Effects of biocontrol bacteria and earthworms on the severity of *Alternaria brassicae* disease and the growth of oilseed rape plants (*Brassica napus*)

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A B S T R A C T

Biological control of plant diseases through the addition of microbial biocontrol agents and the promotion of earthworms can be an environmentally friendly alternative to the chemical control of plant diseases. However, possible risks with biocontrol agents and their interactions with earthworms and other soil biota have not been well studied. The aim of this study was to assess whether the beneficial bacterium *Bacillus amyloliquefaciens* and the earthworms *Aporrectodea caliginosa* or *Aporrectodea longa* could reduce disease in oilseed rape (*Brassica napus*) challenged with the pathogen *Alternaria brassicae*. Plant growth and productivity were measured as plant survival, height, biomass, and flower development as well as disease index. A second objective was to assess whether the presence of the bacterium at high concentrations would influence the survival, growth, and reproduction of the earthworms. One outdoor and one greenhouse experiment were performed with *Br. napus* plants challenged with *Al. brassicae* inoculated to the plant leaves in the presence or absence of *Bacillus amyloliquefaciens* inoculated to the root environment and in the presence or absence of earthworms (*Ap. caliginosa* or *Ap. longa*) added to the soil. All treatments were replicated three times. In the outdoor experiment, inoculation with *Al. brassicae* reduced the growth of plants and the addition of *Ap. caliginosa* increased plant height. In the greenhouse experiment, pairwise comparisons of plants challenged with *Al. brassicae* showed that treatment with *B. amyloliquefaciens* led to significantly lower disease index than the treatment with *Ap. caliginosa* plus *B. amyloliquefaciens*, while other treatments had intermediate disease indices. The addition of *Al. brassicae* or *B. amyloliquefaciens* increased the survival and mass increment of *Ap. caliginosa* as a main effect when used separately but not when used in combination.

This study did not give any clear indication of the usefulness of *B. amyloliquefaciens* for biocontrol of plant pathogens such as *Al.* brassicae when growing plants in natural soil. In addition, no significantly positive effects from the tested earthworm species were seen.

1. Introduction

Plant diseases are among the major factors limiting crop production worldwide. The fungal genus *Alternaria*, a member of Deuteromycetes, comprises many saprophytic and endophytic species and contains destructive plant pathogens. The *Alternaria* species are facultative pathogens causing disease on most of the common and economically important crop species. These fungi are commonly found on plant debris as well as on living plants. Their spread is aggravated by the fact that they are spread through seeds and soil as well as in the air (Chauhan et al., 2009; Kumar et al., 2014). For instance, *Alternaria*

brassicae (Berk.) causes dark spot disease on leaves of *Brassica* spp., and it is virulent against oilseed rape plants (Conn et al., 1988; Danielsson et al., 2007) with up to 70% losses in yield reported (Conn et al., 1990; Ram and Chauhan, 1998). Possible control measures against this pathogen include the use of chemical pesticides, although major concerns about their effects on human health and the environment limit their use (Kumar et al., 2014). Biological control strategies that involve the use of beneficial microorganisms (*e.g. Pseudomonas* and *Bacillus* species) as biocontrol agents (BCAs) offer a practical eco-friendly solution to the management of plant diseases (Shoda, 2000; Van Wees et al., 2008; Gera Hol et al., 2013). Some microorganisms in

the soil can suppress plant diseases, and positive correlations have been observed between high microbial diversity and disease suppression (Garbeva et al., 2004). The mechanisms behind the ability of beneficial rhizobacteria to protect plants against parasitic microorganisms include the priming of induced systemic resistance and the production of enzymes such as chitinases, peroxidases, and proteases as well as several types of antibiotics (Pieterse et al., 2014). Earlier studies have suggested the high potential of *Bacillus amyloliquefaciens* strain UCMB5113 (Reva et al., 2004) as a BCA. It has been shown to provide protection to oilseed rape plants (*Brassica napus* L.) against a number of fungal pathogens (Choudhary and Johri, 2009; Sarosh et al., 2009), including *Al. brassicae* (Danielsson et al., 2007).

