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Potential side effects of biocontrol and plant-growth promoting *Bacillus amyloliquefaciens* bacteria on earthworms

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ABSTRACT

Many bacteria strains are now successfully used for plant-growth promotion (PGPR) and as biocontrol agents (BCA) against plant diseases. Mechanisms behind their action involve production of enzymes and antibiotics, which in high concentrations could also affect non-target organisms hence the biodiversity and processes in the soil. Despite these potential negative side effects, there is little research done on the subject to confirm whether they are significant. In three laboratory experiments, we tested the effect of the bacterial BCA *Bacillus amyloliquefaciens* UCMB5113 (BA) on two earthworm species, common in agricultural soils in temperate regions of the world and representing different ecological groups; one anecic (*Aporrectodea longa*) and one endogeic species (*Aporrectodea caliginosa*). The earthworms were kept in replicated pots containing soil from local agricultural fields. They were fed on cow manure, and exposed to BA by (1) dipping into a BA solution (short-term external exposure in high concentration), (2) mixing BA solution into the soil (long term external and internal exposure) and (3) feeding earthworms with BA infested plant litter (internal exposure of the gut).

After 1–2 months, survival, growth and reproduction of the earthworms were recorded. We found no effect of the treatments as compared to control without BA amendments. We conclude that the use of high doses of BA with concentrations at the same magnitude as maximally expected when the bacteria are used as PGPR and BCA, is not harmful to the soil dwelling earthworms tested in this project. Further studies of the ecological effects of PGPR and BCA bacteria on other non-target soil organisms are encouraged. The development of sustainable agricultural systems, where ecosystem services are optimized, has to be aided by a deeper knowledge of the combined effect of bacteria and earthworms on the promotion of plant health.

1. Introduction

In recent years, scientific attention has been drawn to the effects of rhizobacteria as beneficial to plants: plant-growth promoting rhizobacteria (PGPR), enhancing plant tolerance against abiotic stress, and biological control agents (BCA) against plant diseases and insect pests (Dimkpa et al., 2009; Lugtenberg and Kamilova, 2009; Pieterse et al., 2014). Several bacteria, including strains of the genera *Pseudomonas* and *Bacillus*, are now available commercially as BCAs and are successfully used instead of chemical pesticides in crop production (Choudhary and Johri, 2009). PGPRs can stimulate plant growth in different ways, e.g. enhance seed germination and emergence, stimulate root development and thus mineral, nutrient and water uptake, as well as suppress diseases. The underlying mechanisms of beneficial rhizobacteria for protection of plants against parasitic root colonizing microorganisms include priming of induced systemic resistance and production of enzymes such as chitinases, peroxidases and proteases, and many types of antibiotics (Pieterse et al., 2014). This production does not only affect microorganisms and their interactions with plants but is also known to suppress nematodes and techniques for use of bacterial BCA against plant parasitic nematodes are being developed (Abally 2012; Mutua et al., 2011; Niazi et al., 2014 Wepuhkhulu et al., 2011).

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It is suspected that the use of bacterial BCAs would also affect many other non-target soil organisms and therefore influence soil processes and biodiversity. This has so far not received much attention. For example, earthworms, like nematodes, have chitin in their cuticle, especially in their setae (Jamieson, 1992; Miller and Harley, 1999), and therefore could be negatively affected by addition of microorganisms producing chitinase. Although biocontrol bacteria occur naturally in soil, amending them in large concentrations to soils and plants could imply environmental risks. Therefore, thorough assessment of environmental impacts of BCAs needs to be carried out prior to their development and registration for use in plant production to avoid ecotoxicological effects at different trophic levels in the local ecosystem.

Many *Bacillus* species are ubiquitously present in soil and can become enriched in the rhizosphere depending on root exudates. Phenotypically high ecological diversity has been found among different *Bacillus* species with plant interaction resulting both in epiphytic and endophytic colonization (McSpadden Gardener, 2004). Many strains of *Bacillus subtilis*, *Bacillus cereus* and *Bacillus amyloliuefaciens* have been found to interact with plants and produce beneficial effects including disease suppression (Choudhary and Johri, 2009). The type strain of plant-associated *B. amyloliquefaciens* FZB42 has been shown to produce a variety of secondary metabolites involved in microbial antagonism and thus supporting disease suppression of plants (Chen et al., 2009), and this also includes chitinase (Niazi et al., 2014).

