Research Article

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Endophytic *Beauveria bassiana* induces biosynthesis of flavonoids in oilseed rape following both seed inoculation and natural colonization

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Abstract

Background: Cultivation of oilseed rape *Brassica napus* is pesticide-intensive, and alternative plant protection strategies are needed because both pesticide resistance and legislation narrow the range of effective chemical pesticides. The entomopathogenic fungus *Beauveria bassiana* is used as a biocontrol agent against various insect pests, but little is known about its endophytic potential and role in plant protection for oilseed rape. First, we studied whether *B. bassiana* can establish as an endophyte in oilseed rape, following seed inoculation. To evaluate the plant protection potential of endophytic *B. bassiana* on oilseed rape, we next examined its ability to induce plant metabolite biosynthesis. In another experiment, we tested the effect of seed inoculation on seedling survival in a semi-field experiment.

Results: *Beauveria bassiana* endophytically colonized oilseed rape following seed inoculation, and, in addition, natural colonization was also recorded. Maximum colonization rate was 40%, and generally increased with inoculation time. Seed inoculation did not affect the germination probability or growth of oilseed rape, but *B. bassiana* inoculated seeds germinated more slowly compared to controls. Endophytic colonization of *B. bassiana* induced biosynthesis of several flavonoids in oilseed rape leaves under controlled conditions. In the experiment conducted in semi-field conditions, inoculated seedlings had slightly higher mortality compared to control seedlings.

Conclusion: *Beauveria bassiana* showed endophytic potential on oilseed rape *via* both natural colonization and seed inoculation, and it induced the biosynthesis of flavonoids. However, its use as an endophyte for plant protection against pests or pathogens for oilseed rape remains unclear.

Supporting information may be found in the online version of this article.

Keywords: biocontrol; pest management; mycopesticide; seed inoculation; flavonoids; metabolites

1 INTRODUCTION

With total area in production exceeding 36 million hectares in 2021, oilseed rape, *Brassica napus*, is one the of the most important oilseed crops in the world.[1–3] Conventional cultivation of oilseed rape is pesticide-intensive, and plant protection products are applied against a variety of insect pests and diseases. Though pest and disease distribution varies regionally, oilseed rape production globally is currently affected by 37 insect pests, various diseases, several species of nematodes, and slugs.[4] Even with the use of synthetic pesticides, losses to insect pests pose substantial challenges and are economically significant.[3–5] Furthermore, cultivation-based integrated pest management and biocontrol still require substantial development efforts because they are either considered ineffective or are under-supported or unavailable.[4,6]

In Europe, systemic insecticides such as neonicotinoids, which were commonly used as seed dressing in oilseed rape production, are now banned (EU Regulation No 485/2013 of 24 May 2013, updated in 2018) due to their reported harmful effects on non-target

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organisms including pollinators.[3–5,7] Pesticide restrictions have resulted in both higher losses to pests and reduction in area under oilseed rape cultivation in some European countries.[3,5,6,8,9] Insecticide resistance among major oilseed rape pests such as flea beetles (Psylliodes)[3,5] and pollen beetles (*Brassicogethes aeneus*)[10–12] to pyrethroids has made pest management without neonicotinoids increasingly challenging.[13–15] Thus, alternatives to chemical pesticides are needed to control pests in oilseed rape crops.

The potential of beneficial microorganisms to reduce producer reliance on synthetic pesticides is increasingly recognized as a means to improve agricultural sustainability.[16] Beneficial organisms can increase the health of crop plants via several mechanisms, including: providing plant protection by inducing resistance in the host plant to pests and pathogens, increasing plant tolerance to abiotic stress, and improving plant nutrition and promoting growth.[17–19] Research on beneficial microorganisms with the aim of unlocking potential applications for enhancing crop productivity and protection has increased in recent years.[20,21] Despite the growing number of commercially available microbial biofertilizers and biostimulants, the number of microbial biocontrol products is still small compared to the conventional synthetic product market.[22–25] Van Lenteren et al.[26] identified just over 200 registered microbial biocontrol products, representing 57 different genera, used for augmentative biocontrol in different parts of the world. Challenges related to the use of microbes as biocontrol agents have hindered commercial development of plant protection products containing fungal (and other) entomopathogens. These challenges include abiotic factors such as susceptibility to ultraviolet light, unfavorable moisture conditions, technical challenges in field application, and lack of information on the cost-effectiveness.[27,28] Further, the lack of broad host ranges as well as studies showing variable field efficacy are both limiting factors for deployment of plant beneficial microbes generally, and deeper mechanistic knowledge is needed.[22]