The term 'biological control' (often abbreviated to biocontrol) refers to the use of living organisms or their derivatives to reduce the population density or the impact of a specific pest organism (Eilenberg et al., 2001). Parasitoids, predators, pathogens, herbivores, and/or antagonists are all used as biocontrol agents (BCAs) for the reduction of pest populations and for reducing their effects. Earthworms and other soil invertebrates can also act as BCAs because they can reduce pest organisms both by direct predation and by indirectly strenthening the plants' defence mechanisms, but research in this area is still at an early stage (Clapperton et al., 2001; Brown et al., 2004; Elmer, 2009; Friberg et al., 2005; Wolfarth et al., 2011a,b; Bertrand et al., 2015). As one of the most important soil-dwelling invertebrate groups, earthworms are an indicator of healthy soil (Doran et al., 1996). For instance, earthworms are considered ecosystem engineers for their role in modifying the soil environment and making resources available for other organisms (Jouquet et al., 2006) through their impact on soil structure and soil organic matter dynamics (Lavelle et al., 2001). Apart from speeding up the initial breakdown of organic residues, earthworms also incorporate organic matter into their casts and can thereby protect it against further rapid decomposition (Scullion and Malik, 2000; Bossuyt et al., 2005; Pulleman et al., 2005a,b). Crop performance can also be affected through the impact of worm-made aggregates and biopores on soil water dynamics and root growth (Friberg et al., 2005; Van Groenigen et al., 2014). Although the positive effects of earthworms on plant growth are widely recognized (Friberg et al., 2005; Van Groenigen et al., 2014), the mechanisms involved are still poorly understood. However, such information is vital to determining how earthworms can be explored as a latent ecosystem service to promote more sustainable agriculture. Possible pathways through which earthworms can positively influence plant growth do not rely solely on physical and chemical improvements of the soil quality, but also include biocontrol of pests and diseases and the stimulation of microbial plant symbionts and the production of plant growth-regulating substances (Clapperton et al., 2001; Brown et al., 2004; Bonkowski et al., 2009; Elmer, 2009).

The earthworm species *Aporrectodea caliginosa* (Savigny) and *Aporrectodea longa* (Ude) are among the most common in Swedish agricultural fields (Boström, 1988; Lagerlöf et al., 2012). They represent different ecological groups of earthworms and are therefore interesting as model organisms for studies of interactions with plants and microorganisms. Organisms used for biocontrol might be affected by and affect the environment and the community that they are introduced into. For example, the production of chitinases and other bioactive compounds by *B. amyloliquefaciens* might affect earthworms because they have chitin in their cuticle and setae (Jamieson, 1992; Miller and Harley, 1999). Therefore, to be able to develop BCAs, there needs to be an understanding of the different interactions at different ecological levels (Handelsman and Stabb, 1996).

Although the role played by microbial communities in disease suppression is beginning to unfold (Elmer 1995, 2003; Clapperton et al., 2001; Postma et al., 2005; Sharma and Sharma, 2008), the effects and mechanisms of interactions between these microbes and other soil organisms such as earthworms, and how these interactions in turn affect plant growth and productivity, are poorly understood (Elmer,

2009). The primary objective of this study was to assess whether the bacterium *B. amyloliquefaciens* and the earthworms *Ap. caliginosa* and *Ap. longa*, either separately or in combination, will influence *Al. brassicae* disease severity and growth and productivity of oilseed rape plants (*Brassica napus*). The secondary objective was to assess whether the presence of *Al. brassicae* or *B. amyloliquefaciens* at high concentrations influences the survival, growth and reproduction of the earthworms.

2. Material and methods

2.1. Study area

The study was conducted at the Swedish University of Agricultural Sciences (SLU), Ultuna Campus, Uppsala (59° 49′05″ N, 17° 39′28″ E) and consisted of two different box experiments performed from July to December 2014. The first experiment was performed outdoors in a net-enclosed experimental area that excludes birds but lets in sunshine and rain to mimic a field-like environment in summer (ca. 9 weeks in July–September). The plants were exposed to natural weather conditions and watered at least once a week. One earthworm species (*Ap. caliginosa*) was used. Because disease symptoms caused by the *Alternaria* infection were not obvious in the first experiment, a second pot experiment was performed in the greenhouse over ca. 8 weeks in October–December 2014, but under more controlled conditions concerning water and temperature regime and insect pest control. In this second experiment, two earthworm species (*Ap. caliginosa* and *Ap. longa*) were used in separate treatments.

