Microbial Ecology

Protection of Oilseed Rape (*Brassica napus*) Toward Fungal Pathogens by Strains of Plant-associated *Bacillus amyloliquefaciens*

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Abstract

In this report, four *Bacillus* strains were tested for effects on plant fitness and disease protection of oilseed rape (*Brassica napus*). The strains belonged to newly discovered plant-associated *Bacillus amyloliquefaciens* and a recently proposed species, *Bacillus endophyticus*. The fungal pathogens tested represented different infection strategies and included *Alternaria brassicae*, *Botrytis cinerea*, *Leptosphaeria maculans*, and *Verticillium longisporum*. The *B. amyloliquefaciens* strains showed no or a weak plant growth promoting activity, whereas the *B. endophyticus* strain had negative effects on the plant as revealed by phenological analysis. On the other hand, two of the *B. amyloliquefaciens* strains conferred protection of oilseed rape toward all pathogens tested. *In vitro* experiments studying the effects of *Bacillus* exudates on fungal growth showed clear growth inhibition in several but not all cases. The protective effects of *Bacillus* can therefore, at least in part, be explained by production of antibiotic substances, but other mechanisms must also be involved probably as a result of intricate plant–bacteria interaction. The protective effects observed for certain *Bacillus* strains make them highly interesting for further studies as biocontrol agents in *Brassica* cultivation.

Introduction

Plants exist in a complex ecological community where, e.g., inorganic nutrients, water, temperature, and light are parameters that can be measured and analyzed to describe the system. A more complex growth effect is the interaction of various organisms with plants where especially the soil is a rich source of various microorganisms [8]. Whereas most microorganisms do not interact with a given plant, some microorganisms may be beneficial or detrimental to the host plant. Of vital importance for many plants are, e.g., nitrogen fixation symbionts [6] in intimate association with plant tissues. Another habitat colonized by various microorganisms is the surface of plant tissues or the phyllosphere [15]. Certain bacteria function as plant growth promoting rhizobacteria (PGPR), i.e., they support the growth of plants [23]. Whereas such positive growth effects to plants originally was observed for Rhizobacteria, several other kinds of bacteria such as *Bacillus*, *Pseudomonas*, and *Serratia* strains [16] have also been found to be beneficial to plants. The PGPR effect can be due to, e.g., production of plant hormones like cytokinins and gibberellins or by increasing the amount of minerals and nitrogen available for the plant [22].

Microorganisms isolated from soil or plant tissue mediates protection of various plants toward different pathogens and insect pests in several different studies [1, 18, 19, 20, 24, 33, 36]. Bacteria and fungi that have been shown to protect plants include several genera where *Streptomyces*, *Pseudomonas*, and *Bacillus* are some representatives [10]. A wide spectrum of pest control by beneficial bacteria has been reported including protection against oomycetes as *Peronospora parasitica* [28], bacteria as *Pseudomonas syringae* [12], viruses as Cucumber mosaic virus [24], and insects as Cucumber beetles [35]. This biocontrol is effective in several unrelated plant species where cucumber, bean, oilseed rape, and tomato are examples of crops that have been protected by bacteria that are experimentally applied [18, 28]. Accordingly, the use of beneficial bacteria as environmental friendly pest control to increase yield and quality of crop plants has great potential.

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Protection based on biocontrol agents can be mediated in many ways. Bacteria living in vascular tissue of plants or in the rhizosphere are in an appropriate position to protect the plant from deleterious organisms. Competition for growth space and nutrients by the beneficial bacteria can indirectly protect the plant from harmful microorganisms. Siderophores and phytic acid [13] have been shown to be important for some bacteria to protect plants through competition for nutrients. Bacteria can produce a wide array of antibiotics that can be effective against many different fungi and bacteria. Peptide antibiotics represent the predominant class of bacterially produced antibiotics [10]. Alteration of the plant cell wall that causes an increased protection to pathogens has been found to occur with both *Bacillus subtilis* and *Pseudomonas aeruginosa* [2, 3, 30]. Production of salicylic acid by bacteria can make the plant more tolerant to pests and pathogens by stimulating systemic acquired resistance (SAR), a common defense program induced in plants to combat pathogens [5]. Some bacteria have been shown to be able to induce a plant defense system called induced resistance (IR) or induced systemic resistance (ISR) [5, 28]. This defense is activated by jasmonate- and ethylene-dependent but salicylate-independent signaling and has been shown to be effective against insects, viruses, bacteria, and fungi [28].

