Colonization of maize roots by *P. protegens* CHA0 was found to enhance the production of *E*βC after 72 h of feeding by *D. balteata* larvae. Consistent with this enhanced production of the EPN attractant, we found a higher expression of the terpene synthase gene Zm-*tps*23 after 72 h of insect infestation in colonized roots. The gene expression data revealed a positive effect of both *Pseudomonas* strains. Undamaged roots colonized by *P. chlororaphis* PCL1391 also had a slightly enhanced expression of the terpene synthase gene. The mechanisms that are involved in this enhanced production of *E*βC are still unclear. The same is true for the observed differences in larval growth and mortality on roots of the different treatments. The application of beneficial pseudomonads bacteria and EPN appears to be promising, yet it remains to be tested if root-colonization by bacteria affects EPN attraction towards insect-damaged maize roots.

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AUTHOR CONTRIBUTION STATEMENT

XCM, HG, TCJT and R.C.-H. conceived the experiments, XCM and RC-H analyzed the data and wrote the paper, NI and GR provide technical assistance for microbiology techniques and GC-MS analysis, respectively. CK, MM and TCJT edit the text and approve the paper for publication.

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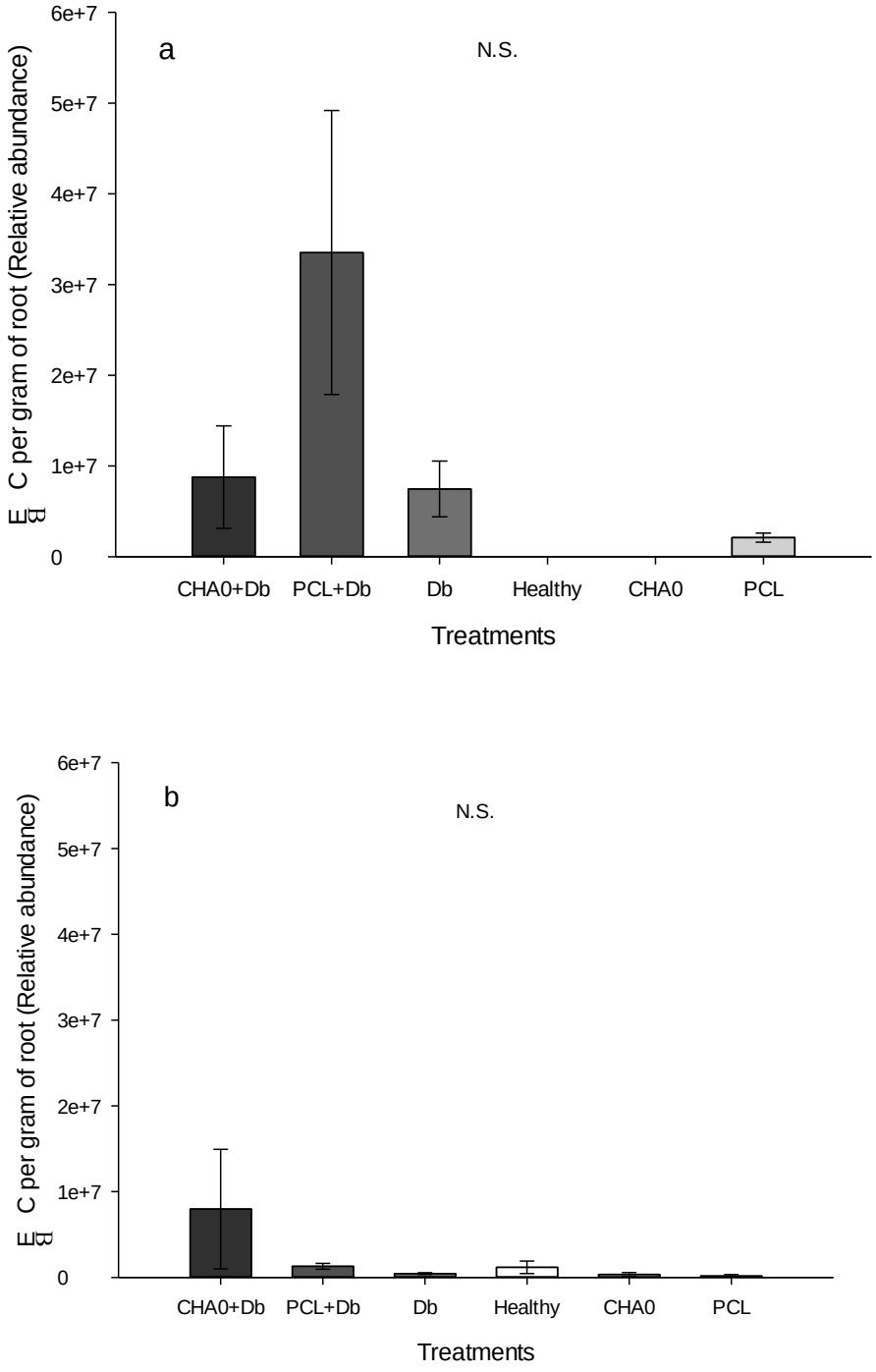
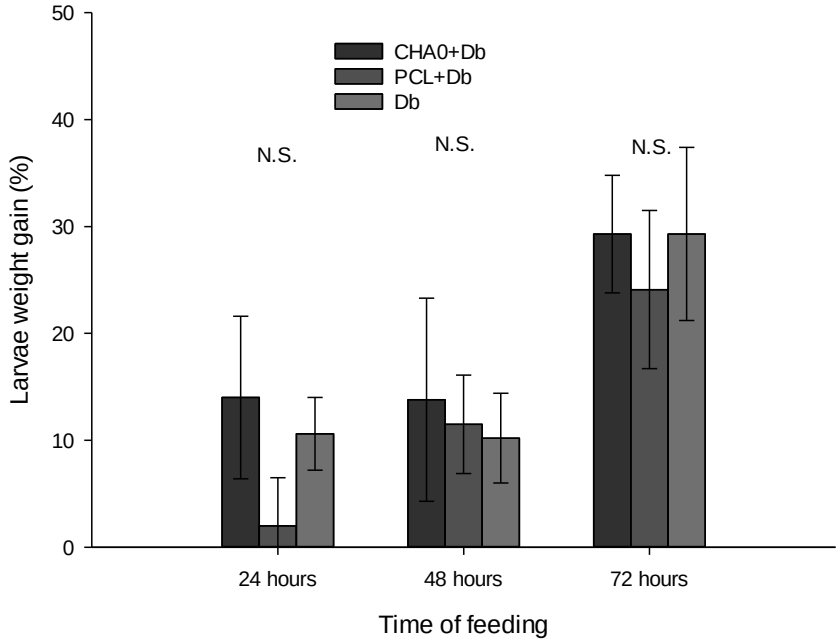
SUPPLEMENTARY DATA