In the present study we have tested the effect of the bacterial BCA *B. amyloliquefaciens* UCMB5113 (here after abbreviated as BA) on the survival, growth and reproduction of two earthworm species that are common in agricultural soils in temperate regions of the world and represent two different ecological groups (Bouché, 1977). Although the BA bacteria are not yet available as a commercial BCA, substantial research has been done on its effect on plant growth and health as well as the underlying mechanisms of action (Danielsson et al., 2007; Sarosh et al., 2009) and genomic and phenotypic analysis infer a close relationship with the type strain FZB42 (Niazi et al., 2014).

The aim of the study was to ascertain whether *B. amyloliquefaciens* UCMB 5113 (BA) has any effect on earthworms when exposed directly to a solution of the bacteria, or to soil or feed inoculated with the bacteria.

2. Material and methods

2.1. Test organisms

The tested earthworm species were *Aporrectodea longa* (Ude) and *Aporrectodea caliginosa* (Savigny). The former belongs to the ecological category of anecic earthworms. It generally feeds on plant litter on the surface, buries litter into the soil and creates burrows from the surface down through the soil profile. The latter is an endogeic species that lives and feeds in the soil profile where it consumes large quantities of soil and organic matter but are not so selective towards fresh litter. The earthworms used were collected from agricultural and garden soils in the vicinity of Uppsala by digging and hand sorting. Prior to their use in the experiments, the earthworms were maintained in a climate chamber at 18 °C for up to two months, in 6-l boxes with soil of the same quality as used in the experiments (see description of soil below), and were fed with rehydrated dry cow dung added once a month and mixed into the superficial layer of the soil. We used new earthworms for each experiment. They were adults with fully developed clitellum or subadults with early signs of clitellum development and all chosen specimens were in full vigour.

B. amyloliquefaciens subsp. *plantarum* UCMB5113 (Borriss et al., 2011) (BA) was grown in LB medium at 28 °C with agitation until stationary phase was reached. The suspension was heat shocked for 5 min at 65 °C and surviving spores collected by centrifugation. After washing the pellet in sterile MilliQ water, the density was determined using colony forming unit counts and the concentration adjusted with sterile water to 10^7 ml^{-1}.

2.2. Experimental set up

The study was conducted in laboratories, based at the Swedish University of Agricultural Sciences (SLU), Uppsala (59°49′05″N, 17°39′28″E). In mesocosm experiments, we exposed earthworms to BA by (1) dipping into a bacteria solution (short term external exposure in high concentration), (2) mixing the bacteria into the soil where the earthworms were kept (long term external and internal exposure) and (3) feeding earthworms with bacteria infested plant litter (internal exposure of the gut).

Three different experiments were done with various combinations of exposition methods and earthworm species, summarized in Table 1. The experiments were preceded by preliminary studies where soil mixture, moisture level and feeding were tested. Water content appeared to be the most critical since the soil became hard and impenetrable for the earthworms if allowed to dry out. The vessels used in experiments 1 and 2, were cylinders made from PVC plastic sewage pipes with 14.5 cm inner diameter and 30 cm height. At the bottom of the cylinders, nylon mesh (mesh size 1 mm) was attached with a rubber band to allow good drainage of the soil and prevent earthworms from escaping. The walls of the cylinders extended ca 15 cm above the level of the soil surface, to prevent earthworms from escaping. The top of the cylinders were loosely covered with transparent polyethene plastic bags in order to minimize evaporation. For experiment 3, opaque plastic boxes (27 cm × 17 cm wide × 13 cm deep) were used. They were perforated in the bottom to allow drainage and the internal base of the vessels was covered with nylon net to prevent escape of

Table 1
Characteristics of three laboratory experiments testing effects of the biocontrol and plant-growth promoting *Bacillus amyloliquefaciens* UCMB5113 bacteria to the earthworms *Aporrectodea longa* and *Aporrectodea caliginosa*.