One group of beneficial microorganisms are entomopathogenic microbes including fungi, viruses and protozoa that are utilized through epiphytic application (surface/foliar application or soil amendment) to provide crop protection due to their ability to infect and kill pest insects.[27] An example of an epiphytic application for pest control is foliar spraying of a solution containing biologically active conidia of an entomopathogenic fungus. In addition to this surface contact mechanism, some entomopathogenic fungi can colonize plants systemically by growing as endophytes in the plant's intercellular spaces, and this may provide continuous plant protection in comparison to epiphytic application. Endophytic fungi can synthesize an array of specialized metabolites, which are subsequently released and transported through the plant transport systems. These metabolites can directly affect plant interactions with the biotic environment by functioning as repellents to herbivores or in pathogen defense.[29–32] While the mode-of-action of entomopathogens as endophytes may be different to epiphytic application, endophytic entomopathogenic fungi have been shown to mediate the severity of disease symptoms, improve plant resilience and promote plant growth.[23,33,34] Endophytic entomopathogenic fungi can produce or induce the biosynthesis of specialized metabolites which can induce plant resistance to insect pests and pathogens, and they can enhance plant growth, which enhances the plant's ability to compensate for biomass lost due to herbivory.[27,35] Thus, they are considered promising tools for biological plant protection.[16,27,36]

The entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) is a common, naturally occurring fungus in soils. It is used as an epiphytic biocontrol agent against various agricultural pests, and several commercial products are available.[18,27,37] In addition, *B. bassiana* is capable of endophytically colonizing a range of host plants,[23] and it can be artificially inoculated in several plant species via seed inoculation, soil watering, root dipping, or foliar spraying.[18,27,38,39] Following artificial inoculation, *B. bassiana* has been shown to endophytically colonize several important crop species such as broad bean (*Vicia faba*), maize (*Zea mays*), potato (*Solanum tuberosum*), date palm (*Phoenix dactylifera*), banana (*Musa acuminata*), soybean (*Glycine max*), tomato (*Solanum lycopersicum*), cassava (*Manihot esculenta*) and even oilseed rape.[27] In some studies, endophytic colonization by *B. bassiana* has not increased host plant resistance against herbivores while, in other cases, it has been shown to increase the host plants' resistance to herbivorous insects and plant pathogens.[40,41] For instance, endophytic *B. bassiana* infection in cucumber resulted in elevated levels of specialized metabolites which increased cucumber plants' repellency against herbivorous insects.[42] The ability of endophytic entomopathogenic fungi to induce defense-related metabolites is a promising way to increase plant resistance.

Here, we studied, via seed inoculation, the endophytic potential of *B. bassiana* in oilseed rape and investigated whether endophytic *B. bassiana* has plant protection potential. Previous studies have shown that *B. bassiana* can establish as an endophyte on oilseed rape via leaf sprays.[23] However, nothing is known about the plant protection potential of endophytic *B. bassiana* on oilseed rape. In fact, in their review of beneficial endophytic microorganisms of *Brassica spp*., Card *et al*.[33] note there are no alternative endophytic pest control products for *Brassica* species. We examined: (1) endophytic potential of *B. bassiana* on oilseed rape following seed inoculation, (2) effect of endophytic *B. bassiana* on oilseed rape growth and induction of plant metabolites, and (3) effect of seed inoculation on germination and seedling survival in semi-field conditions.

2 MATERIAL AND METHODS

2.1 Plant material, fungal culture conditions and seed inoculation

The endophytic potential of *B. bassiana* strain ATCC74040, from the commercial product Naturalis® (Bioguard, CBC Europe, Italy) was tested for endophytic potential via seed inoculation on oilseed rape (*B. napus*) cultivar 'Cleopatra^{BOR}'. The untreated oilseed rape seeds subsequently inoculated with *B. bassiana* were sourced from Avena Nordic Grain Oy. Prior to use, seeds were stored at +4 °C.