Supplementary Fig. 1 Relative abundance of EβC (mean±SEM) released by maize roots after different treatments: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0+Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D. balteata* (PCL+Db), infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL); (n = 6). Abundance of *E*βC was analyzed with One-way ANOVA. **a** maize *var*. Delprim, **b** maize *var.* F268. Treatments (CHA0 and healthy) in which EβC was not detected were excluded from the analysis. N.S. indicate no significance



Supplementary Fig. 2 Weight gain (percentage, mean ± SEM) of *D. balteata* larvae after 6, 48 and 72 hours of feeding on maize roots *var.* F268 with different treatments: inoculated with *P. protegens* CHA0 (CHA0), inoculated with *P. chlororaphis* PCL1391 (PCL) and control non-inoculated plants (Db), (n = 12). Weight gain of larvae was analyzed with Two-way ANOVA. N.S. indicate no significance

FINAL DISCUSSION, CONCLUSIONS AND OUTLOOKS

In this thesis, I have focused on understanding the biotic and abiotic factors that affect the emission and function of the root-maize signal (*E*)-β-caryophyllene (*E*βc), which may serve as an indirect defense against root feeders by attracting entomopathogenic nematodes (EPN). Furthermore, I evaluated the possibility of applying EPN and other beneficial soil organisms to enhance wheat crop performance under realistic field conditions, tracing their persistence after augmentation and assessing plant traits related to growth and yield.