Experiment	1	2	3
Species	*A. longa*	*A. longa* *A. caliginosa*	*A. longa* *A. caliginosa*
Exposition methods	short term external; long term external and internal exposure	long term external and internal exposure; internal exposure of the gut	short term external
Vessels	3 l cylinders	3 l cylinders	6 l boxes
Moist soil[a] (kg)	1.5	1.5	4.0
Treatments	4	10	4
Replicates	6	6	3
Starting date	February 2, 2014	July 28, 2014	August 20, 2014
Duration (days)	57	28	28

[a] 15% water content.

earthworms. The boxes had no lid and were covered with a net and a nylon sheet in order to avoid excessive evaporation and infection of Sciaridae flies (See Table 1). The boxes provided a greater soil volume than the cylinders allowing for a higher number of earthworms and less laborious handling.

The vessels were filled with a moist soil mixture (15% water) consisting of 60% clay–loam soil and 30% sandy soil and 10% cow manure. The clay–loam soil contained 36.5% clay, total carbon content was 1.5%, pH 6.6, and was classified as Eutric cambisol (Kirchmann et al., 1994). The sandy soil contained 2.7% carbon and pH was 6.3. Both soils were collected from the experimental farm area of the SLU University in the vicinity of 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. This would be efficient for reduction of macro- and mesofauna but not for nematodes and other microfauna (Sulkava and Huhta, 2003). Dried cow manure (Weibulls® concentrated, dried organic cow manure) was wetted to 50% moisture content before being mixed into the experimental soil as feed for the earthworms. The particle size of the manure was on average less than 1 mm with no particles larger than 3 mm. In experiment 1, an additional amount of 100 g of wetted cow manure was added per cylinder at day 29 of the experiment as feed for the worms. The manure was evenly mixed into the soil in all experiments and also when additional manure was added in experiment 1. The water content of the mineral soil was ca. 15% by wet weight at the start of the experiment and the soil mixture was wetted to field capacity before introducing the earthworms.

The procedure for the three exposure methods was as follows: in the dipping method (treatments DS and D in experiments 1 and 3) earthworm specimens were dipped for 15 s into a BA spore solution in sterile water with 1×10^7 cells ml^{-1}. In the Control (C), worms were dipped into deionised water for 15 s before being added to soil-filled cylinders. In treatments with BA mixed into the soil (Experiments 1 and 2; treatments S, DS, SL, and SL+) 150 ml of BA spore solution in sterile water (1×10^7 cells ml^{-1}) was poured over the soil. To distribute it more evenly, we did not pour the whole solution on top of the soil. Instead, it was added in three portions; after filling 1/3, 2/3 and 3/3 of the whole soil volume. Amendment to leaves (treatment L+ in experiment 2) was done by keeping leaves in the BA solution for 1 min and then the excess liquid was shaken off gently to mimic spray administration of *Bacillus* with subsequent runoff. Leaves treated with water only, served as a control (treatment L). In a similar way to what we did with the BA solution to distribute it more evenly, we added 4 g of amended or control leaves on top of the first third of the total amount of soil mixture, then another third of soil was added to the cylinder and 4 g more of amended leaves, and so with the third portions of soil and leaves. An additional 4 g of leaves was added on the surface after one and two weeks in L+, L and SL+ treatments (see Table 2).

Two earthworm specimens were added to each experimental cylinder and four *A. longa* or six *A. caliginosa* to each box. The experimental units were arranged in a randomized design and kept in darkness at 17–19 °C in a climate chamber for the duration of the experiments (see Table 1). They were covered with transparent plastic bags in order to prevent excessive evaporation, and watered regularly. They were moved around every second week in order to minimize effects due to any local differences in temperature and evaporation rates. Each individual earthworm was weighed at the start and end of the experiments after being washed in cold tap water and dried on paper tissue. The individual fresh mass was also recorded at day 29 in experiment 1. At the end of the experiments, all cocoons produced were sorted out by wet sieving of the soil over a mesh (mesh size 2 mm) and counted.

