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

J. Lagerlöf[a,*], F. Ayuke[a,e], S. Bejai[b], G. Jorge[c], E. Lagerqvist[a], J. Meijer[b], J. John Muturi[d], S. Söderlund[a]

[a] Dept. of Ecology, SLU, Sweden
[b] Dept. of Plant Biology, SLU, Sweden
[c] Facultad de Agronomía, Universidad de la República, Uruguay
[d] Embu University College, Kenya
[e] Dept. of Land Resource Management & Agric. Technology, University of Nairobi, Kenya

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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).

* Corresponding author. Fax: +46 18672890.
E-mail address: jan.lagerlof@slu.se (J. Lagerlöf).

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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.