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Effects of biocontrol bacteria and earthworms on *Aphanomyces euteiches* root-rot and growth of peas (*Pisum sativum*) studied in a pot experiment

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ABSTRACT
The role of below-ground interactions between microbial biocontrol agents and soil fauna for combatting soil-borne plant diseases have not been studied sufficiently. This study tested the hypothesis that the beneficial bacterium *Bacillus velezensis* UCMB5113 and the anecic earthworm *Lumbricus terrestris* positively influence health and growth of peas (*Pisum sativum* L.) infested with the pathogen *Aphanomyces euteiches* causing root-rot disease. A greenhouse fully factorial experiment studied the effects of *A. euteches*, *B. velezensis* and *L. terrestris* on the emergence, growth and health of pea plants. The factors *B. velezensis* and *L. terrestris* resulted in taller plants (p = .003 and p = .030). *B. velezensis* treatment resulted in a higher biomass of shoots and roots ($p \leq .001$ and p = .005). The effects increased with the presence of both factors (p = .036). Earthworms reduced the disease symptoms significantly (p = .032). The decreased disease symptoms caused by the earthworms might be due to the consumption of *A. euteiches* (direct effect) as well as soil disturbance (indirect effect). Interactions between the microorganisms added and the earthworms were shown. *B. velezensis* and *L. terrestris* can be useful for enhancement of plant growth and for biological control of root-rot in peas.

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Bacillus; below-ground interaction; biological control; *Lumbricus terrestris*; plant disease

Introduction

Protection of crops against plant pathogens is of paramount importance since these organisms cause substantial yield loss worldwide. Protection against soil-borne plant pathogens is especially difficult since such organisms can seldom be effectively managed by use of chemical pesticides (Mihajlović et al. 2017). Soil-borne obligate fungal parasites are among the most difficult to target, as they do not grow outside their host plants and may produce long-lived spores.

Root-rot of legumes caused by the soil-borne obligate parasite *Aphanomyces euteiches* Drechs is the most devastating disease in peas globally and a major limiting factor in pea production (Heyman 2008). Disease symptoms begin with healthy white roots turning honey brown. In later stages, roots turn brown, the hypocotyl darkens at the soil line and eventually the plants wilt (Gaulin et al. 2007; Wu et al. 2018). *A. euteiches* is a filamentous plant pathogen belonging to the Oomycetes with both asexual and sexual stages. The oospores are sexual unicellular resting structures, which can remain viable in the soil for decades to withstand unfavourable conditions. Germination of oospores is triggered by root exudates from host plants. Oospores can form infective mycelia, but the formation of a short germ tube that releases high numbers of root-infecting asexual zoospores is more common. Upon germination and root penetration, a mycelium is formed inside the plant that releases new zoospores spawning a new generation of oospores in the rotting root tissue (Gaulin et al. 2007). The whole life cycle is completed within hours for zoospores and within a few days for oospores in a suitable host (Heyman 2008). The most secure management option to minimise root rot in pea is to avoid cultivation of susceptible (and alternative host) plants for several years since neither effective fungicides (with acceptable environmental impact) nor fully resistant germplasm are available (Gaulin et al. 2007; Hughes and Grau 2007; Wu et al. 2018). Genes associated with pea immunity have been identified, which can support breeding of disease-resistant pea cultivars (Hosseini et al. 2015). Good drainage, less compact soils, the addition of calcium, use of cover crops and biofumigation with *Brassicaceae* plant residues are also promising control strategies (Heyman et al. 2007; Hossain et al. 2012).

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For soil-borne pathogens, biological control and management of crop and field conditions are of special interest to prevent disease (Matthiessen and Kirkegaard 2006). The use of belowground ecosystem services provided by soil biota and the addition of biological control agents (BCAs) has large unexplored potential (Raaijmakers et al. 2009). Many BCAs originally found to stimulate plant growth and denoted as plant growth-promoting rhizobacteria (PGPR) were later found to also improve stress management (Bhattacharyya and Jha 2012). Mechanisms of disease suppression by bacterial BCAs include the production of enzymes and antibiotics (Bhattacharyya and Jha 2012) as well as priming of induced systemic resistance (ISR) in the host plant (Pieterse et al. 2014). Several publications report the use of microorganisms as BCAs in controlling *A. euteiches* infection of leguminous plants. Wakelin et al. (2002) found that several spore-forming bacteria were able to control the pathogen and suggested that inhibition of zoospore germination, lysis of germ tubes and the production of antibiotics served as control mechanisms. Xue (2003) showed that a *Clonostachys rosea* strain was effective in controlling pea root-rot caused by a complex of pathogens, including *A. euteiches*. Thygesen et al. (2004) found that Arbuscular mycorrhiza fungi reduced root-rot by *A. euteiches* in peas. Oubaha et al. (2018) found two *Streptomyces* strains out of a large collection to display antimicrobial activity against *Aphanomyces* and significantly reduce damping-off on pea. Godebo (2019) screened 184 rhizosphere bacteria for potential antagonism to *A. euteiches* and identified several strains that inhibited zoospore germination *in vitro* and suppressed *Aphanomyces* root rot in field pea.

Besides microorganisms, soil fauna affects plant growth and health through direct and indirect interactions in the environment of plant roots (for review see Friberg et al. 2005; Bonkowski et al. 2009; Schrader et al. 2013). Earthworms, in particular, are known to interact with the soil biota (Brown 1995; Scheu et al. 2002; Postma-Blaauw et al. 2006; Gómez-Brandón et al. 2012). Microorganisms are common food sources for many earthworms (Moody et al. 1996; Byzov et al. 2007). Consumption of soil-borne plant pathogens has thus positive effects on plant health (Bi et al. 2018; Puga-Fretas et al. 2016; Elmer 2009; Elmer and Ferrandino 2009; Meghvansi et al. 2011; Wolfarth et al. 2011; Hume et al. 2015). Many fungal pathogens are attractive to most species of earthworms, for example, *R. solani* and *Microdochium nivale* (formerly *Fusarium nivale*) (Bonkowski et al. 2000). *Plasmodiophora brassicae*, causing clubroot disease in *Brassica* plants, produces very durable resting spores and passage through the gut of earthworms reduced the disease rate in *Brassica* plants (Nakamura et al. 1995). On the other hand, if spores survive gut passage earthworms can spread the disease (Brown 1995). For pathogens that grow saprophytically, earthworms may actively consume the whole crop residue infected with pathogens and thereby reduce disease pressure on plants. In addition, the incorporation into the soil of such plant material reduces disease pressure (Wolfarth et al. 2011). The impact of earthworms on plant pathogens such as *A. euteiches* that do not grow actively outside the plants could thus be through the consumption of resting spores or infested tissue as well as indirectly by strengthening of plant defence. Mechanisms underlying plant disease suppression by earthworms may accordingly involve both direct and indirect effects.

