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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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ABSTRACT

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

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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* × 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.