The genus *Bacillus* is characterized by rod-shaped, facultative aerobe, endospore-forming bacteria that live in soil and often colonize the plant rhizosphere [20]. There is great genetic diversity in this genus, which include strains that have been found to live as endophytes inside plant vascular tissues but also as human pathogens like *Bacillus anthracis*. Several *Bacillus* spp. produce different kinds of antibiotics [14, 22, 29, 30, 32, 34] and some *Bacillus* strains, mainly *B. subtilis*, *Bacillus cereus*, and *Bacillus amyloliquefaciens*, are known to mediate protection against pathogens on plants [1, 19, 30, 33, 34].

The aim of this study was to evaluate the effects of treatment of oilseed rape (*Brassica napus*) with certain strains of *Bacillus*, which were shown in our earlier studies to belong to the genetically separated taxa of plant-associated bacteria referred to as *Bacillus endophyticus* [21] or *B. amyloliquefaciens* [20]. One goal was to evaluate the strains as potential biocontrol agents against four serious fungal pathogens to *Brassica* species, *Alternaria brassicae*, *Botrytis cinerea*, *Leptosphaeria maculans*, and *Verticillium longisporum* [11, 17, 26]. Another goal was to investigate if and how these strains might differ in the effect they have on the plants to provide a better understanding of how the bacteria–plant interaction operates. Is plant protection provided by all related bacterial strains? How is this protection mediated? How specific is this protection, and does it respond the same way toward all the pathogens or in a pathogen species-specific way? To approach these questions, we used three closely related strains of *B. amyloliquefaciens* originally isolated by Reva [20]. *Bacillus* UCMB-5033 and UCMB-5036 were isolated from inner tissues of cotton plants (*Gossypium barbadense*) in Tadjikistan, whereas UCMB-5113 is a red pigmented bacteria isolated from the soil of Karpaty mountains, Ukraine. Their plant colonization abilities on *B. napus* differ from high, for UCMB-5113, to very low for UCMB-5033, and none, for UCMB-5036 [20], which prompted further studies of protection abilities. The bacterial strain UCMB-5715 is the type culture of a newly proposed species, *B. endophyticus* [21]. Although this *Bacillus* strain was also isolated from the inner tissue of cotton plants together with *B. amyloliquefaciens* strains, it represents a phylogenetic lineage that is quite distant from other *Bacillus* species. Comparative analysis of sequences of 16S rRNA genes showed that this species is closely related to *Bacillus sporothermodurans* and *Bacillus gibsonii* and that they all are close to *Jeotgalibacillus alimentarius* (data not shown). This strain was included in our investigation to serve as a reference strain for *B. endophyticus*. To investigate how protection was mediated, production of antifungal compounds by the bacteria was investigated. A study of plant phenology was also performed to investigate effects of bacterial treatment on plant growth and development.

Materials and Methods

Bacillus Spore Solution. *Bacillus* strains: UCMB-5715, UCMB-5033, UCMB-5036, and UCMB-5113, were all grown in Luria–Bertani (LB) media at 28°C with agitation for three days to allow for production of spores. The bacterial cultures were then heat treated at 75°C for 10 min to select for *Bacillus* spores and to kill possible contaminants. The spore concentration was determined by viable count analysis of an aliquot on LB plates and the stock spore solution was kept refrigerated until use.

Seed Treatment. Oilseed rape (*B. napus*, cv. Westar) seeds were surface sterilized for 20 min in 20% sodium hypochlorite followed by a brief rinse with 50% methanol. The seeds were mixed with each *Bacillus* spore solutions with a concentration of 10 spores ml^{-1}. LB or water served as control but were not different from each other. The seeds were then left on agitation for two h until planting in soil. Standard soil (K-jord Weibulls) was sterilized in an autoclave for 30 min, and after allowing the soil to cool, the process was repeated.

Effects of Bacillus Inoculation on Plant Development. Sterile soil was put into 200 11×11×5 cm pots. One seed from separate treatments was put into each pot with a forceps. The pots were then arranged

into five trays for every treatment, eight pots on each tray, and grown in a greenhouse using a 16/8 h photoperiod with minimum temperature settings at 22/18°C. All pots were given a similar amount of water to exclude differences between treatments depending on water status. The plants were observed continuously to identify any phenotypic differences between treatments. The characteristics that were measured included germination efficiency, number of true leaves developed at week two, three, and four, how many plants that were flowering at week three and four, and how many plants that survived seed development. Plants were harvested and the seeds were pooled for each treatment and were weighted. The germination rate of the harvested seeds was tested by planting 40 seeds from each treatment into sterile soil.