The combined effects of plant-beneficial bacteria and earthworms on plant production and plant health have so far been studied only in a few cases (Ayuke et al. 2017). In order to develop sustainable agricultural systems with optimal use of ecosystem services and biological control the interaction of these below-ground organism groups is of high interest. Earthworms could modify the effects of plant-beneficial bacteria in either positive or negative ways. Because of their massive production of bioactive substances, applying bacterial BCAs in high concentrations may interfere with non-target organisms such as earthworms. However, Lagerlöf et al. (2015) found no negative effects of the bacterial BCA *Bacillus velezensis* on two species of earthworms (*Aporrectodea caliginosa* and *A. longa*), and Söderlund (2015) found no effects on the tropical earthworm *Pontoscolex corethrurus* when exposed to the bacterial BCA *Bacillus subtilis* at doses comparable with the highest probable exposure dose when used as BCAs.

In this paper, we present a study of the influence of the plant pathogen *A. euteiches*, the BCA and PGPR *B. velezensis* UCMB5113 and the anecic earthworm *Lumbricus terrestris* L. on plant health and growth of peas. We hypothesised that pea plant emergence, growth and health would be positively influenced by the added BCA bacteria and earthworms, and that hampered plant growth and disease symptoms caused by the plant pathogen would be counteracted. We also tested the effect of the added microorganisms *A. euteiches* and *B. velezensis* on growth and survival of earthworms.

Material and methods

Experimental setup

The influence of *A. euteiches*, the causal agent of pea rot, the BCA gram positive bacterium *B. velezensis* subsp. *plantarum* UCMB5113 (formerly *B. amyloliquefaciens*) and the anecic earthworm *L. terrestris* on growth and health of peas was studied in a fully factorial pot experiment in a greenhouse at SLU in Uppsala (59°49′05′′ N, 17°39′28′′ E) during the period 29 October–16 December 2014. The influence of the added microorganisms on survival and growth of *L. terrestris* was also studied. The presence of *A. euteiches* in the soil at the end of the experiment was tested using qPCR analysis. Peas were sown at the start of the experiment and grown in soil fertilised with cow manure.

The experiment was fully factorial with three factors and two levels of each factor: *Aphanomyces* (no, yes), *Bacillus* (no, yes) and Earthworms (no, yes), thus resulting in eight treatments, which were applied in five replicates. The test groups were denoted as:

- **C**, Control: no organisms added to soil or seeds
- **E**, Earthworms: *L. terrestris* added to soil
- **B**, *Bacillus*: pea seeds coated with *B. velezensis*
- **BE**, *Bacillus*-Earthworms: pea seeds coated with *B. velezensis* and *L. terrestris* added to soil
- **A**, *Aphanomyces*: *A. euteiches* spores mixed into soil
- **AE**, *Aphanomyces*-Earthworms: *A. euteiches* spores mixed into soil and *L. terrestris* added to soil
- **AB**, *Aphanomyces-Bacillus*: *A. euteiches* spores mixed into soil and pea seeds coated with *B. velezensis*
- **ABE**, *Aphanomyces-Bacillus*-Earthworms: *A. euteiches* spores mixed into soil, pea seeds coated with *B. velezensis,* and *L. terrestris* added to soil

In addition to the eight treatments mentioned above, one more treatment was set up in five replicates in order to analyse the effect of the pea plants on the presence of *A. euteiches* in the soil, namely

- Soil fertilised with cow manure without plants, with addition of *A. euteiches*.

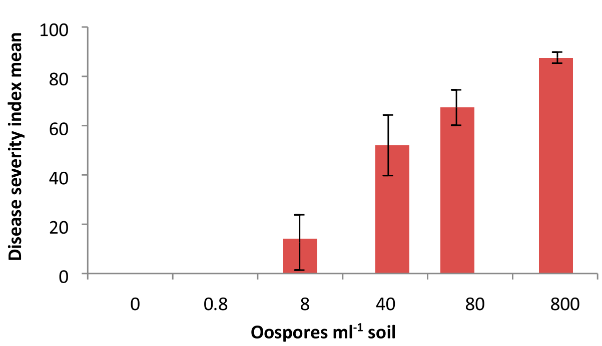

Figure 1. *Aphanomyces euteiches* infection in peas with increasing oospore concentration in planting soil. Mean disease severity index and SE in 6 replicated pots. Based on this pre-experiment, the concentration of *A. euteiches* spores in the experimental soil was set at 15 oospores ml^{-1} soil.