(*E*)-β-caryophyllene: function and application

The sesquiterpene *E*βc is found in many plants (Maffei et al. 2011) and is emitted by several plant organs such as flowers (Knudsen at al., 2006), leaves (Degen et al., 2004; Köllner et al., 2008) and roots (Rasmann et al., 2005). The release of this compound is induced by insect herbivory and is part of the volatile blend that is attractive to parasitic wasps when lepidopteran larvae feed on maize leaves (Köllner et al., 2008) and to entomopathogenic nematodes (EPNs) when beetle larvae attack maize roots (Rasmann et al, 2005). However, the release of herbivore-induced plant volatiles (HIPVs) is likely to have evolved first to serve other functions than may have gradually evolved into communication signals between organisms (Steiger et al. 2011), initially to benefit the emitter. For instance, over evolutionary time *E*βc may first have functioned as a direct defense compound to intoxicate and deter herbivores, or as antibiotic against pathogens, and indeed it is still effective as such against many antagonists (Huang et al. 2012).

*E*βc is a versatile compound that can readily diffuse in the air and belowground, but the latter had only been studied in sand and not in the complex soil matrix (Hiltpold and Turlings 2008). Using agricultural soils, we could show that *E*βc can also diffuse in soils, but less efficiently and only at relatively high moisture levels. We found good diffusion at a 20% moisture (common for most cropping conditions), independent of soil texture. However, at 10% water content, diffusion was reduced and differed for different soil textures, with reduced *E*βc movement in soils with high clay content (Chapter 1). *E*βc is a non-polar compound that dissolves poorly in water and it can diffuse through the gaseous phase of the soil, which is probably why in sand it moves best under dry conditions (Hiltpold and Turlings 2008). However, in real soil, organic matter may interfere with diffusion due to its strong adsorption properties (Steinberg and Kreamer 1993), and water may limit adsorption of *E*βc by coating the soil particles (Ruiz et al. 1998). In contrast, sand has little affinity

for volatile compounds and with its high porosity it allows for rapid diffusion under dry conditions. Therefore, we suggest that a balance between soil pores, adsorption particles and water retention capacity is needed for an ideal diffusion of *E*βc in real soils and this balance can be found in clay loam soils. This was supported by the results from experiments in soil-filled glass trays. In these trays the recruitment of the EPN *H. megidis* by plants that produce *E*βc was found to be more efficient in clay loam soils than in any other soil types (Chapter 1). Interestingly, the first field test that showed that EPN are attracted to *E*βc was done in a clay loam soil (Rasmann, 2006). Finally, experiments with dispensers containing synthetic *E*βc did not result in an enhanced ability of *H. megidis* to infect sentinel host (*G. melonella*) larvae (Chapter 1).

Maize lines from North America have lost the capacity to produce *E*βc, but it is still produced by lines of European breeding programs (Degen et al., 2004; Rasmann et al., 2005; Köllner et al., 2008). Genetic transformation of maize plants to restore this ability resulted in the constitutive emission of *E*βc and α-humulene and was found to enhance EPN attraction and the protection that the nematodes offer against rootworm damage (Degenhardt et al., 2009). However, the transformation compromises seed germination, plant growth and yield and also makes the plants attractive to the aboveground pest *Spodoptera frugiperda* (Robert, 2012a). An additional drawback of *E*βc emissions is that *D. virgifera* larvae are also attracted to *E*βc and use the signal to aggregate on a root system (Robert et al. 2012b). A constitutive release of *E*βc is therefore undesirable, but making it inducible by insect attack might still be a way to optimize the use of EPN as biocontrol, especially if the emissions are high enough to repel rootworm larvae. This might be achieved by inserting an herbivore-inducible promotor in front of the *E*βc synthase gene (Robert, 2012b). In addition, habitat management strategies, as further discussed below, may be optimized to enhance the presence of beneficial soil organisms and thereby ensure the control of roots feeders.