Plant Growth for Pathogen Studies. One seed was put into each pot (8×8×5 cm pots with sterile soil) with forceps. The pots were then arranged into different trays depending on treatment. The plants were grown in a growth chamber under 18/6 h photoperiod at 22/18°C using fluorescent light of approximately 200 μmol m^{-2} s^{-1}. All pots received similar amounts of water to exclude differences between treatments depending on water availability.

Preparation of Fungal Spores. The fungal strains, *A. brassicae* (980:3), *V. longisporum* (Vd11), *B. cinerea* (30158), and *L. maculans* (1245) were grown on potato dextrose agar (PDA) plates (Sigma Chemical Co., St Louis, MI) in a growth chamber (16/8 h photoperiod at 21/16°C) for a month or until spores had been produced. The plates were harvested by addition of sterile water to the plates followed by scraping the agar surface with a pipette tip to release the spores. This procedure was repeated once, and the spores were pooled and filtered through miracloth. The concentration of spores was measured with a Bürkner chamber, and the final concentration was adjusted to 10^7 spores ml^{-1}. The spore solution was stored at 4°C until use.

Inoculation of Plants with Pathogens. Plants that had a similar size (five true leaves) were chosen from each treatment. The plants were coded and mixed into trays in a random design. The inoculation of pathogen was done differently according to the fungus studied. *A. brassicae* and *B. cinerea* spores were sprayed on three-week-old plants. The *A. brassicae* infection was performed with 15 plants from each treatment and was replicated. *B. cinerea* infection experiments were replicated thrice with 8, 7, and 10 plants in each experiment. In the case of *V. longisporum*, 400 ml of spore solution was put into the tray containing the protruding roots of two-week-old plants as disease development is slower. This experiment was first done with 10 plants from each treatment, and then, was repeated with varying amounts of plants from each treatment. *L. maculans* was applied by puncturing two leaves on each plant twice with a pipette tip without damaging the mid-vein. Then, 10 μl spore solution was applied on each puncture. This experiment was first performed with 15 plants and then repeated twice with 10 plants from each treatment. Control plants were always treated the same way as other samples with the exception that sterile water was applied. Plants were kept in mini greenhouses or wrapped in plastic, 12 h before and 12 h after inoculation to maintain high humidity and facilitate infection.

Scoring. All plants were scored before decoding the material. *A. brassicae*- and *B. cinerea*-infected plants were divided into four groups. The groups were; uninfected, (1); <1/2 of the leaves infected, (2); >1/2 of the leaves infected, (3); and the last step, dead plants, (4). The scoring took place one week after inoculation. *V. longisporum* were scored after two weeks by two parameters; growth retarded compared to negative control or unaffected plants. *L. maculans* infection was scored after one week. Presence of necrotic lesions at the punctures was used to score plants as either infected or uninfected.

Antifungal Test. *Bacillus* strains, UCMB-5715^T, UCMB-5033, UCMB-5036, UCMB-5113, were all grown in LB media at 28°C with agitation for 24 h when stationary growth had been achieved for all bacteria. The cultures were diluted to a concentration of 10 spores ml^{-1}. The cultures were then sterile filtered through 0.45-μm filters to remove the bacteria.

Multiwell plates with PDA media were used. Each well was filled with 100 μl spore solution from the different fungi. Six rows encompassing four wells were filled with 200 μl of water, 190 μl water and 10 μl culture filtrate, 180 μl water and 20 μl culture filtrate, 150 μl water and 50 μl culture filtrate, 100 μl water and 100 μl culture filtrate, or 0 μl water and 200 μl culture filtrate, respectively. This was repeated with all combinations of strains and fungi. The plates were incubated in a growth chamber (16/8 h photoperiod at 21/16°C) and scored after two weeks. Relative efficiency was estimated according to the amount of culture filtrate needed to completely inhibit fungal growth. Culture filtrate pH was measured to assure that no differences in pH were responsible for the inhibition.