The pots were made from PVC plastic sewage pipes with 14.5 cm inner diameter and 30 cm height. Nylon mesh (1 mm mesh size) was attached with a rubber band at the bottom of the pots in order to allow drainage but prevent escape of earthworms. Each pot was filled with 2 L of a moist soil mixture, up to approx. 15 cm of the height of the cylinder. The soil mixture was composed of 60% clay-loam soil (36.5%, clay, 1.5% C-content, pH 6.6), 30% sandy soil (C-content 2.7%, pH 6.3), and 10% cow manure (Weibulls® concentrated, dried organic cow manure, particle size mostly <1 mm and not more than 3 mm). Soil was collected from two different sites on SLU's experimental farm outside Uppsala, close to the greenhouse where the experiment was performed. Soil was hand-sorted to eliminate stones, plant debris and macrofauna and frozen and thawed twice at −20°C for 24 h before use. The cow manure was wetted to 50% moisture content before being added to serve as fertiliser for the plants and as feed for the earthworms. An additional amount of 70 g of wetted cow manure was added superficially to each treatment after four weeks to ensure enough feed for the earthworms and plant nutrients, also in the treatments without earthworms. *A. euteiches* oospores from a pure culture, applied in a dry talcum powder mixture (Persson et al. 1999) containing 1.3*104 spores g^{-}1 powder was added to the soil. The inoculum dose used was 15 oospores ml^{-1} soil, and the soil was thoroughly mixed with a cement mixer. The oospore inoculum batch used was first tested on peas in a dose–response experiment (Figure 1) to select a dose that resulted in clear disease symptom development but not death of the plants during the course of the experiment. The dose ranged from 0.8 to 800 spores ml^{-1} soil, and disease symptoms were observed at 8 spores ml^{-1} (disease index mean 14 of 6 pots) and increased progressively with higher inoculum (disease index mean 87 at 800 spores ml^{-1}). Weak disease symptoms were only observed on parts of the roots, while more severe disease affected the whole root system, then, in addition, stems and stipples.

At the onset of the experiment, six pea (*P. sativum* L., cv. Clara) (Lantmännen Lantbruk, Malmö, Sweden) seeds were sown in each pot at 1 cm depth. *B. velezensis* UCMB5113 (formerly referred to as *B. amyloliquefaciens*, Dunlap et al. 2016) has earlier been shown as an efficient BCA towards *Brassica* pathogens (e.g. Danielsson et al. 2007; Sarosh et al. 2009). UCMB5113 was grown in LB medium at 28°C until stationary phase and after heat shock, centrifugation and washing with phosphate-buffered saline the concentration was determined using colony-forming unit counts. The pots were watered when needed, at least twice a week. In treatments with *B. velezensis* UCMB5113, the pea seeds had been coated with a layer of *Bacillus* spores (10^7 ml^{-1}) (Danielsson et al. 2007) prior to sowing.

The earthworms *L. terrestris* were collected from the Ultuna Park at SLU campus in Uppsala by means of extraction from the soil after watering with a mild detergent, whereupon they were thoroughly rinsed in cold tap water. The earthworms were kept in a soil mixture similar to the experimental soil for at most two weeks before being used in the experiment. Two *L. terrestris* specimens per pot were weighed and then added on November 3, five days after sowing the peas. All individuals used were adults with developed clitellum. The soil was watered to field capacity prior to the addition of earthworms and the worms could easily submerge into the soil. We observed during the course of the experiment that there was very little visible activity of earthworms at the soil surface, e.g. casts or visible channel openings. Therefore, on November 28, one more earthworm individual was added to each pot of the earthworm treatments. This was to ensure that there would be at least one live individual per pot throughout the experimental period. However, all individuals survived and gained weight during the experimental time period. The pots were placed randomly in the greenhouse with light regime 18 h full daylight and 6 h night, and the air temperature (recorded continuously) was 19–22°C at daytime and 16–18°C at night, with a mean temperature for the whole period of 19.1°C. Overheating of air and soil was not a problem since the study was done in late autumn and winter when outdoor temperatures and sun exposures were low.

Data collection and analysis

At the end of the experiment, plant height, above ground and root biomass, disease severity index as well as earthworm survival and biomass were recorded. The pea plants were in florescence and no plants had started to wilt when the experiment was terminated. We presumed based on earlier experiments (Heyman et al. 2007) that the clearest differences in symptom development (and disease severity index) among treatments would be shown at this stage. At a later stage, the symptoms would be more extreme without nuances. Therefore, the experiment was not run until maturity of peas and crop yield was not measured.

Plants were dried at 70°C for 48 h before recording dry biomass. The disease severity index was scored as 0% = no symptoms, 25% = symptom on parts of roots (brownish colour), 50% = symptoms on the whole root system, 75% = symptoms on the whole root system and on the stem, 100% = symptoms on the whole root system and all stipes below top senescent (Heyman et al. 2007). Earthworms were retrieved from the pots and weighed live after having been washed in tap water and dried on a paper tissue. The presence of *A. euteiches* in the soil at the end of the experiment was analysed with qPCR using specific primers (Heyman 2008). Soils of all treatments of the experiment, except for *Bacillus*-Earthworms, were analysed. In addition to this, control soils without peas and with the addition of *A. euteches* was analysed. Genomic DNA was isolated from soil using the NucleoSpin® Soil kit (Macherey-Nagel GmbH, Düren, Germany) and DNA amount and purity measured using a Nanodrop instrument (A260/280 was 1.81 ± 0.05 and yield 2.40 ± 0.63 mg for 35 samples). The primers used were Ae169F (5′-TCAGGGCTAGCCGAAGGTT-3′) and Ae169R (5′-ACAAGCTTCATTTCTGATGCTAGTTTA-3′) at 400 nM final concentration with 25 ng DNA as a template (Heyman 2008). The amount of pathogen DNA was quantified using a dilution series of standard target DNA included in each run. The standard contained a cloned 524 base pair sequence of the target gene. The real-time PCR reaction used EvaGreen supermix (Bio-Rad Laboratories, Hercules, CA). Post-run melting curve analysis was performed to ensure the specificity of the amplification reaction, which amplifies a 96 bp fragment of the ITS1 region (Heyman 2008). Two technical replicates of each treatment was analysed. The number of *A. euteiches* target copies in soil sample extracts was quantified according to Wallenhammar et al. (2012). Potential inhibition of the PCR reaction by the isolated DNA fractions due to soil contaminants was tested by dilution analysis. Samples were considered positive if the technical replicates showed Ct ≤ 32 in the same qPCR reaction.