2.2. Experiment set up

2.2.1. Soil preparation

Clay and sandy soil were collected at SLU's experimental farm at Ultuna, Uppsala. The soils were hand-sorted to remove roots, debris, stones, and macrofauna (*e.g.* earthworms and beetles) and thereafter frozen (48 h, −20 °C) and thawed (48 h, +20 °C) twice to reduce the remaining indigenous fauna. Such treatment is effective for reducing macrofauna and mesofauna, but not for nematodes and other microfauna (Sulkava and Huhta, 2003). During each experiment, the soils were mixed in a ratio of 6:3:1 by volume, 60% clay-loam soil, 30% sandy soil, and 10% rehydrated dried organic cow manure (Weibulls Concentrated® pelletized, NPK 2-1.5-1.7). The two mineral soils had 15% water content by weight, and the rewetted cow manure contained 50% water by weight when mixed into the experimental soil. The clay-loam soil contained 36.5% clay with a total carbon content of 1.5% and a pH of 6.6, and it was classified as Eutric Cambisol (Kirchmann et al., 1994). The sandy soil contained 2.7% carbon and had a pH of 6.3. The cow-manure was used as feed for the earthworms and as a nutrient supply for the plants.

2.2.2. Plants and microorganisms

Br. napus cv. Banjo (winter hybrid variety from SW Seed), the beneficial bacterial strain *B. amyloliquefaciens* subsp. *plantarum* UCMB5113 (Reva et al., 2004; Borriss et al., 2011), and the fungal pathogen *Al. brassicae* 980:3 were used for the experiment. *B. amyloliquefaciens* was grown in LB medium at 28 °C with agitation until a stationary phase was reached. The suspension was heat shocked for 5 min at 65 °C, and surviving spores were collected by centrifuging (10000 $g \times 5$ min). After washing the pellet in sterile MilliQ water, the spore density was determined using colony forming unit counts and the concentration was adjusted to 10^7 spores ml^{-1}. *Al. brassicae* was maintained on potato dextrose agar (PDA) medium at 4 °C and activated on PDA at 25 ± 1 °C. Fungal cultures grown overnight on PDA were used as the inoculum for the experiments.

2.2.3. Earthworms

Ap. caliginosa was used in the outdoor experiment, and both *Ap. caliginosa* and *Ap. longa* were used for the experiments in the greenhouse. *Ap. caliginosa* is endogeic and is the most common species in the agricultural soil of the area, while *Ap. longa* belongs to the functional group of anecic earthworms and is less abundant than *Ap. caliginosa*, especially in intensively cultivated soil (Lagerlöf et al., 2012). The worms were collected from agricultural and garden soils in the vicinity of Uppsala by digging and hand sorting. The earthworms were stored in 6 L opaque plastic boxes (measuring 55 cm long × 35 cm wide × 26 cm deep) with a moist soil mixture in a climate chamber at 17 °C for a maximum time period of 3 weeks (in June 2014 and in September 2014) and were fed with cow manure wetted to 50% moisture content and added at the soil surface on a need basis. The earthworms were gently rinsed in tap water immediately prior to their use in the experiment. New adult (clitellate) and sub-adult (tubercula pubertatis only) earthworms were used for each experiment (Fründ et al., 2010).

2.3. Experimental design

Both experiments were fully factorial with three factors: *Al. brassicae* (two levels: with, without); *B. amyloliquefaciens* (two levels: with, without) and earthworms (three levels: without, *Ap. caliginosa*, or *Ap. longa*). The outdoor experiment, where only *Ap. caliginosa* was used, thus consisted of 8 treatments, and the greenhouse experiment, where both earthworm species were used, consisted of 12 treatments. Both experiments were replicated three times. The outdoor experiment was set up in a net-enclosed area where 24 opaque plastic boxes (55 cm × 35 cm × 26 cm) were used and filled with soil up to about 20 cm height ($\sim$27 L). The boxes had holes in the bottom to drain excess water, and nets (1 mm mesh) to prevent the earthworms from escaping. In the greenhouse experiment, the same type of box was used. The boxes with different treatments were laid out in a completely randomized design in both experiments.

In each box, a 1 cm layer of potting soil was added as the top dressing where oilseed rape (*Br. napus)* seeds were sown. Seeds were sown in 10 positions in each box with five seeds per position. After emergence, 10 plants per box were allowed to grow with a spacing of 10 cm between rows and plants. The earthworms were weighed with gut contents as total fresh biomass per box before being added to the containers four days after sowing. To each box receiving the relevant earthworm treatment, 50 individuals of *Ap. caliginosa* or 25 individuals of *Ap. longa* were added. For *Ap. caliginosa*, this corresponds to 250 individuals m^{-2} and approximately 30 g fresh mass m^{-2}, and for *Ap. longa* this corresponds to 125 individuals m^{-2} and 50 g fresh mass m^{-2}. This is within the normal range of earthworm density in agricultural soil in Northern Europe (Lagerlöf et al., 2012; Christensen et al., 1987).