The three experiments were arranged as indicated in Tables 1 and 2. Experiment 1 included four treatments with *A. longa* as follows: (1) DS: dipping earthworms into BA solution + mixing BA into the soil; (2) D: dipping earthworms in BA solution + no mixing; (3) S: no dipping + mixing BA into the soil; (4) C: control, no dipping + no mixing (Table 2). To repeat and extend experiment 1, we added treatments with another earthworm species (*A. caliginosa*) and an alternative exposure method, where the earthworms were exposed to BA amended plant material (*Brassica napus* leaves) as food. In this case, both the external and internal tissues of the earthworms were exposed to the BA bacteria. Since results from experiment 1 had shown considerable earthworm weight increase and cocoon production during the first month, we judged that a shorter period would give reliable results. Hence, the experimental duration was shortened to 28 days for experiments 2 and 3. The treatments for experiment 2 were: control (C) without addition of BA; addition of BA by pouring 150 ml of bacteria solution into the soil (S), like in the earlier experiment; addition of BA-amended *B. napus* leaves (L+); addition of leaves treated with water only (L); addition of BA to the soil and addition of BA amended leaves (SL+). These five treatments were set up with *A. longa* (treatment 1–5) and with *A. caliginosa* (treatment 6–10). Experiment 3 included 4 treatments, dipping *A. longa* and *A. caliginosa* in BA solution and their respective controls (Tables 1 and 2).

Table 2
Treatments in the three lab experiment testing effects of *Bacillus amyloliquefaciens* UCMB5113 (BA) on earthworms.

Experiment: treatment	1	2	3
1	DS.long	C.long	D.long
2	D.long	S.long	C.long
3	S.long	L+.long	D.cal
4	C.long	L.long	C.cal
5		SL+.long	
6		C.cal	
7		S.cal	
8		L+.cal	
9		L.cal	
10		SL+.cal	

Notes: D = dipping earthworms into BA solution; S = addition of BA by pouring 150 ml of bacteria solution into the soil; C = control; L+ = addition of BA-amended *Brassica napus* leaves; L = addition of leaves treated with water only; long = *Aporrectodea longa*; cal = *Aporrectodea caliginosa*.

2.3. Statistical analysis

Data for earthworm mass, relative mass increase and cocoon production were analysed using a general linear model (GLM) with treatments as model components. When significant effects were found ($P < 0.05$), Tukey's pairwise comparisons was used to compare treatment means. Minitab 16 Software was used for all analyses.

3. Results

3.1. Experiment 1

The mortality of earthworms was rather high in this experiment. However, it did not differ significantly between treatments ($P = 0.903$). In Table 3, column n shows the number of populated mesocosms (with one or two live worms per mesocosm). The surviving earthworms grew well and had on average increased from 2.2 g fresh mass at the start to 3.9 g at the end of the experiment (Table 3). There were no significant differences in earthworm individual growth between treatments after 29 days or 57 days from the start ($P = 0.25$ and $P = 0.69$, respectively). Relative

Table 3
Experiment 1: survival, biomass evolution and cocoon production of the earthworm *Aporrectodea longa* in a mesocosm experiment testing influence of *Bacillus amyloliquefaciens* UCMB5113 (BA).

Treatment	Start		29 days			57 days			Cocoons
	n	Fresh mass (g ind^{-1})	*n*	Fresh mass (g ind^{-1})	Relative increment%	*n*	Fresh mass (g ind^{-1})	Relative increment%	
1. DS	6	2.20 (0.38)	4	3.37 (0.17)	53.2 (23.9)	4	4.51 (0.65)	105 (38.8)	0.12
2. D	6	2.10 (0.39)	5	2.73 (0.38)	30.0 (6.6)	5	3.62 (0.40)	72.4 (25.9)	0.20
3. S	6	1.99 (0.34)	4	3.78 (0.41)	89.9 (16.7)	4	3.82 (0.86)	92.0 (37.1)	0.25
4. C	6	1.82 (0.35)	4	3.33 (0.40)	83.0 (10.7)	4	4.33 (0.44)	137.9 (10.6)	0.25
P value		0.91		0.25	0.16		0.69	0.70	

Note: Mean individual fresh mass (g per individual), relative increment from the start (Relative increment%), and SE (within brackets) of the number of mesocosms per treatment with live earthworms (*n*), which decreased during the course of the experiment; at the start, and at 29 days and 57 days after start. Treatments: 1. DS = dipping into BA solution, mixing BA into the soil; 2. D = dipping into BA solution; 3. S = mixing BA into the soil; 4. C = control, no dipping or mixing into the soil. *P* value = testing differences between treatments with Anova.

increment of earthworm biomass did not differ between treatments, either after 29 days ($P = 0.16$) or after 57 days ($P = 0.70$). Cocoon production amounted to a maximum of 0.25 cocoons per earthworm.