Differences between treatments and the factors *Aphanomyces*, *Bacillus* and Earthworms in plant emergence, growth and disease severity index were analysed with three-way general linear model (GLM) ANOVA. Earthworm survival and growth as a function of the factors *Aphanomyces* and *Bacillus* were analysed with two-way GLM ANOVA. When significant effects were found ($p<0.05$), Tukey's pairwise comparisons were used to compare treatment means. ANOVA was done on log10-transformed data in order to fulfil the assumption of normal distribution. A correlation analysis was done for the correlation between plant growth and disease index response factors. Minitab 16 Software was used for all analyses.

Results

Growth of pea plants and disease symptoms

Tukey's pairwise comparisons between treatments

The number of emerged plants per pot remaining at the end of the experiment was lower if *Bacillus* was added

(3.4 plants per pot) compared with the treatment with both *Aphanomyces* and Earthworms (6.0 plants per pot, $p < .05$). An average number of remaining plants in the other treatments was intermediate to these values. None of the treatment combinations was, however, significantly different from the control group (Table 1).

The pairwise comparisons showed that plants grew significantly higher ($p < .05$) in the treatment if *Aphanomyces*, *Bacillus* and Earthworms were added simultaneously (738 mm) compared to control (560 mm). If *Aphanomyces* was present in the pot, adding *Bacillus* also significantly increased plant growth (700 mm). Plant height of other treatments did not differ significantly from the control.

The dry mass of individual shoots was larger in the treatments *Aphanomyces-Bacillus* (2.15 g) and *Aphanomyces-Bacillus*-Earthworms (2.26 g) than in control (1.21 g) and *Aphanomyces* (1.29 g). Values of the other treatments were in between.

Root dry mass of individual plants was significantly lower ($p < .05$) in *Aphanomyces* (0.15 g) and *Aphanomyces*-Earthworms (0.17 g) than in *Aphanomyces-Bacillus* (0.29 g), while the other treatments were in between and did not differ significantly among each other.

The disease symptoms on pea plants measured as disease severity index were significantly higher ($p < .05$) in *Aphanomyces* (52.2) than in the uninfected treatments control (0.00), *Bacillus* (0.00), Earthworms (2.25) and *Bacillus*-Earthworms (0.00) as well as in the infected treatment *Aphanomyces*-Earthworms (1.67). The average disease-severity index was less than half as high in *Aphanomyces-Bacillus* (22.2) and *Aphanomyces-Bacillus*-Earthworms (25.8) as compared to *Aphanomyces*, but the differences were not significant.

Influence of the factors Aphanomyces, Bacillus and earthworms on plant growth and health

The GLM ANOVA showed that the factor *Bacillus* had a significantly negative effect on the number of remaining plants ($p = .031$). Interaction between Bacillus, *Aphanomyces* and Earthworms shows that the negative influence was moderated by the other factors (Table 2).

Further, the analysis showed that the factors *Bacillus* and Earthworms had a significantly positive effect on plant height ($p = .003$ and $p = .03$).

The factor *Bacillus* increased shoot mass ($p < .001$). Interaction between Earthworms and *Bacillus* ($p = .036$) indicated a reinforced effect with both factors present.

The factor *Bacillus* enhanced root dry mass significantly ($p = .005$).

The factor *Aphanomyces* increased the disease severity index significantly and it was reduced by the factor Earthworms. There was the interaction between the factors *Bacillus* x Earthworms and *Aphanomyces* x *Bacillus* × Earthworms, indicating that earthworm reduced the disease severity index caused by *Aphanomyces*.

Correlations between factors

The number of remaining plants at the end of the experiment was not correlated to plant growth variables or disease severity index. Plant height was significantly positively correlated with shoot dry mass and root dry mass per plant. Shoot individual dry mass was significantly positively correlated with plant height and root dry mass per plant. Root dry mass of individual plants was significantly positively correlated with plant height and shoot dry mass per plant. There were no significant correlations between disease severity index and any of the other individual response factors (Table 3).

Aphanomyces euteiches in soil at the end of the experiment

At the end of the experiment, the qPCR analysis (Table 4) showed the presence of *A. euteiches* at least in one or a few of the replicates of the treatment where the pathogen had been added. In treatments without addition of *A. euteiches*, no *A. euteiches* DNA was detected. In all positive reactions, melting curve analysis displayed only one product with a symmetrical peak at 76.0°C, indicating specific amplification of the target gene. In treatment *Aphanomyces*, three samples were clearly positive and two showed a weaker reaction. In the treatment with A. *euteiches* without pea plants, the qPCR reaction was weakly positive in three out of five samples. In the rest of the treatments, the reaction was in general weakly positive. Due to high variation in Cq values, we restricted analysis of qPCR results as presence or absence of the pathogen in the soil in the different treatments.

Earthworm growth and survival

All earthworm individuals survived the experiment and increased in biomass by 30–50% in the different treatments (Table 5). There was no significant difference between treatments, but GLM ANOVA showed that weight increase was higher where the factor *Bacillus* was present than without (Table 5, $p = .039$).

Discussion

The greenhouse experiment showed that the added BCA bacteria *B. velezensis* and anecic earthworms of the species *L. terrestris* enhanced plant growth, mostly resulting in taller plants and higher biomass of above- and below-ground tissues. The effects were increased by the presence of both factors. Our hypothesis was therefore valid in this respect and could not be rejected.

Table 1. Influence of the factors *Aphanomyces euteches* (A), *Bacillus velezensis* (B) and *Lumbricus terrestris* (E) on survival (plants pot^{-1}), growth (shoot height, shoot and root dry mass) and health (disease severity index) of pea plants (*Pisum sativum*) in a factorial pot experiment in the greenhouse.