Ten days after sowing, 5 mL of a water solution with *B. amyloliquefaciens* strain 5113 (10^7 spores ml^{-1}) was added around the root zone of the plant seedlings in the relevant treatment using a syringe. Fourteen days after sowing, plant seedlings in the relevant treatment were infected with *Al. brassicae* pathogen in solution form by putting 50 μL drops on four separate spots per leaf per plant in all *Alternaria* treatments. At that time, each plant had four developed leaves and the drops were put on to the surface of each developed leaf.

The outdoor experiment was run for 63 days between 18 July and 19 September 2014, and the greenhouse experiment was run for 53 days between 24 October and 16 December 2014. In both experiments, an additional 100 g per box of cow manure, wetted to 50% moisture content by weight, was provided at the soil surface as food for the earthworms after 4 weeks. The same amount was added in all treatments with or without earthworms. The plants were watered as needed at least twice a week for the first month, and thereafter once a week. In the greenhouse experiment, temperature was set at 22 °C during the day and 18 °C at night. Light was on for 18 h and off for 6 h at night.

The outdoor experiment was subjected to normal prevailing summer weather conditions with additional watering as described above. Daily mean temperature for the experimental period (18 June–19 September) was +16.8 °C, with an average daily maximum temperature of +23.1 °C and an average daily minimum temperature of +10.5 °C. Total precipitation was 114.7 mm, and total insolation was 986.8 MJ/m^2 (SLU Climate station in Uppsala, 2016). In the outdoor experiment, larger insects such as Pieridae butterfly larvae were removed manually, but otherwise no pest control was performed. In the greenhouse experiment, no pest problems were seen except for Sciaridae flies that were controlled with sticky-paper traps.

2.4. Data collection

The presence of some disease symptoms on the plants in the outdoor experiment were difficult to distinguish from damage possibly caused by other outdoor factors such as insect pests and other pathogens as well as weather conditions. Therefore, disease scoring is not presented in this paper for the outdoor experiment, but only for the greenhouse experiment. Pathogen infection was scored for the greenhouse experiment by assessing the number and size of lesions on the leaves of each individual plant 21 days after pathogen inoculation. Disease severity was based on a scale of 0 to 5 (0 = no infection, 1 = 1–5% of the surface area covered by the disease, 2 = 6–10% covered, 3 = 11–20% covered, 4 = 21–30% covered, 5 = 31–100% covered) (Shrestha et al., 2005). The disease scores for all leaves of each plant were averaged per plant and calculated for each box as one observation.

At the end of the experiments, the aboveground plant biomass was harvested, and plant height measurements of each plant were taken. Harvested plants were oven-dried for 48 h at 70 °C and reweighed. The frequency of flowering or budding plants was also calculated in the greenhouse experiment in order to assess development stage. Earthworms from each box were sorted (as adults and juveniles), washed in tap water, counted, and then weighed together (all earthworms per pot in each age category) for biomass determination. Earthworm cocoons were retrieved by wet sieving of the soil through a steel wire net (mesh size 2 mm).

2.5. Statistical analyses

Data collected on plant height and biomass, flowers and buds, and earthworm survival, mass increments, juvenile number and mass, and cocoon number were subjected to analysis of variance (ANOVA GLM) using the Minitab 17 statistical package. Disease indices were analysed using the SAS statistical software package. Mean values of all plants or earthworms per box were used as one treatment replicate. The effect of the presence and absence of the added organisms (*Alternaria, Bacillus,* earthworms) on plant growth variables and their interactions were analysed by three-way ANOVA. Effects of the added microorganisms on earthworms' growth and reproduction were analysed by two-way ANOVA. Log transformed (LN + 1) values were used in the analyses, but normal values are shown in the results. The statistical significance was determined at $p < 0.05$, and significant differences among the different factors and treatments were evaluated using Tukey's post hoc multiple comparisons test.

In the greenhouse experiment, the disease score of all infected leaves was noted and the mean disease index per leaf for each plant was calculated. A mean disease index per leaf per plant, calculated for each box containing ca. ten plants, was used as one treatment replicate. The disease indices of all *Alternaria*-infested treatments were compared using two-way ANOVA in the GLM procedure in SAS. Pair-wise comparisons of treatments were made by Least Square Means calculations. The plants of the non-infected treatments were inspected and found not to have any signs of *Alternaria* infection, and thus they were not included in the statistical analysis of disease index.