3.2. Experiment 2

In this experiment all earthworms survived and gained mass during the four week experimental period (Table 4). The results confirmed earlier observations in experiment 1 where there was no significant effect of adding a solution of BA to the soil ($P > 0.05$). In addition, offering leaves amended with the BA solution as food did not affect either growth or cocoon production of any of the two species ($P > 0.05$). However, relative increment in mass of *A. caliginosa* was larger in treatment SL+.cal ($P = 0.029$), with the combined addition of BA amended leaves and BA to the soil, as compared to the control. Cocoon production was considerably higher than in experiment 1. For *A. longa*, mean for the different treatments was between 2.92 and 4.17 cocoons per individual but did not differ significantly among treatments ($P = 0.921$). The corresponding value for *A. caliginosa* was between 6.50 and 9.58 and it did not differ significantly among treatments either ($P = 0.421$).

3.3. Experiment 3

Effects of dipping earthworms into the BA solution are shown in Table 5. Growth of earthworms in absolute or relative terms did not differ between treatments ($P = 0.778$ and $P = 0.768$ for *A. longa* and $P = 0.880$ and $P = 0.976$ for *A. caliginosa*) and mean values were larger than in experiment 2. Cocoon production did not differ between treatments either ($P = 0.417$ for *A. longa* and $P = 0.613$ for *A. caliginosa*), but mean values were considerably lower than in experiment 2 (Table 5).

4. Discussion

We aimed to conduct the experiments in soil conditions similar to the soil where the earthworms were collected, which was the agricultural soil of the Uppsala area. In a preliminary study, we had some initial problems with the experimental conditions and found that the clay dominated soil got very hard and impenetrable when drying out, which affected earthworm survival. Therefore keeping moisture within favourable limits is a must for successful lab experiments with earthworms. Lowe and Butt (2005) suggest a moisture content of 25% wet weight for cultures of *A. longa* and three other earthworms of the same family (Lumbricidae).

Table 4
Experiment 2: individual fresh mass (g per individual) and individual cocoon production of the earthworm *Aporrectodea longa* or *Aporrectodea caliginosa* in a mesocosm experiment testing influence of *Bacillus amyloliquefaciens* UCMB5113 (BA).

	Aporrectodea longa			
Treatment	Initial fresh mass (g ind^{-1})	Final fresh mass (g ind^{-1})	Relative increment%	Cocoons per worm
1. Control	2.71 (0.20)	4.44 (0.23)	63.8 (8.7)	2.92 (0.93)
2. S	2.75 (0.20)	4.10 (0.22)	49.1 (5.4)	3.42 (0.80)
3. L+	2.53 (0.20)	4.15 (0.29)	64.0 (10.6)	3.67 (0.99)
4. L	2.72 (0.23)	4.71 (0.26)	73.2 (9.8)	3.33 (0.79)
5. S L+	2.86 (0.31)	4.81 (0.29)	68.2 (10.8)	4.17 (1.25)
Anova *P* values	0.894	0.223	0.358	0.921
	Aporrectodea caliginosa			
Treatment	Initial fresh mass	Final fresh mass	Relative increment%	Cocoons per worm
6. Control	1.71 (0.13)	2.40 (0.17)	40.4 (3.9) B	9.58 (2.22)
7. S	1.61 (0.08)	2.62 (0.14)	62.7 (7.8) AB	9.75 (1.45)
8. L+	1.56 (0.10)	2.45 (0.13)	57.1 (5.3) AB	8.92 (1.08)
9. L	1.58 (0.06)	2.42 (0.10)	53.2 (3.7) AB	6.83 (1.48)
10. S L+	1.40 (0.07)	2.32 (0.13)	65.7 (13.7) A	6.50 (1.85)
Anova *P*	0.245	0.627	0.029 *	0.421