Factor/	*Aphanomyces* (A)							
	No				Yes			
	Bacillus (B)				Bacillus (B)			
	No		Yes		No		Yes	
	Earthworm (E)		Earthworm (E)		Earthworm (E)		Earthworm (E)	
Variable	No	Yes	No	Yes	No	Yes	No	Yes
Treatment	C	E	B	BE	A	AE	AB	ABE
Plants pot^{-1}	5.4ab	4.0ab	3.4b	3.8b	4.4ab	6.0 a	4.8ab	4.2ab
Plant height (mm)	560bc	631abc	641abc	630abc	530c	660abc	700ab	738a
Shoot DM (g)	1.21b	1.58ab	1.93ab	1.80ab	1.29b	1.79ab	2.15a	2.26a
Root DM (g)	0.21ab	0.21ab	0.22ab	0.24ab	0.15b	0.17b	0.29a	0.22ab
Disease severity index (%)	0.00b	2.25b	0.00b	0.00b	52.2a	1.67b	22.2ab	25.8ab

Note: Mean and SE of plants in five replicated pots. GLM ANOVA and Tukey's pairwise comparisons between treatments. Means that do not share a letter are significantly different ($p < .05$). Six pea seeds were sown per pot, *A. euteches* spores were mixed into the planting soil. Seeds were coated with *B. velezensis* before sowing. Two *L. terrestris* individual were added per pot 5 days after sowing and one additional individual 33 days after sowing. The experiment was running for 49 days. Disease severity index was classed as 0 = no symptoms, 25% = symptom on parts of roots (brownish colour), 50% = symptoms on the whole root system, 75% = symptoms on the whole root system and on stem, 100% = symptoms on the whole root system and all stipes below top senescent.

Addition of *B. velezensis* did, however, reduce seed germination and subsequently the number of remaining plants at the end of the experiment. Disease symptoms caused by the addition of *A. euteches* to the soil were significantly reduced by the presence of the earthworms but not by *B. velezensis*. Therefore, the hypothesised disease-reducing effect of the *Bacillus* strain alone could not be confirmed using this particular system. The inoculation of pea plants by *A. euteiches* oospores added to the soil was successful and caused symptoms recorded as high disease indices, while none of the control treatments showed any signs of infection. The qPCR analysis at the end of the experiment confirmed the presence of *A. euteiches*. DNA in the soil of the treatments with this organism added, and its absence in the non-infested treatments indicated the integrity of the samples with no cross-contamination occurring during the experimental period.

Table 2. Influence of the factors *Aphanomyces euteches* (A), *Bacillus velezensis* (B) and *Lumbricus terrestris* (E) on survival (plants pot^{-1}), growth (shoot height, shoot and root dry mass (dm)) and health (disease severity index) of pea plants (*Pisum sativum*) in a factorial pot experiment in the greenhouse.

	Plants pot−1	Plant height (mm)	Shoot dm (g plant^{-1})	Root dm (g plant^{-1})	Disease Severity Index (%)
Aphanomyces (A)	0.071	0.139	0.054	0.446	**<0.001**
Bacillus (B)	**0.031**	**0.003**	**<0.001**	**0.005**	0.683
Earthworms (E)	0.901	**0.030**	0.058	0.589	**0.032**
A × B	0.469	0.162	0.635	0.062	0.852
A × E	0.171	0.365	0.456	0.246	**0.019**
B × E	0.771	0.056	**0.036**	0.385	**0.014**
A × B × E	**0.007**	0.786	0.859	0.261	**0.008**

Note: Three-way GLM ANOVA, factors and *p*-values. Significant *p*-values at $p < .05$ are in bold.

The plants were grown under controlled conditions in a greenhouse. *A. euteiches* was present in the soil at the end of the experiment only in treatments where this organism had been added but not in other treatments. The introduced earthworms survived to 100% and increased in biomass during the experimental time. These results and the fact that we used five replicates of each treatment prove the validity of the results and the reproducibility of this experiment.

A. euteiches caused no negative effects on plant height or biomass production as compared to the control, and disease severity indices were not correlated to any of the recorded aspects of plant growth (Table 3). Probably because of optimal plant growth conditions concerning water and nutrient status, the disease had not yet resulted in impeded plant growth at the time when the experiment was terminated. The reason for terminating the experiment at the florescence stage was that we assumed that the clearest differences in disease severity would be evident between treatments.

Table 3. Correlation analysis (correlation index and *p*-values) between response factors of pea plants in *Aphanomyces euteiches* and *Bacillus velezensis* infection experiment with earthworms (*Lumbricus terrestris*).

	Height	S DM	R DM	DSI
S DM	0.864			
	<0.000			
R DM	0.398	0.568		
	0.011	<0.000		
DSI	−0.036	0.070	−0.051	
	0.825	0.669	0.754	
Number	0.122	−0.076	−0.178	0.072
	0.453	0.642	0.273	0.657

Note: Shoot dry mass per individual plant (S DM plant^{-1}), Root dry mass per individual plant (R DM plant^{-1}), Disease severity index (Dsi), Number of remaining plants per pot out of maximum 6 plants (Number).

Table 4. Result of qPCR analysis of *Aphanomyces euteiches* DNA in soil under the experimental treatments at the end of the experiment and in soil inoculated with *A. euteiches* but without pea plants.

Treatment	Positive qPCR out of 5 samples[a]
Soil + A	0/3/2
C	0/0/5
A	3/2/0
B	0/1/4
E	0/1/4
AB	1/3/1
AE	0/3/2
EB	0/0/5
ABE	0/3/2

Notes: Soil + A = Soil fertilised with cow manure without plants, with addition of *A. euteiches*. For other treatments, see Matherial and methods.
[a]Results presented as samples above/detectable (but unreliable)/below a reliable detection limit (<32/32 to 0/>40 cycles). The lowest standard was detected after 32 cycles but no amplification was recorded after >40 cycles.