Table 1
Height (cm) and dry mass (g/plant) of *Brassica napus* plants at the end of the outdoor experiment. Treatment factors: control without addition of organisms (C), infested with *Alternaria brassicae* (A), addition of the beneficial bacteria *Bacillus amyloliquefaciens* (B), and addition of the earthworm *Aporrectodea caliginosa* (E1). Mean and SE of three replicates. Significant *p*-values at *p* < 0.05 are in bold.

Treatment/factors	Height (cm)	Dr mass/plant (g)
C	71.0 (6.5)	9.9 (0.4)
A	78.6 (4.2)	5.9 (0.4)
B	66.3 (5.5)	7.0 (0.8)
E1	81.3 (0.5)	9.0 (1.6)
A + B	73.2 (4.4)	6.1 (0.5)
A + E1	83.8 (4.3)	7.7 (0.7)
B + E1	74.9 (3.7)	8.2 (0.9)
B + A + E1	76.4 (3.9)	7.4 (0.8)
Three-way ANOVA, factors and p-values:		
A	*0.166*	***0.010***
B	*0.093*	*0.172*
E1	***0.047***	*0.209*
*A*B*	*0.950*	*0.276*
*A*E1*	*0.367*	*0.249*
*B*E1*	*0.831*	*0.593*
*A*B*E1*	*0.985*	*0.273*

Note: Taller plants in treatments with E1 than without: *p = 0.047* (mean for E1 – containing treatments 78 cm vs. not 72 cm). Lower mass/plant in treatments with A than without: *p = 0.010* (mean for with 6.7 g vs. without 8.5 g). No differences among treatments (*p* > 0.05).

3. Results

3.1. Plant growth and productivity in the outdoor experiment

Table 1 shows growth parameters measured across the different treatments. Only plant height and weight were assessed, and no disease scoring was done. Comparison of treatments with and without the factors *Alternaria*, *Bacillus*, and earthworms showed a significant effect of earthworms (*p* = 0.047) on height – *i.e.* there were taller plants with earthworms present than without earthworms. Treatments with *Alternaria* had lower mass than those without (*p* = 0.010). There were no significant interactions, which indicates that neither the earthworms nor the BCA bacteria influenced the effects of the *Alternaria* infection.

3.2. Plant growth, productivity, and disease severity in the greenhouse experiment

No significant differences in height, weight, or flowers were observed among any of the 12 treatments of the greenhouse experiment, and no effects of combinations of the soil organisms on the growth parameters could be shown (Table 2).

Analysis of the disease index on plants infected with *Al. brassicae* by two-way ANOVA (Table 3) showed a significant interaction between the factors *Bacillus amyloliquefaciens* inoculation and earthworm addition (*p* = 0.045). Pair wise comparisons of treatments by Least Square Means revealed that the treatment with *Alternaria* + *Bacillus* added had significantly lower disease index than the treatment with *Alternaria* + *Bacillus* + *Ap. caliginosa*. Other treatments had intermediate values that did not differ significantly from these two treatments (Table 3).

3.3. Earthworm survival, growth, and reproduction

The earthworms used in the outdoor experiment (*Ap. caliginosa*) had conditions favourable for their survival, growth, and reproduction (Table 4). Survival increased when either *Bacillus* or *Alternaria* were added, but not when both were added together (interaction between *Alternaria* and *Bacillus*; *p* = 0.003) (Table 4). Survival ranged between 90% and 98%, and weight increments ranged between 229% and 259%. Cocoon production per worm was in the range of 4.3–5.6 among

Table 2
Height (cm), dry mass (g/plant), and flower bud/flowering frequency (proportion of plants with flower buds/flowers) of *Brassica napus* plants at the end of the greenhouse experiment. Treatment factors: control without addition of organisms (C), infested with *Alternaria brassicae* (A), addition of the beneficial bacteria *Bacillus amyloliquefaciens* (B), and addition of the earthworm *Aporrectodea caliginosa* (E1) or *Aporrectodea longa* (E2). Mean and SE of three replicates. No significant differences among treatments and treatment factors (*p* > 0.05).