Outlook:

1. High water content, correspondent to field capacity of several soils types, favored *E*βc diffusion. It remains to be investigated how other factors that characterize soils such as pH, organic matter content and porosity affect the diffusion of *E*βc.

2. There is a great diversity of EPN species and each one may display its own foraging behavior and each species may adapt its foraging strategy to specific soil conditions. It would be interesting to test whether other EPN species (different from *H. megidis* and *H.*

bacteriophora) are attracted to *E*βc emitting roots and might have potential to improve biocontrol efficiency of Diabroticine larvae in field conditions.

3. HIPV not only affect the attraction of insect herbivores and their natural enemies, but also other members of the associated soil food web, such as kleptoparasites, and competitors. It is important to also evaluate if these organisms also respond to *E*βc. If so, this may potentially have negative consequences for the effectiveness of the biocontrol agents.

Combined application of beneficial soil organisms in wheat cropping systems

Soil microorganisms, in particular *Pseudomonas* bacteria and arbuscular mychorrizal fungi (AMF), are already being used in agriculture to boost plant performance and to control pests and diseases (Berg, 2009, Lerh 2010). Entomopathogenic nematodes (EPN) are also successfully employed in a number of crops to control certain insect pests (Georgis et al. 2006). Usually, only one of these beneficial soil organisms is applied on field crops and often with success (Berg, 2009; Kupferschmied et al., 2013; Lacey et al., 2015; Campos-Herrera, 2015). Combined inoculation of soil with multiple beneficial organism (BeSO) is less common and has had contrasting results in terms of benefits for plants (Frey-Klett et al., 2007; Walker et al., 2011; Couillerot et al., 2012; Lemanceau et al., 1991; Fogliano et al., 2002; Ansari et al., 2010; Tarasco et al., 2011). In a series of three field trials we showed that single applications of Pseudomonas bacteria, EPN and AMF, the prevalence of organisms depended on the species (Chapter 2). For example, for EPN the species that most persisted and had higher activity in the soil after application was *Steinernema feltiae*. In the case of bacteria, *Pseudomonas protegens* CHA0 was the strain that persisted best after 4 months of application. A high dosage of the commercial AMF strains of *Rhizoglomus irregulare* INOQ-Top and SAF 22 showed the most successful colonization. The EPN *H. bacteriophora* tended to persist better when combined with *P. protegens* CHA0 and the AMF *R. irregulare*. Also, *Pseudomonas protegens* CHA0 persisted best in combination with *H. bacteriophora*. In combined treatments, the success of AMF augmentation was variable. Overall, we observed that: 1) the introduced BeSO persisted until the end of the cropping seasons, although populations declined considerably with time, 2) the introduced BeSO showed no cross-contamination between plots, 3) augmented BeSO (temporarily) displaced the original community of soil organisms depending on strain/population and dosage, 4) the combination of *Pseudomonas protegens* CHA0 and *P. chloraphis* PCL and their combination with *H. bacteriophora* significantly benefitted wheat plants (seed survival) and combination of the three BeSo benefitted yield only when the plants were

attacked by frit flies, and 5) tripartite combinations did not provide beneficial effects on wheat performance compared to the individual applications of BeSO.

Overall, our results are in agreement with the results from previous studies, in which combinations of BeSO were found to have beneficial effects under certain conditions (Frey-Klett et al., 2007), and in most cases had similar effects as single applications (Tarasco et al., 2011). Under field conditions a number of abiotic factors such as: soil chemistry, physical soil properties and climate can strongly affect the impact of BeSO inoculants on plants and their persistence in the soil (Ritz & van der Putten, 2012). BeSO inoculants are also subjected to interactions with the resident soil organism community, and the latter can contribute to buffering, masking and suppressing beneficial effects of inoculations (Lee and Pankhurst, 1992). Thus, selection of a suitable BeSO should take into account their biology, ecology and biogeographical distribution. Indeed, the augmented BeSO species that naturally occurred in our experimental field persisted better than those that were not present in the original community. As we only found positive effects of *Pseudomonas* spp. combined with *Heterorhabditis megidis* on wheat yield when plants suffered from frit fly attack, we suggest that positive effects of BeSO inoculants are more evident when plants are suffering from biotic stress or adverse abiotic conditions.