Note: Mean and SE (within brackets), n = 6. 28 days experimental time (28/7–25/8 2014). Treatments: 1. Control = no application of BA or *Brassica napus* leaves; 2. S = mixing BA into the soil, no leaves added; 3. L+ = leaves with BA added, no BA into the soil; 4. L– = Leaves without BA added, no BA into the soil; 5. SL+ = mixing BA into the soil, leaves with BA added. Testing differences between treatments = Anova P value (* = significant difference). All earthworms in all treatments survived the experimental time. Values with different letters in a column indicate significant differences ($P < 0.05$).

Table 5
Experiment 3: dipping earthworms (*Aporrectodea longa*,*Aporrectodea caliginosa*) into a bacteria solution of *Bacillus amyloliquefaciens* (10^7 cells ml^{-1}) and into water (control).

Treatment/species	Initial fresh mass (g ind^{-1})	Final fresh mass (g ind^{-1})	Relative increment%	Cocoons per worm
A. longa				
Water dipping	2.18 (0.18)	4.47 (0.35)	105.0 (9.44)	0.68 (0.55)
Bacteria dipping	2.23 (0.17)	4.35 (0.23)	95.1 (13.9)	0.17 (0.08)
Anova *P* values	0.826	0.778	0.768	0.417
A. caliginosa				
Water dipping	0.98 (0.046)	2.03 (0.079)	107.1 (9.8)	0.67 (0.17)
Bacteria dipping	0.99 (0.051)	2.01 (0.075)	103.0 (13.0)	0.83 (0.26)
Anova *P* values	0.906	0.880	0.976	0.613

Note: Mean and SE (within brackets) of fresh mass of earthworms at the start and after 29 days, relative increment and cocoon production per individual. Mean of 4 worms per container for *A. longa* and 6 worms per container for *A. caliginosa*, replicated in 3 containers with 4 l of soil. Testing differences between treatments = Anova *P* value.

The conditions and viability of the worms is also a delicate issue. In experiment 1, the *A. longa* specimens used were collected from the field in October–November and had been kept in cultivation boxes for two months before the start of the experiment in February. High mortality and low cocoon production could be due to less favourable conditions of the worms during storage and perhaps also, because they were at the end of their life cycle. In experiments 2 and 3, which were done during the summer, the worms were in good conditions and moisture was regularly controlled. This ensured a 100% survival and high reproduction with little variation among replicates.

In all experiments, the earthworms were provided with sufficient amounts of feed. This is necessary when studying the interaction of earthworms with their environment since they would otherwise go into diapause or try to escape from the experimental soil units. Boström (1988) and Boström and Lofs-Holmin (1986) found that *A. caliginosa* went into estivation in an earthworm growth experiment, as soon as the added food resource was depleted. The feed was mixed into the soil of the mesocosms of all treatments, although *A. longa* is an anecic species that feeds mainly on the soil surface. According to some authors (e.g. Boyle, 1990; Lowe and Butt, 2002), earthworms, especially anecic and epigeic species, but also endogeics, grow better if the feed is placed on the soil surface. Lofs-Holmin (1983) however, found that mixing of feed into the soil gives just as good growth and reproduction, and it is practical since the risk of drying out of fodder is minimized and infection of the substrate with, e.g. Sciaridae fly larvae is less likely to occur. Increase in mass for both species gives an indication that experimental conditions were favourable for their activity. In the case of *A. caliginosa* this increase ranged between 41 and 112%, which is lower than the average 196% increase reported by Eriksen-Hamel and Whalen (2006), and by Vercesi et al. (2006). If relative mass increase of the earthworms is a response factor, it is important to have specimens within the same mass range since relative growth rate decreases as the animals become larger. Based on this, it should be noted that, whereas juveniles were used in these experiments, in our experiment only adults and sub-adults were used, hence lower growth rates are expected. In the case of *A. longa*, their relative increase in body mass, ranging 50–138%, more than doubled the 25.81% obtained by Butt (1993) in a 3-month long study. The higher relative increment in treatment 5 of experiment 2 (Table 4) could also be a result of somewhat smaller worms used in that treatment as compared to the other treatments. Cocoon production, which ranged 0.027–0.287 and 0.004–0.104 cocoon $worm^{-1}$ day^{-1}, for *A. caliginosa* and *A. longa*, respectively, showed a higher production for the former than for the latter. Reported values for cocoon production in similar temperatures as in our study for *A. caliginosa* include averages of 0.09 and 0.221 cocoon $worm^{-1}$ day^{-1} (Boström, 1988; Garvín et al., 2002; Vercesi et al., 2006); the lowest value may also be due to the inclusion of juveniles in the study, while the highest value is within our range. Butt (1993) and Holmstrup (1999) report that *A. longa* produced an average of 0.052 and 0.090 cocoon $worm^{-1}$ day^{-1} in their experiments, respectively. The former being included in our range, while the latter is slightly higher. The low cocoon production in experiment 3 (Table 5) could also be a result of smaller specimens used as compared to those used in experiment 2—the earthworms may not yet have reached their full maturity and was still allocating most resources to body mass increase. Growth of individuals decline asymptotically with increasing body mass (Lowe and Butt, 2005) and there is a trade-off between body-mass increase and reproduction.