Treatments/factors	Height (cm)	Dry mass/plant (g)	Bud and flower frequency
C	36.9 (2.4)	4.6 (0.7)	0.2 (0.4)
A	43.2 (3.9)	5.6 (0.7)	0.5 (0.8)
B	47.5 (5.8)	4.2 (0.4)	0.5 (0.7)
E1	49.1 (6.5)	7.1 (1.1)	0.7 (0.8)
A + B	46.7 (10.2)	4.3 (0.7)	0.6 (0.8)
A + E1	43.3 (2.6)	5.2 (0.6)	0.7 (0.7)
B + E1	49.5 (9.9)	5.2 (1.5)	0.4 (1.0)
A + B + E1	48.6 (3.5)	6.1 (0.7)	0.4 (0.8)
E2	54.0 (1.1)	6.1 (0.7)	0.8 (0.8)
A + E2	40.7 (1.8)	4.6 (0.6)	0.6 (0.7)
B + E2	39.2 (2.0)	4.5 (0.4)	0.3 (0.4)
A + B + E2	49.2 (8.3)	5.7 (0.7)	0.5 (0.7)
Three-way ANOVA, factors and p-values:			
A	*0.856*	*0.825*	*0.361*
B	*0.591*	*0.227*	*0.307*
E	*0.535*	*0.133*	*0.730*
*A*B*	*0.317*	*0.105*	*0.419*
*A*E*	*0.797*	*0.791*	*0.238*
*B*E*	*0.430*	*0.844*	*0.153*
*A*B*E*	*0.144*	*0.166*	*0.466*

Table 3
Disease index of *Brassica napus* plants in treatments infected with *Alternaria brassicae* (A) in the greenhouse experiment. Mean index per leaf per plant in boxes with 10 plants per box and SE for individual treatments (n = 3), and two-way ANOVA analysis (GLM) of disease index. Treatment factors: B = beneficial bacteria (*B. amyloliquefaciens*) inoculation, E = addition of earthworms (*Aporrectodea caliginosa* E1 or *Ap. longa* E2). Significant *p*-values at *p* < 0.05 are in bold. Tukey's pairwise comparisons: treatment mean values followed by the same letters are not significantly different at *p* < 0.05.

Treatment/factor	Disease index
A	2.09 (0.1) ab
A + B	1.11 (0.3) b
A + E1	2.04 (0.3) ab
A + B + E1	2.82 (0.4) a
A + E2	1.50 (0.1) ab
A + B + E2	1.87 (0.4) ab
Two-way ANOVA, factors and p-values:	
B	*0.694*
E	*0.054*
*B*E*	***0.045***

all boxes over the entire time period of the experiment (Table 4).

In the greenhouse experiment (Table 4), earthworm survival ranged between 96% and 99% for *Ap. caliginosa* and was 100% for *Ap. longa*. The difference in survival between the two species was significant (*p* < 0.05). The mass increment of the added earthworms was significantly higher for *Ap. caliginosa* than for *Ap. longa* (*p* < 0.05). Earthworm reproduction measured in terms of the number of cocoons per individual earthworm ranged between 6.5 and 8.3 for *Ap. caliginosa* and between 7.3 and 10.6 for *Ap. longa* among all of the boxes. At the end of the experiment, *Ap. caliginosa* had produced 87–157 juveniles per box in the different treatments and *Ap. longa* had produced 27–39 juveniles. There were no significant difference in earthworm survival, growth and reproduction due to the experimental treatments. However, there was a tendency of higher mass increment of *Ap. caliginosa* in treatments with *Alternaria* or *Bacillus* (interaction *Alternaria* and *Bacillus*; *p* = 0.059) than in the control without these organisms added,

Table 4

Earthworms' survival, growth and reproduction in the outdoor (1) and greenhouse (2) experiments recorded at the end of the experiments. Survival (%) and individual mass increment (%) of 50 individuals of *Aporrectodea caliginosa* (E1) or 25 individuals of *Ap. longa* (E2) per box introduced from start (adults or sub adults). Reproduction is presented as number of egg cocoons and juveniles (only greenhouse experiment) produced per adult earthworm. Mean and SE of three replicates. Significant *p*-values at $p < 0.05$ are in bold. Columns with the same lower case letters are not significantly different at $p < 0.05$. For description of treatments and treatment factors see Table 1 and 2.