To further understand these interactions, we performed an additional laboratory study in which we applied pseudomonad bacteria and EPN together or separately in pots with squash plants infested by the rootworm *Diabrotica balteata* (APENDIX-Chapter 2). The combination of *P. protegens* plus *H. bacteriophora* did not cause a higher rate of rootworm mortality than the bacteria alone; moreover, a potential antagonism may be exerted by the bacteria against EPN and-or its enteric bacteria. In contrast, the combination *P. chloraphis* plus *S. feltiae* resulted in enhanced larval mortality comparing with bacteria alone. A good understanding of the mechanisms that are responsible for this effect may help to develop ways to improve biological treatments against soil pests.

Outlook:

1. To have efficient BeSO inoculants it is necessary to know which ecological conditions (e.g. soil, climatic factors and biotic factors) are the most suitable for each of them. Based on this knowledge, protocols can be designed to select the best BeSO strains and combinations for specific biogeographical areas.

2. The outcomes of studies on BeSO combinations are variable and context-dependent. Therefore, future studies should focus on obtaining more insight into how BeSO interact in the rhizosphere and how these interactions affect plant health and performance.

3. In a combination of the EPN *S. feltiae* and the root-colonizing bacteria *P. chlororaphis* we found enhanced effectiveness in the control of *D. balteata* larvae. This promising result warrants further studies to determine the combination of bacteria and EPN with the highest virulence against this and other insect root herbivores.

The effect of root colonization by *Pseudomonas* spp. on the release of *E*βc by maize roots

Plant roots are associated with a diverse microbial community, including soil-borne bacteria and fungi. These microbes can be beneficial or pathogenic to plants (Sugio et al. 2015). Recently it was discovered that certain strains of *Pseudomonas* spp. carry an insecticidal toxin that is able to kill lepidopteran insect larvae (Ruffner et al. 2013; Péchy-Tarr et al., 2008). In addition to directly affecting insect, the bacteria may also have an effect on the plant defensive chemistry. It is for instance known that root-associated bacteria affect the emissions of HIPV's in different and sometimes contrasting ways (Pineda et al. 2013; Pangesti et al., 2015b), depending on the plant, insect and bacterial species. Root colonization by soil bacteria may also alter the attractiveness of plants to insect herbivores. For example, the generalist root feeder *D. speciosa* are more attracted to non-inoculated maize roots than to maize roots inoculated with *Azospirillum brasilense*, which appears to be adaptive, as they gain less weight when feeding on inoculated roots (Santos et al. 2014).

This thesis addressed, for the first time, the question whether root-colonizing bacteria *Pseudomonas* spp. affect the release of *E*βc by maize roots, this sesquiterpene is produced by the roots in response to rootworm feeding and is attractive to EPN. It was found that *E*βc emissions induced by *D. balteta* feeding are enhanced when maize roots are colonized by *P. protegens* CHA0, this effect was absent in roots colonized by *P. chloraphis* PCL1391 (Chapter 3). The results from chemical analyses matched with measurements of the expression of the corresponding terpene synthase gene *Zm*-TPS23, which was higher in rootworm-damaged roots colonized by *P. protegens* CHA0, but also those colonized by *P. chloraphis* PCL1391 (Chapter 3). We suggest that that these changes in the chemistry of maize roots are the consequence of increased JA-signaling. We also found a slight production of *E*βc and expression of TPS23 in undamaged roots that were colonized by the *Pseudomonas* species.