We know from earlier repeated experiments that *B. velezensis* may affect seed germination plant varieties, while a growth stimulatory effect is often registered for the plants germinating. Such observations were reported in e.g. Asari, Tarkowská, et al. (2017) and Danielsson et al. (2007). *B. velezensis* often stimulates above- and below-ground tissue growth, which also was observed in this study as increases in some plant root and shoot parameters. This effect may be due to changed phytohormone status of the host plant by plant or *Bacillus* generated biosynthesis of growth hormones (e.g. Asari, Tarkowská, et al. 2017). For *Bacillus* interactions, the negative effect on plant emergence was attenuated in combined treatments. This may be due to chemical or structural effects caused by the other test organisms, for example on root exudation, which is known to be an important factor interacting with soil microbiota (Tkacz and Poole 2015; Lareen et al. 2016). Plant growth above and below ground and plant height was not significantly negatively correlated to the number of plants per pot (Table 3). This means that increased shoot and root dry mass in treatments with *Bacillus* as compared to without *Bacillus* was not due to fewer plants, and subsequently more space per plant and less interplant competition, but could be due to positive stimulation effects by *Bacillus*. The slight stimulatory effect on plant height by *Bacillus* had a tendency to be enforced by *Aphanomyces*, which could be due to stress growth effects on plants. Combinations of PGPR and different stressors have been observed to stimulate plant growth in other systems (e.g. Barriuso et al. 2008; Pandey et al. 2017). The observed stimulation of shoot biomass by *Bacillus* seemed to be potentiated in combination with *Aphanomyces*, indicating a stress effect that may be due to less control of phytohormone balance as a stress reaction or as a strategy by *Aphanomyces* to weaken plant defence and obtain more biomass in line with the cost of resistance model (Bergelson and Purrington 1996). The combination of *Bacillus* and *Aphanomyces* also stimulated root growth compared to single treatments, suggesting similar systemic growth effects by these microorganisms.

Table 5. Influence of the factors *Aphanomyces euteches* (A) and *Bacillus velezensis* (B) on the relative fresh mass increase (%) of the earthworm *Lumbricus terrestris* (E) during an experimental period of 49 days.

Factor	*Aphanomyces*		*Aphanomyces*	
	No		Yes	
	Bacillus		*Bacillus*	
	No	Yes	No	Yes
Treatment	E	BE	AE	ABE
Earthworm fresh mass increase (%)	30.7 ± 8.2	50.0 ± 2.5	37.4 ± 4.1	50.2 ± 5.7

Note: No significant differences between treatments (GLM ANOVA; $p > .05$, Tukey's pairwise comparisons).
Two-way GLM ANOVA.

Earthworm relative fresh mass increase (%) *vs*. the factors *A. euteches* (A) and *B. velezensis* (B). Significant *p*-values at $p < .05$ are in bold.

Factor	*p*-value
A	0.372
B	**0.039**
A × B	0.346

Earlier reports have demonstrated priming of ISR in many host plants by different rhizobacteria (Bhattacharyya and Jha 2012; Pieterse et al. 2014). The *B. velezensis* UCMB5113 strain has proven to be effective against several but not all *Brassica* pathogens (Danielsson et al. 2007; Sarosh et al. 2009; Asari, Ongena, et al. 2017). The present study was done in a more complex environment and with a novel host plant and pathogen, and apparently, this *Bacillus* strain does not influence the disease development in this particular setup. Earthworm treatments increased the height of plants and reduced disease-severity index values. In an earlier study of effects of the endogeic earthworm species *A. caliginosa* and *B. velezensis* on the fungal plant pathogen *Alternaria brassicae* on *Brassica napus* plants, Ayuke et al. (2017) found, as in the present study, significantly taller plants in treatments with earthworms than those without. Possibly the altered soil properties due to earthworm movement and secretion could enhance plant height growth (see below). We have earlier noted, in experiments with other crops and pathogens, that earthworms can stimulate plant growth (Söderlund 2015). This is the first study where the effect of earthworms on *A. euteiches* is studied, and the ability of *L. terrestris* to reduce disease symptoms is encouraging for the use of this earthworm species in the control of pea root-rot. Most studies of this earthworm's ability to reduce plant diseases have involved fungal plant parasites that are facultative saprophytic (Elmer 2009; Schrader et al. 2013; Wolfarth et al. 2011).

In these studies, earthworms were able to reduce the abundance of pathogens by their consumption of fungal biomass growing on plant residues or by burying plant residues where the pathogens in many cases are outcompeted by saprophytic fungi. In this way pathogen inoculum density is decreased. *A. euteiches* is an obligate plant parasite that does not grow and reproduce unless a suitable living host plant is present. The mechanism by which earthworms could reduce the abundance of the pathogen, and subsequent infection of plant roots, may, therefore, involve consumption of resting spores in the soil and consumption of zoospores searching for roots of suitable host plants. Large anecic earthworms such as *L. terrestris* have longer gut passage time than endogeic and epigeic species (Brown 1995) and, therefore, their ability to influence ingested biological material is greater. Friberg et al. (2008) did not find that the endogeic earthworm *A. caliginosa* influenced concentration or infection ability of *P. brassicae*, an obligate plant parasite on the *Brassicaceae* species. However, Nakamura et al. (1995) found that the anecic earthworm *Pheretima hilgendorfi* did. The infection risk could also be reduced if the earthworms could strengthen plants by improving the nutrient availability and soil structure. This reduction may also include the production of plant hormone-like substances by the earthworms themselves or in combination with soil and rhizosphere microorganisms (Bonkowski et al. 2009). Coeolomic fluid that is expelled by earthworms into the exterior environment has antimicrobial properties. Plavšin et al. (2017) found in an *in vitro* experiment that coelomic fluid from two epigeic earthworm species (*Eisenia foetida* and *Dendrobena veneta*) had an inhibitory effect on the plant pathogenic fungus *Fusarium oxysporum*. The fluid contains coelomocytes as well as a variety of molecules with antimicrobial properties (Plavšin et al. 2017). The increased earthworm growth in treatments with *Bacillus* is in line with our earlier studies on the effects of *B. velezensis* on earthworm growth and survival (Lagerlöf et al. 2015; Söderlund 2015). This also indicates that this bacterium is not harmful to earthworms (Lagerlöf et al. 2015) and supports the usefulness of certain microbes in developing improved biocontrol strategies for various crops. Developing further combinations of organisms from different trophic levels in order to improve crop stress management and plant growth seems to be a promising approach ultimately improving yield and contributing to more sustainable crop production. For the production of peas and other leguminous crops, coating of seeds with BCA bacteria and enhancement of earthworms by adding organic matter to the soil and reducing soil cultivation (Lagerlöf et al. 2012) would be useful components of sustainable production systems that take advantage of common ecosystem services.