Treatment	Survival (%)	Mass increment (%)	Cocoon prod./ worm	Juv. prod./ worm
1: Outdoor experiment				
Ap. caliginosa (E1)				
E1	90 (2) b	229.3 (24)	4.9 (1.0)	–
E1 + A	98 1.2) a	243.5 (21.8)	5.6 (1.8)	–
E1 + B	97 (1.3) a	259 (29.6)	4.3 (0.7)	–
E1 + A + B	93 (0.7) ab	254.7 (27.2)	6.4 (0.5)	–
Two-way ANOVA, factors and *p*-values				
A	*0.184*	*0.819*	*0.382*	–
B	*0.625*	*0.464*	*0.984*	–
A*B	***0.003***	*0.724*	*0.784*	–
2: Greenhouse experiment				
Ap. caliginosa (E1)				
E1	96 (2)	92.5 (7.8)	7.6 (3.0)	3.3 (0.9)
E1 + A	99 (1.3)	150.2 (13.6)	8.2 (1.9)	1.8 (0.6)
E1 + B	98 (1.2)	123.3 (24.6)	6.5 (1.0)	1.9 (0.3)
E1 + A + B	97 (1.8)	92.8 (21.2)	8.3 (1.7)	2.6 (0.7)
Two-way ANOVA, factors and *p*-values				
A	*0.926*	*0.349*	*0.389*	*0.630*
B	*0.922*	*0.456*	*0.551*	*0.898*
A*B	*0.541*	*0.059*	*0.691*	*0.123*
Ap. longa (E2)				
E2	100	25.6 (14.3)	7.3 (1.3)	1.1 (0.3)
E2 + A	100	45.5 (20.7)	9.7 (1.0)	1.6 (0.5)
E2 + B	100	37.5 (9.8)	10.6 (2.8)	1.4 (0.4)
E2 + A + B	100	39.1 (7.1)	9.9 (1.7)	1.4 (0.4)
Two-way ANOVA, factors and *p*-values				
A	*0.356*	*0.705*	*0.964*	*0.539*
B	*0.356*	*0.909*	*0.977*	*0.761*
A*B	*0.985*	*0.608*	*0.983*	*0.573*

Note: In greenhouse experiment, survival larger in E2 than in E1; mass increment larger in E1 than in E2 ($p < 0.05$).

but not when both microorganisms were present (Table 4).

4. Discussion

This study showed negative effects of the *Al. brassicae* pathogen on *Br. napus* plant growth under outdoor conditions. Although plants in the treatment with addition of *B. amyloliquefaciens* in the greenhouse experiment had the lowest disease index among the *Alternaria*-infected treatments, the disease reducing effects of this BCA was not statistically significant and therefore could not be demonstrated by this experiment (Table 3). Also, *B. amyloliquefaciens* did not increase plant height or biomass in either of the two experiments. These results are not consistent with Danielsson et al. (2007) who found that the same strain of *B. amyloliquefaciens* used in this study protected *Br. napus* against several fungal pathogens, including *Al. brassicae*, when tested in an *in vitro* experiment. They found that the selected bacterial strain gave protection against both soil-borne pathogens that infect roots and foliar pathogens, such as *Al. brassicae*. In addition, they found that *B. amyloliquefaciens* had a positive effect on seed set but did not affect growth.

Our results show that in a more complex environment – containers with soil instead of *in vitro* – effects of BCAs can be difficult to demonstrate or can be blurred by additional factors. This has been found also in many other studies when laboratory results have been tested in soil environments (Friberg et al., 2006; Marschner et al.,

2004). The interaction between earthworms and *B. amyloliquefaciens* found in the analysis of disease index, with the highest disease index observed in the treatment with *Ap. caliginosa* added (Table 3; A + B + E1), could be an additional example of such an inhibition of expected effects when the environment is diversified.

The results of this study showed positive effects of earthworms on plant height, but they showed no significant contributions of *Ap. longa* or. *Ap. caliginosa* in reducing *Al. brassicae* disease. Several studies have reported significant contribution of earthworms in reducing the effects of pathogens on plants, which therefore do not agree with our findings. Earlier positive results of pathogen suppression by earthworms used different combinations of plants, pathogens and earthworm species than we did. For instance, Elmer (2009) reported 60–80% increase in plant mass production and 50–70% reduction in disease symptoms in vegetable crops infested with different *Fusarium* and *Verticillium* species, when the anecic earthworm *Lumbricus terrestris* was added to the soil. Although *Ap. longa* that was used in our greenhouse study also is a deep burrowing anecic species, it is not as large as *L. terrestris* and might therefore have less influence on soil conditions. Clapperton et al. (2001) found that the endogeic earthworm *Ap. tuberculata* increased yield of wheat infested with *Geomannomyces graminis* var. *tritici,* but it did not affect infection symptoms of the parasitic fungus. Their results indicate that the positive effect mediated by the earthworms would have resulted from changes of the soil microflora that in turn increased plant growth. *Ap. caliginosa* is similar in size and behaviour to *Ap. tuberculata* and could have had a similar effect although we could only detect significant effects on height of plants. Stephens and Davoren (1997) studied the effects of the endogeic earthworm species *Ap. trapezoids* and *Ap. rosea* on root infection and plant production of subterranean clover and ryegrass grown in soil inoculated with *Rhizoctonia solani*. They found that the added earthworms did increase above and below ground plant production and in most cases decreased root infection by *R. solani*. In treatment without addition of *R. solani*, the earthworms had no effect on plant production.