We did not detect a clear impact of *Pseudomonas* spp. on rootworm larvae, but we found a lower overall biomass for roots colonized by *P. protegens* CHA0 and better weight gain of the insect larvae that fed on that roots; and the opposite for larvae that fed on, and roots that were colonized by, *P. chloraphis* PCL1391. We found no effect of *Pseudomonas*-colonization on larval mortality (Chapter 3), but *P. chloraphis* PCL in combination with the EPN *S. feltiae* showed a tendency to increase mortality of *D. balteta* larvae (APENDIX-Chapter 3).

It should be noted that soil quality may play an important role in the outcome of these kinds of studies. As mentioned by Biere and Bennett (2013), the protective effect of beneficial microbes against herbivory and their effect on tolerance to biotic or abiotic stresses appear to be most pronounced under the most stressful abiotic conditions. Pangesti et al. (2015a) also acknowledged the role of soil composition in relation to the variable outcomes of plant-mediated effects of root-associated microbes on insect herbivore performance. Interestingly, the plants of our experiments were grown in a relatively poor substrate, mix of sand and potting soil (1:1).

Our results suggest that the biocontrol of Diabroticine larvae with EPN to protect the roots of *E*βc emitting maize varieties, may be most effective in fields with the right soil type (Chapter 1) and could be enhanced by choosing the right species of root-colonizing bacteria (Chapter 3) (Fig 1.). Further habitat management strategies "belowground" can also be designed as proposed by Robert et al. (2012). In real field conditions, we can predict different possible outcomes in relation to EPN attraction and *Diabrotica* feeding preferences. Considering that *Diabrotica* larvae did not show a preference between bacteria-colonized and non-colonized plants, the enhanced release of *E*βc in *P. protegens* CHA0-colonized plants might increase the attraction of EPN towards *Diabrotica*-damaged maize roots, resulting possibly in enhanced biocontrol. If maize roots colonized by *P. protegens* CHA0 are as attractive to EPN as non-colonized roots, there may not be an enhanced control of the insect herbivore, but the plant may benefit from the presence of the root bacteria, because it could protect against soil-borne diseases, increased biomass and/or induced systemic resistance. We also should consider the possibility that *D. balteata* larvae might prefer to feed on roots colonized by *P. protegens* CHA0, resulting in increased damage to roots colonized by the bacteria, but also increased release of *E*βc signaling and increased attraction to EPN. Such highly attractive plants to both insect pest and biocontrol agent, could be employed as sentinel plants with a "pull" effect, whereby colonized plants lure the insect herbivore away from the main crop and attract EPN to kill the majority of the pest larvae. In the case of *P. chloraphis* PCL-colonized roots, they could be employed because *D. balteata* larvae perform poorly and/or feed less on colonized roots, resulting in reduced root damage.

However, considerably more research needs to be done to fully determine the potential of root colonization by *Pseudomonas* spp. for enhanced control of Diabroticine larvae in maize and other crops, either in combination with EPN or by other indirect effects on the insect herbivore larvae. For this, we propose to answer the following questions:

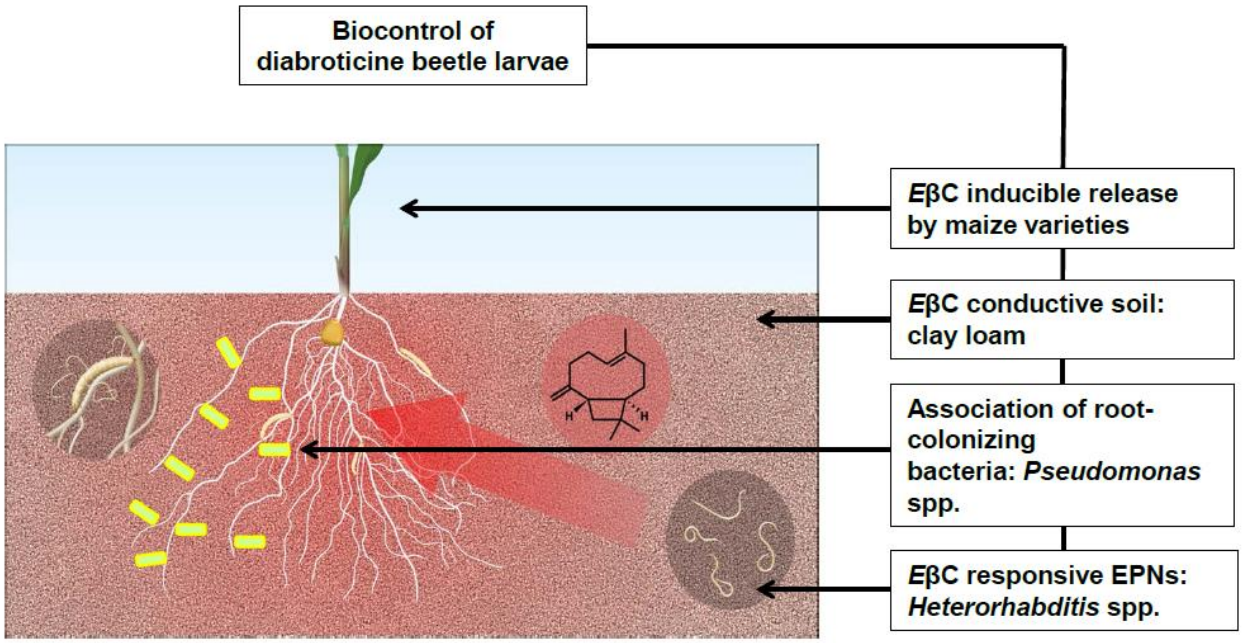


Fig. 1 A picture envisioning application of *E*βC knowledge: in maize cropping systems biocontrol of diabroticine beetle larvae may be achieved by matching cultivation in clay loam soils, association of root colonizing bacteria (*Pseudomonas* spp.) and *E*βC responsive strains of entomopathogenic nematodes (*Heterorhabditis* spp.).

Outlook:

1. Production of *E*βc and the expression of the terpene synthase Zm-TPS23 are likely to be triggered by jasmonic acid (JA). It remains to be investigated if enhanced production of *E*βc in roots colonized by *P. protegens* CHA0 and *P. chloraphis* PCL1391 is indeed the result of increased JA-signaling in the colonized plants.
2. Soil composition is expected to greatly influence how microbe-mediate enhanced volatile signaling in plants affects insect performance. Therefore, a key question to be answered is whether the effect of Pseudomonas root-colonizing bacteria on the emission of *E*βc is consistent in real soil conditions.

3. With our method we found the same volatiles, but in different amounts, in Pseudomonas-colonized roots and non-colonized root attacked by *D. balteata*. It would be interesting to find out if some (other) volatiles are produced by bacteria themselves upon root colonization and if these volatiles directly affect interactions with soil organisms (e.g. entomopathogenic nematodes and/or root herbivores).
4. We observed a trend for increased weight gain in larvae that fed on *P. protegens* CHA0 roots and for lower root biomass in these plants. The opposite was found for *P. chloraphis* PCL roots. To confirm and explain these effects it would be necessary to investigate whether there is a modification of the nutritional quality of colonized roots for the larvae. It would also be interesting and relevant for application to find out if potential changes in root biomass due to *Pseudomonas* colonization have any consequences for tolerance responses of maize plants to insect feeding.

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In the majority of "developing" countries, things are 50 years behind than in "developed" countries. Research is not an exception. Might it be the reason for which many students yearn to develop research topics needed in their regions and contribute -with their handwork- to build up the knowledge required to solve problems there?

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Chiriboga M., X, Campos-Herrera, R., Jaffuel, G., Roder, G. Turlings, T.C.J. 2017. Diffusion of the maize root signal (E)-β-caryophyllene in soils of different textures and the effects on the migration of the entomopathogenic nematode *Heterorhabditis megidis*. *Rhizosphere.* 3:53-59. http://dx.doi.org/10.1016/j.rhisph.2016.12.006

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Submitted or to be submitted for publication

Chiriboga M., X., Guo, H., Campos-Herrera R., Röder G., Imperiali N., Keel C., Maurhofer, M., Turlings, T.C.J. Root-colonizing bacteria enhance the levels of (E)-β-caryophyllene produced by maize roots in response to rootworm feeding. Submitted to *Oecologia*.