In Vitro Protection of Plants. *Bacillus* UCMB-5715^T, UCMB-5033, UCMB-5036, UCMB-5113, and *L. maculans* spore solutions were prepared, and *B. napus* seeds were surface sterilized as described above. Seeds were

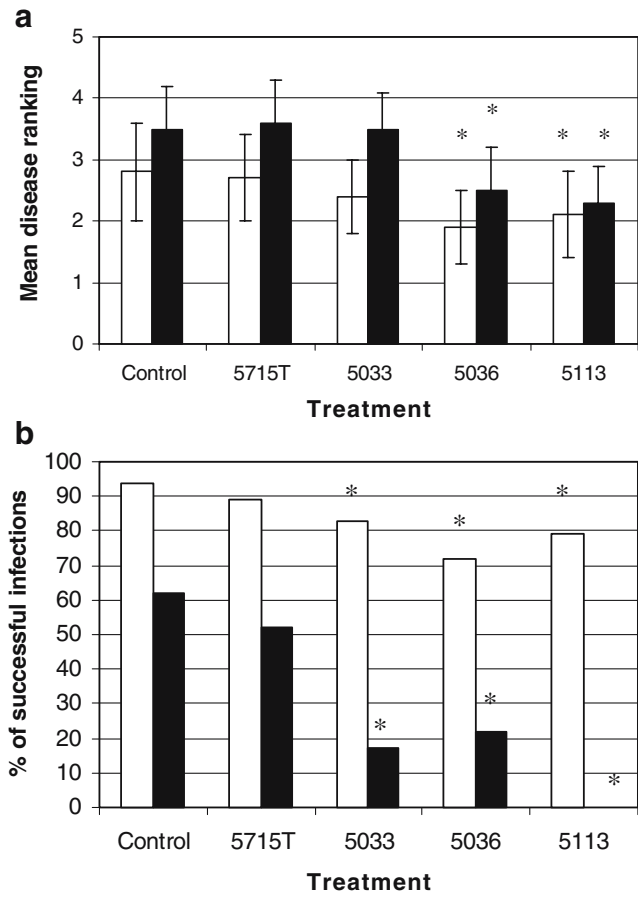

Figure 1. Effects of *Bacillus* on oilseed rape protection to different pathogens. Plants were grown on soil and challenged with different pathogens and scored as described in the Materials and Methods section using different scales. (a) *Alternaria brassicae* (*white bars*, *N*=30), *Botrytis cinerea* (*black bars*, *N*=25). *Asterisks* (*) depicts significant differences ($P<0.05$) from control. *Variation bars* on the histograms correspond to standard deviation. (b) *Leptosphaeria maculans* (*white bars*, *N*=35), *Verticillium longisporum* (*black bars*, *N*=18–21). *Asterisks* (*) depicts significant differences ($P<0.05$) from control.

treated with the bacterial spore solution as mentioned earlier. Multiwell plates with MS media (Duchefa, Harlem, Netherlands) were filled with two seeds in each well. To each well, except for the negative control, 20 µl of *L. maculans* spore solution (10^6 spores ml^{-1}) was added. A positive control was also included comprising of *L. maculans* spores added to seeds that had been treated with water instead of bacteria. The plates were incubated in a growth chamber (16/8 h photoperiod at 21/16°C), and surviving seedlings were counted after 10 days.

Statistical Analysis. Two-sample *t*-tests and Andersson Darling normal variance tests were made using Minitab 14. *P* values were calculated and values <0.05 were used as a level for significant differences between control and *Bacillus* treatment and as a between-experimental repeats.

Results

The pathogen screenings revealed that treatment of oilseed rape seeds with *Bacillus* strains UCMB-5113 and UCMB-5036 resulted in significant protection against all fungal pathogens tested (Fig. 1). *Bacillus* strain UCMB-5033 showed protection against *V. longisporum* and efficient protection to *L. maculans*, but had no effect toward *B. cinerea* and *A. brassicae*. The use of *B. endophyticus* strain UCMB-5715 did not result in any significant protection against the pathogens except *V. longisporum*, but this effect was very close to being not significant. The protection seemed to be a combination of lower infection frequency and less severe disease symptoms on treated plants.