In addition to different plant and earthworm species compared to our study, the fungal pathogens involved in the above examples were inoculated to the soil of the experimental vessels. The pathogen *Al. brassicae* used in our study was inoculated to the above-ground parts of the plants and was therefore not in direct contact with the earthworms in the soil or with the biocontrol bacteria that were added to the rhizosphere. Interactions between the pathogen and the BCA, giving effects on plant growth and health, would therefore have been indirect.

Biocontrol bacteria can increase plant resistance through priming of induced systemic resistance, and this is a common mechanism of action for many beneficial bacteria (Choudhary and Johri, 2009). *Bacillus* could also have enhanced the plants' ability to take up nutrients from the soil by strengthening of the root system in a manner common to many plant growth promoting rhizobacteria (Lugtenberg and Kamilova, 2009). The *B. amyloliquefaciens* strain 5113 can stimulate plant growth as well as prime induced systemic resistance thus providing plants with more resources and improved vigour and disease resistance (Asari, 2015).

In our experiments, nutrients and water were probably not limiting factors for plant growth hence additional mineralisation due to earthworm activities would not have induced higher plant growth to a large extent, especially not in the greenhouse experiment where water supply was optimal and disturbing factors such as insects and weather-related factors were at a minimum. In the outdoor experiment, plants were relatively more stressed and the effects on plant growth of the BCA bacteria and earthworms were more obvious.

The earthworms used in the experiment had favourable conditions for their reproduction, growth, and activity in all treatments, as demonstrated by the high survival and substantial fresh mass increments and reproduction observed in this study. This is an indication that the experiment was conducted under good conditions as suggested by Fründ et al. (2010), hence the results of this study ought to be

considered robust. The fact that no negative effects of the added microorganisms were detected in growth and reproduction among treatments supports the findings by Lagerlöf et al. (2015), who demonstrated that exposure of earthworms to the biocontrol bacterium *B. amyloliquefaciens* does not harm them. On the contrary, the added microorganisms enhanced the survival and growth of *Ap. caliginosa*. We can only speculate on the cause of this stimulation by the added microorganisms. Possibly *B. amyloliquefaciens* could be used as an additional food source and perhaps both microorganisms created an improved soil chemical environment for earthworms – while in combination such an improvement did not occur. Earthworms are in all cases affected by the interaction between plant roots and rhizosphere microorganisms that very actively influence the soil environment (Bonkowski et al., 2009). However, based on this study we cannot exclude negative effects of the added microorganisms on earthworms under more natural conditions where the earthworms might be more stressed.

The *B. amyloliquifaciens* 5113 strain has earlier been shown to improve disease resistance in *Br. napus* (Danielsson et al., 2007) and was therefore chosen for this study. Our study is the first to test the effects of *B. amyloliquefaciens* on *Brassica* in box experiments with soil, and it is the first study to test the combined effects of earthworms and BCA bacteria on plant growth and health. We had only three true replicates in the experiments. Although each replicate represents the mean of the effects on several plant individuals and earthworms, we were probably not able to point out some significant treatment effects because of a small sample size.

5. Conclusions

This study did not give any clear indication of the usefulness of *B. amyloliquefaciens* for biocontrol of plant pathogens such as *Al.* brassicae when growing plants in natural soil. In addition, no significantly positive effects from the tested earthworm species were seen. Although, no significant contributions were detected of the combined effects among the tested soil organisms on plant growth and productivity, earthworms alone showed to significantly promote height growth. Earthworm growth and reproduction were neither affected by *B. amyloliquefaciens* nor *A. brassicae*. These microorganisms had no synergic effect on earthworm survival, but acting separately, they significantly increased earthworm survival in the outdoor experiment. The next step in taking advantage of these finding for the development of sustainable agricultural systems that use ecosystem services would be to scale up the research and to perform experiments under field conditions. Further studies of interactions between plants, BCAs, and the wider communities of soil organisms are also needed for understanding of underlying mechanisms leading to biological control of plant diseases.

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