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References

Asari S, Tarkowská D, Rolčík J, Novák O, Palmero DV, Bejai S, Meijer J. 2017. Analysis of plant growth-promoting properties of *Bacillus amyloliquefaciens* UCMB5113 using *Arabidopsis thaliana* as host plant. Planta. 245:15–30.

Asari S, Ongena M, Debois D, De Pauw E, Chen K, Bejai S, Meijer J. 2017. Insights into the molecular basis of biocontrol of *Brassica* pathogens by *Bacillus amyloliquefaciens* UCMB5113 lipopeptides. Ann Bot. 120:551–562.

Ayuke FO, Lagerlöf J, Jorge G, Söderlund S, Muturi John J, Sarosh BR, Meijer J. 2017. Effects of biocontrol bacteria and earthworms on *Alternaria brassica* disease severity and growth of oilseed rape plants (*Brassica napus*). Appl Soil Ecol. 117–118:63–69.

Barriuso J, Solano BR, Gutiérrez Mañero FJ. 2008. Protection against pathogen and salt stress by four plant growth-promoting rhizobacteria isolated from Pinus sp. on *Arabidopsis thaliana*. Phytopathology. 98:666–672.

Bergelson J, Purrington CB. 1996. Surveying patterns in the cost of resistance in plants. Am Nat. 148:536–558.

Bhattacharyya PN, Jha DK. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechol. 28:1327–1350.

Bi Y-M, Tian G-L, Wang C, Zhang Y, Wang D-N, Zhang F-F, Zhang L-S, Sun Z-J. 2018. Differential effects of two earthworm species on Fusarium wilt of strawberry. Appl Soil Ecol. 126:174–181.

Bonkowski M, Griffiths BS, Ritz K. 2000. Food preferences of earthworms for soil fungi. Pedobiologia. 44:666–676.

Bonkowski M, Villenave C, Griffith B. 2009. Rhizosphere fauna: the functional and structural diversity of intimate interactions of soil fauna with plant roots. Plant Soil. 321:213–233.

Brown GG. 1995. How do earthworms affect microfloral and faunal community diversity? Plant Soil. 170:209–231.

Byzov BA, Khomyakov NV, Kharin SA, Kurakov AV. 2007. Fate of soil bacteria and fungi in the gut of earthworms. Eur J Soil Biol. 43:S149–S156.

Danielsson J, Reva O, Meijer J. 2007. Protection of oilseed rape (*Brassica napus*) toward fungal pathogens by strains of plant-associated *Bacillus amyloliquefaciens*. Microb Ecol. 54:134–140.

Dunlap CA, Kim SJ, Kwon SW, Rooney AP. 2016. *Bacillus velezensis* is not a later heterotypic synonym of *Bacillus amyloliquefaciens; Bacillus methylotrophicus, Bacillus amyloliquefaciens* subsp. *plantarum* and '*Bacillus oryzicola'* are later heterotypic synonyms of *Bacillus velezensis* based on phylogenomics. Int J Syst Evol Microbiol. 66:1212–1217.

Elmer WH. 2009. Influence of earthworm activity on soil microbes and soil borne diseases of vegetables. Plant Dis. 93:175–179.

Elmer WH, Ferrandino FJ. 2009. Suppression of *Verticillium* wilt of eggplants by earthworms. Plant Dis. 93:485–489.

Friberg H, Lagerlöf J, Rämert B. 2005. Influence of soil fauna on fungal plant pathogens in agricultural and horticultural systems. Biocontrol Sci Technol. 15:641–658.

Friberg H, Lagerlöf J, Hedlund K, Rämert B. 2008. Effect of earthworms and incorporation of grass on *Plasmodiophora brassicae*. Pedobiologia. 52:29–39.

Gaulin E, Jacquet C, Bottin A, Dumas B. 2007. Root rot disease of legumes caused by *Aphanomyces euteiches*. Mol Plant Pathol. 8:539–548.

Godebo AT. 2019. Evaluation of soil bacteria as bioinoculants for the control of field pea root rot caused by *Aphanomyces euteiches* [thesis]. University of Saskatchewan.

Gómez-Brandón M, Lores M, Domínguez J. 2012. Species-specific effects of epigeic earthworms on microbial community structure during first stages of decomposition of organic matter. PLoS One. 7(2):e31895.

Heyman F, Lindahl B, Pärsson L, Wikström M, Stenlid J. 2007. Calcium concentrations of soil affect suppressiveness against *Aphanomyces* root rot of pea. Soil Biol Biochem. 39:2222–2229.

Heyman F. 2008. Root rot of pea caused by *Aphanomyces euteiches* [doctoral thesis]. Uppsala: Swedish University of Agricultural Sciences. Acta Universitatis agriculturae Sueciae 1652–6880; 2008:24. ISBN 978- 46791-85913-57-2.

Hossain S, Bergkvist G, Berglund K, Mårtensson A, Persson P. 2012. *Aphanomyces* pea root rot disease and control with special reference to impact of *Brassicaceae* cover crops. Acta Agric Scand B Soil Plant Sci. 62:477–489.

Hosseini S, Elfstrand M, Heyman F, Funk Jensen D, Karlsson M. 2015. Deciphering common and specific transcriptional immune responses in pea towards the oomycete pathogens *Aphanomyces euteriches* and *Phtophtora pisi*. BMC Genomics. 16:627.

Hughes TJ, Grau CR. 2007. *Aphanomyces* root rot (common root rot) of legumes. Plant Health Instr. DOI:10.1094/PHI-I-2007-0418-01.

Hume EA, Horrocks AJ, Fraser PM, Curtin D, Meenken ED. 2015. Alleviation of take-all in wheat by the earthworm *Aporrectodea caliginosa* (Savigny). Appl Soil Ecol. 90:18–25.