To study direct effects of bacteria toward fungi, the potential production of antifungal substances in the growth medium of the *Bacillus* strains were tested on fungal growth *in vitro*. The effect of addition of growth medium was more of an all or nothing reaction (Table 1). Either the fungi and the control grew, or otherwise, the wells showed no growth at all even after three weeks. This would suggest that the antifungal substances present in the growth medium either kills or prevents germination of the fungal spores in a concentration-dependent matter. UCMB-5036 was the only strain that produced antifungal substances that showed a strong growth retardation effect against all fungi tested. UCMB-5033 and UCMB-5113 media showed effects that varied between no visible effects to a strong effect on pathogen growth. UCMB-5715^T medium was only effective against

Table 1. Effects of *Bacillus* extracts on fungal growth

Fungus	*UCMB-5715^T*	*UCMB-5033*	*UCMB-5036*	*UCMB-5113*
Alternaria brassicae	+++	+++	+++	–
Leptosphaeria maculans	–	+++	+++	+++
Verticillium longisporum	–	–	+++	+++
Botrytis cinerea	–	+	++	++

Antibiotic effect scale, from *in vitro* experiments was: – no inhibition with 200 µl bacterial supernatant, + complete inhibition with 200 µl bacterial supernatant, ++ complete inhibition with 100 µl bacterial supernatant, +++ complete inhibition with 10 µl bacterial supernatant

Table 2. Phenological analysis of oilseed rape plants treated with *Bacillus* strains

Characteristics	*Control*	*UCMB-5715^T*	*UCMB-5033*	*UCMB-5036*	*UCMB-5113*
Germination rate[a] (%)	80	25*	85	80	65
True leaves after two weeks	1.5	0.4*	1.4	1.2	1
True leaves after three weeks	2.9	1.9*	2.8	2.6	2.7
True leaves after four weeks	4.7	3.8*	4.4	4.3	4.7
Flowering plants after three weeks[b] (%)	71	71	21	24	61
Seed weight (g/plant)	0.34	0.22	0.26	0.39	0.37
Survival[c] (%)	70	75	90*	90*	89*
Germination second generation[d] (%)	97	96	91	98	94

In each case N=40
*Indicates significant difference (P<0.05) from control in t-test
[a]Proportion of seeds that germinated after one week
[b]At least one flower opened to be considered as flowering
[c]Number of plants that survived until seed set
[d]The germination rate of seeds harvested from plants treated with bacteria

A. brassicae. None of the bacterial strains affected the pH of their growth media.

Phenological analysis of plants was undertaken to observe any fitness effects due to *Bacillus* inoculation. The fitness study showed a significantly higher survival rate for the seeds treated with UCMB-5033, UCMB-5036, and UCMB-5113 providing a growth promoting effect (Table 2). Otherwise, there were no significant differences between treatments with the exception of the UCMB-5715^T strain that showed deleterious or growth-retarded symptoms on oilseed rape plants (Table 2). No visible signs of infection such as chlorosis or yellowing were observed on plants that did germinate after bacterial treatment. The germination efficiency was the same as the seeds harvested from the different plants including control plants.

Effects of *Bacillus* strains to *L. maculans* inoculation on oilseed rape seeds were also tested on *in vitro* cultivated plants as this could provide a more convenient high-throughput screening. Treatment with all strains except for UCMB-5715^T gave significantly higher number of surviving oil seed rape plants (Fig. 2) similar to the soil experiments. However, UCMB-5113 had stronger disease suppression with plants on soil compared with agar grown seedlings (Fig. 2).

Discussion

Biocontrol is an interesting durable and potential environmental friendly alternative for plant protection compared with chemical control and its many negative effects on the environment. A number of *Bacillus* strains representing different genotypes were tested for the first time on oilseed rape for their potential usefulness for disease protection and also to evaluate fitness effects on plants. Several fungal pathogens constituting a serious problem to *Brassica* cultivation were tested in this report. *A. brassicae*, *B. cinerea*, *L. maculans*, and *V. longisporum* all constitute severe problems to *Brassicas* but have widely different infection mechanisms, which provide a good opportunity to also study the mechanism for *Bacillus* control, if present.