If laboratory reared earthworms had been used instead of specimens collected from the field, differences in fecundity, growth and survival between experiments due to seasonal changes caused by the phenology of the earthworms could have been avoided. Under constant environmental conditions, earthworms have been shown to maintain both activity and reproductive conditions throughout the year. However, reproductive fatigue and high death rate can occur compared to those kept under fluctuating temperatures (Lowe and Butt, 2005). Although use of laboratory reared earthworms of the same age would have given more reliable and replicable data we chose to use field-collected ones since resources and time were not available to produce the amounts of specimens needed for our experiments.

This is the first study focussing on the impact of BCA bacteria on earthworms and from the results we can conclude that no harmful effects of *B. amyloliquefaciens* UCMB5113 on the tested earthworm species were recorded. Previous studies on the interaction between BCA bacteria and earthworms, focused on the opposite direction of the interaction: earthworm effect on bacteria, rather than bacteria effect on earthworms. These were conducted with the genus *Pseudomonas*, and the only reference made to the effect of these on earthworms was the lack of earthworm mortality during the experiments. No records of weight change or cocoon production have been reported (Stephens et al., 1993; Doube et al., 1994; Schmidt et al., 1997). Further studies of interactions of BCA bacteria and earthworms could concern other species of bacteria and earthworms. Earthworms by themselves also have positive effects on plant production. The underlying mechanisms for these positive effects include (i) biocontrol of pests and diseases, (ii) stimulation of microbial plant symbionts, (iii) production of plant growth-stimulating substances, (iv) soil structure improvements, and (v) increase of soil nutrient availability (Brown et al., 1999). Though recent studies focused on the first three mechanisms, van Groenigen et al. (2014) suggest that the last one is the most important. Earthworm activity influences the microbial community of soils directly by consumption, digestion and distribution of microorganisms and indirectly by modification of the soil environment (Byzov et al., 2007; Postma-Blaauw et al., 2006; Scheu et al., 2002; Schrader et al., 2013). This could either enhance or hamper the effects of bacterial BCAs. Their potential synergy becomes a relevant line for future research since the combined

effects of earthworms and BCA bacteria on plant health and productivity are of great interest for development of sustainable agricultural methods with minimum use of chemical pesticides and optimal use of ecosystem services.

5. Conclusions

From the experiments described above, we can conclude that the use of high doses of BA with concentrations of the same magnitude as maximally could be expected when the bacteria are used as BCA, is not harmful to the soil dwelling earthworms tested in this project. BA does not have negative impact on survival, growth or reproduction of two of the most common earthworm species in Swedish agricultural soils when these earthworms are exposed to BA by short-term external contact with high concentration (dipping), long-term external contact with lower dose (mixing into soil) and internal contact with the gut (feeding with BA-amended leaves). The combined effects of earthworms and BCA bacteria for promotion of plant health are of interest for the development of biological control and sustainable agriculture with reduced use of chemical pesticides.

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