Imperiali, N[†], **Chiriboga, X.**[†], Schlaeppi K., Fesselet M.,Villacrés D., Jaffuel, G., Bender, F., Dennert F., Blanco-Pérez R., van der Heijden, M.G.A., Maurhofer, M., Mascher F., Turlings T.C.J., Keel, C., Campos-Herrera, R.. Combined field inoculations of *Pseudomonas* bacteria, arbuscular mycorrhizal fungi and entomopathogenic nematodes and their effects on wheat performance Submitted to *Frontiers in Plant Science*

Jaffuel, G., Blanco-Pérez, R., Hug, A.-S., **Chiriboga, X.**, Giulio, R., Mascher, F., Turlings, T.C.J. & Campos-Herrera, R. The evaluation of entomopathogenic nematode soil food webs in Swiss soils reveals major differences between agricultural and natural habitats. To be submitted to *Frontiers in Plant Science*

PRESENTATIONS

Poster presentations

Annual PhD Students Meeting — May, 2014
Neuchatel, CH

15th International Symposium on Insect-Plant Relationships (SIP 15)
Neuchatel, CH — Agoust, 2014

Symposium of the Zurich-Basel Plant Science Center — November, 2014
Zurich, CH

Annual Meeting of the International Society of Chemical Ecology (ISCE 2015),
Stockholm, SWD — June, 2015

16th International Symposium on Insect-Plant Relationships (SIP 16)
Tours, FR — July, 2017

Talk presentation

22th Reunión Latinoamericana del Maíz.
Quevedo, ECU — September, 2017

Meetings

AGRIDEA Meeting — November, 2013

AGRIDEA Meeting — Spring, 2015

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Xavier Chiriboga Morales was born in Quito, Ecuador. He got an engineer degree in Agricultural Sciences in the Army Polythecnic School (University of the Armed Forces) in 2003, with the thesis "Determination of the survival time of three antagonistic bacteria of *Moniliophtora roreri* in cocoa pods through serological techniques" presented in the XXVI Congreso Nacional de Control Biológico in Guadalajara (Mexico) in the same year. After working as an Assistant of Laboratories in the School of Agricultural Sciences of the Pontificia Universidad Católica del Ecuador-Sede Ibarra, he got a NUFFIC scholarship to pursue his master studies in Plant Sciences in Wageningen University and Research Center in the Netherlands. He chose the specialization Plant Pathology and Entomology, although his courses were focused in biological and ecological aspects of microrganims, viruses, nematodes and insects. There, he got to know interesting fields such as chemical ecology, behavioral ecology and several ecological-friendly insect pests management options that can be developed through research. He graduated with the thesis "Effects of plant nutritional quality on densities and sizes of herbivores, parasitoids and hyperparasitoids" in 2009. After a short intership in the International Center for Insect Physiology and Ecology in Kenya, where he investigated the feeding habits of cicadellids insect vectors, he returned to Ecuador for four years. Back in Ecuador he worked at the Instituto National de Investigaciones Agropecuarias, in the Estación Experimental Santo Domingo leading a research group in management of insects pests and diseases of oil palm, cocoa and passion fruit. Thereafter, he moved to the Laboratory of Entomology of AGROCALIDAD, where he was responsible of identifying insects pests and mites of quarentine importance. Despite having experience in agricultural entomology, he tries to expand his sight to the infinite biodiversity of entomofauna, moreover, he concerns about environmental issues and thinks that research & development must be oriented by socio-cultural frameworks. In 2013, he was awarded a scholarship of the Swiss government to develop his PhD project in the Fundamental and Applied Resarch in Chemical Ecology Laboratory in the University of Neuchatel. His PhD degree has paved his way to become an insect ecologist.