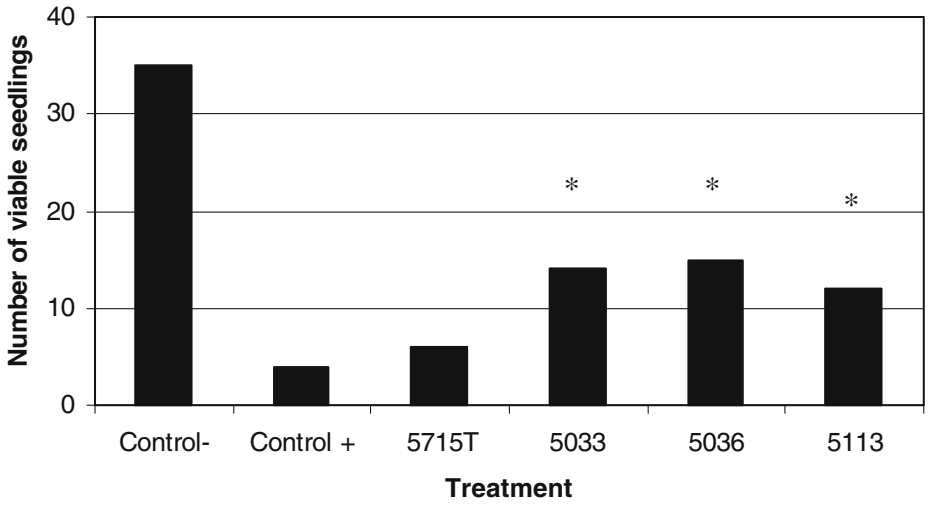

Figure 2. Effects of *Bacillus* on oilseed rape seeds infected by *Leptosphaeria maculans*. Oilseed rape seeds were inoculated with *Bacillus* spores, transferred to PDA agar and challenged with *Leptosphaeria maculans* spores. Survival was scored after 10 days. *Asterisks* (*) depicts significant differences (P<0.05) from control (N=35).

Bacillus strains UCMB-5036 and UCMB-5113 were found to confer protection to oilseed rape against all pathogens tested in this study. Protection was confirmed in spite of the very different infection strategies used by the different pathogens. *V. longisporum* is a soil-borne pathogen that infects roots whereas the other pathogens tested are foliar pathogens [4, 9, 11, 26]. It is tempting to speculate that the difference in protection observed between the *Bacillus* strains is dependent on the fungal lifestyles. UCMB-5033 only provides significant protection against *V. longisporum* and the hemibiotroph, *L. maculans*, but does not provide protection toward the necrotrophic fungi, *B. cinerea* and *A. brassicae.* The UCMB-5715T strain did not confer protection to any of the pathogens tested on oilseed rape.

The production of antifungal substances gave a complex answer, indicating production of structurally different or different amounts of antifungal compounds among the *Bacillus* strains (Table 1). Fungicides produced by the bacteria does not seem to strictly correlate with the resistance as all strains showed antifungal production but no protection, or protection but no antifungal production, except for UCMB-5036 that had antibiotic effect against all fungi tested. It has earlier been shown that the correlation of antiobiotic production on nutrient rich medium as LB media and other medias differ significantly, and this might also be a reason why no correlation is seen in this study [31]. Production of antifungals is probably one of many ways these strains protect the plant, but not the only explanation. Another possibility is inducible defense of plants where IR dependent on jasmonate and phytoalexins has been shown to be effective against pathogens in *Arabidopsis* [27]. The discrepancies between protection in *planta* and *in vitro* suggest that the protection observed here could at least, in part, be dependent on IR, which will be the subject of future research.

The agar-screening assay using plant seeds confirmed the protection mediated by UCMB-5033, 5036, and 5113 to *L. maculans* using pure isolates *in vitro*, but if induction of production of antifungal compounds were responsible for the effect in planta, it could not be evaluated from our investigation. Moreover, biocontrol capacity of the strains relays on their ability to survive on roots and colonize plants. UCMB-5033, 5036, and 5113 treated seeds all showed significantly higher survival rate than control in the greenhouse experiments. This could also be an indicator that seed treatment with these bacteria makes the plants more tolerant. The exception was strain UCMB-5715^T that clearly delayed plant development.

Despite the close phylogenetic relationship of the studied *B. amyloliquefaciens* strains, the antifungal effect pattern differs between all the strains and the protective capability, which indicates that the interactions between plants and bacteria seem to be strain and species specific. The finding that *B. endophyticus* strain UCMB-5715^T delayed development to *B. napus* is interesting especially as this bacterium was isolated from the vascular tissue of cotton plants. This also indicates that the colonization of endophytes is very species specific, as shown previously for other systems [7, 25] and that results for potential biocontrol strains not can be generalized but need to be tested in each context. Differences between colonization patterns by endophytes can be seen even on cultivar level as demonstrated for oilseed rape [9]. *B. amyloliquefaciens* strains, UCMB-5036 and UCMB-5113, showed a broad protective capability on *B. napus* against fungi that has a necrotrophic or biotrophic lifestyle, and are foliar or soil spread pathogens. This gives these two bacteria a good potential as future biocontrol agents, although further studies are needed of various properties before field experiments can be performed.

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