Lagerlöf J, Ayuke F, Bejai S, Jorge G, Lagerqvist E, Meijer J, Muturi John J, Söderlund S. 2015. Potential side effects of biocontrol and plant-growth promoting *Bacillus amyloliquefaciens* bacteria on earthworms. Appl Soil Ecol. 96:159–164.

Lagerlöf J, Pålsson O, Arvidsson J. 2012. Earthworms influenced by reduced tillage, conventional tillage and energy forest in Swedish agricultural field experiments. Acta Agric Scand B Soil Plant Sci. 62:235–244.

Lareen A, Burton F, Schäfer P. 2016. Plant root-microbe communication in shaping root microbiomes. Plant Mol Biol. 90:575–587.

Matthiessen JN, Kirkegaard JA. 2006. Biofumigation and enhanced biodegradation: opportunity and challenge in soil borne pest and disease management. Crit Rev Plant Sci. 25:235–265.

Meghvansi MK, Srivastava RB, Varma A. 2011. Assessing the role of earthworms in biocontrol of soil-borne plant fungal diseases. In: Karaca A, editor. Biology of earthworms. soil biology. Vol. 24. Berlin, Heidelberg: Springer; p. 173–189.

Mihajlović M, Rekanović E, Hrustić J, Grahovac M, Tanović B. 2017. Methods for management of soilborne plant pathogens. Pestic. Phytomed. (Belgrade). 32:9–24.

Moody SA, Piearce TG, Dighton J. 1996. Fate of some fungal spores associated with wheat straw decomposition on passage through the guts of *Lumbricus terrestris* and *Aporrectodea longa*. Soil Biol Biochem. 28:533–537.

Nakamura Y, Itakura J, Matsuzaki I. 1995. Influence of the earthworm *Pheretima hilgendorfi* (Megascolecidae) on *Plasmodiophora brassicae* clubroot galls of cabbage seedlings in pot. Edaphologia. 54:39–41.

Oubaha B, Nafis A, Baz M, Mauch F, Barakate M. 2018. The potential of antagonistic Moroccan *Streptomyces* isolates for the biological control of damping-off disease of pea (*Pisum sativum* L.) caused by *Aphanomyces euteiches*. J Phytopathol. 167:82–90.

Pandey P, Irulappan V, Bagavathiannan MV, Senthil-Kumar M. 2017. Impact of combined abiotic and biotic stresses on plant growth and avenues for crop improvement by exploiting physio-morphological traits. Front Plant Sci. 8:537.

Persson L, Larsson-Wikstrom M, Gerhardson B. 1999. Assessment of soil suppressiveness to *Aphanomyces* root rot of pea. Plant Dis. 83:1108–1112.

Pieterse CM, Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA. 2014. Induced systemic resistance by beneficial microbes. Annu Rev Phytopathol. 52:347–375.

Plavšin I, Velki M, Ečimović S, Vradecić K, Ćosić J. 2017. Inhibitory effect of earthworm coelomic fluid on growth of the plant parasitic fungus *Fusarium oxysporum*. Eur J Soil Biol. 78:1–6.

Postma-Blaauw MB, Bloem J, Faber JH, van Groenigen JW, de Goede RGM, Brussaard L. 2006. Earthworm species composition affects the soil bacterial community and net nitrogen mineralization. Pedobiologia. 50:243–256.

Puga-Fretas R, Belkacem L, Barot S, Bertrand M, Roger-Estrade J, Blouin M. 2016. Transcriptional profiling of wheat in response to take-all disease and mechanisms involved in earthworm's biocontrol effects. Eur J Plant Pathol. 144:155–165.

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y. 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil. 321:341–361.

Sarosh BR, Danielsson J, Meijer J. 2009. Transcript profiling of oilseed rape (*Brassica napus*) primed for biocontrol differentiate genes involved in microbial interactions with beneficial *Bacillus amyloliquefasciens* from pathogenic *Botrytis cinerea*. Plant Mol Biol. 70:31–45.

Scheu S, Schlitt N, Tiunov AV, Newington JE, Jones TH. 2002. Effects of the presence and community composition of earthworms on microbial community functioning. Oecologia. 133:254–260.

Schrader S, Wolfarth F, Oldenburg E. 2013. Biological control of soil-borne phytopatogenic fungi and their mycotoxins by soil fauna. Bull UASMV Agric. 70:291–298.

Söderlund, S. 2015. Biocontrol of *Fusarium* wilt on tomatoes – use of *Bacillus subtilis* and interactions with the earthworm *Pontoscolex corethrurus* in a Kenyan highland soil. Independent project/Degree project/SLU, Department of Ecology, Uppsala, 2015:6.

Thygesen K, Larsen J, Bødker L. 2004. Arbuscular mycorrhizal fungi reduce development of pea root-rot caused by *Aphanomyces euteiches* using oospores as pathogen inoculum. Eur J Plant Pathol. 110:411–419.

Tkacz A, Poole P. 2015. Role of root microbiota in plant productivity. J Exp Bot. 66:2167–2175.

Wakelin SA, Walter M, Jaspers M, Stewart A. 2002. Biological control of *Aphanomyces euteriches* root-rot of peas with spore-forming bacteria. Australas Plant Pathol. 31:401–407.

Wallenhammar A-C, Almquist C, Söderström M, Jonsson A. 2012. In-field distribution of *Plasmodiophora brassicae* measured using quantitative real-time PCR. Plant Pathol. 61:16–28.

Wolfarth F, Schrader S, Oldenburg E, Weinert J, Brunotte J. 2011. Earthworms promote the reduction of *Fusarium* biomass and deoxynivalenol content in wheat straw under field conditions. Soil Biol Biochem. 43:1858–1865.

Wu L, Chang K-F, Conner RL, Strelkov S, Fredua-Agyeman R, Hwang S-F, Feindel D. 2018. *Aphanomyces euteiches*: A threat to Canadian field pea production. Engineering. 4:542–551.

Xue AG. 2003. Biological control of pathogens causing root rot complex in field pea using *Clonostachys rosea* strain ACM941. Phytopathology. 93:329–335.