Conidia for seed inoculations were produced by culturing *B. bassiana* in Petri dishes (90 × 15 mm) containing 20 mL of Potato Dextrose Agar (PDA; Sigma-Aldrich). Cultures were kept in an incubator (Sanyo, MLR-350, Tokyo, Japan) in total darkness at 25 ± 2 °C for 3 to 4 weeks. Conidia from one Petri dish were harvested with a sterile spatula and suspended in 500 µL sterile distilled water supplemented with 0.05% Triton X-100 (Sigma-Aldrich, Darmstadt, Germany), after which the suspension was first vortexed for 2 min and then centrifuged at 3000 RPM for 3 min to remove hyphal fragments, conidial clumps, and bits of agar. After centrifuging, 250 µL of suspension was carefully pipetted to a clean 1.5 mL Eppendorf tube. Conidial concentration was estimated from 20 µL of each sample using a BLAUBRAND® counting chamber 'Burker' under the light microscope (Olympus BH-2) by completing four counts (0.2 × 0.2 mm square) randomly across the counting chamber. For seed inoculation, the suspension was diluted to contain

1×10^8 spores mL^{-1}. Conidial viability was checked by transferring 200 µL of the suspension onto a PDA plate and counting conidia germination after 24 h at 25 ± 2 °C. Suspension was only used if germination rate exceeded 95%.

Prior to seed inoculation, the oilseed rape seeds were surface sterilized by submerging them in 1.5% NaClO for 3 min followed by rinsing them three times in autoclaved MilliQ water for 1 min. A quantity of 500 µL of the final rinse water was pipetted and plated on PDA to assess the surface sterilization efficacy. For seed inoculation, surface sterilized seeds were soaked in *B. bassiana* spore suspension (1×10^8 spores mL^{-1}; 40 seeds/10 mL suspension) and kept in an incubator (Sanyo, MLR-350, Tokyo, Japan) in total darkness at 25 ± 2 °C for 2 h in the case of the semi-field experiment (see Section 2.4) and 2 and 6 h, respectively, in the growth chamber experiment in which the effect of inoculation time on the endophytic establishment and colonization of *B. bassiana* on oilseed rape as well as the effect of endophytic *B. bassiana* on plant growth and metabolism was assessed (see Section 2.2). Control seeds were soaked (2 h in experiment 2.4, and 2 and 6 h in experiment 2.2) in sterile distilled water containing 0.05% Triton X-100 (40 seeds/10 mL).

2.2 Effect of endophytic *Beauveria bassiana* on plant growth

Results by Jaber and Enkerli[18] indicate that longer seed inoculation time can enhance the endophytic establishment and colonization success of *B. bassiana* in *Vicia faba*. Thus, the effect of inoculation time on the endophytic establishment and colonization of *B. bassiana* on oilseed rape was tested in a growth chamber experiment. In addition, the effect of inoculation time (2 and 6 h; see 2.1 for more details) on plant growth and the effect of endophytic *B. bassiana* on plant metabolite concentration was studied. After inoculation, 60 seeds per treatment and length of inoculation time were sown as two seeds per pot in 90 mL plastic pots (11 × 11 × 7.5 cm, Schetelig Oy, Vantaa, Finland) containing sterilized potting soil (Kekkilä Viherkasvimulta no. 10046; mixture of peats, sand, silts, silty clay, NPK 6-12-24 and pH 6.5, Kekkilä-BVB, Finland). The potting medium was sterilized before use by autoclaving the soil to 120 °C for 1 h. Altogether, there were 30 pots (i.e. 60 seeds) per treatment for a total of 120 pots (240 seeds). The pots were randomly arranged on trays that were placed in two growth cabinets each of which used a photoperiod of 16:8 h L:D and at 21:18 °C and light intensity of approximately 300 µmol m^{-2} s^{-1} (Sanyo, MLR-350, Tokyo, Japan). Pots were watered every third to fourth day with tap water.

The pots were monitored daily to record time from sowing to germination. A seed was considered germinated when the cotyledon leaves were open. Next, sampling was conducted at 13 days (DNA), 28 days (DNA, metabolites) and 60 days (biomass) after sowing (Table 1). To detect whether *B. bassiana* had established as an endophyte on oilseed rape, tissue samples were taken for DNA analysis 13 and 28 days after sowing. For the sample taken after 13 days, 10 seedlings per treatment were randomly uprooted (no more than one seedling per pot). Remaining soil was carefully washed from the roots, and the entire seedling was surface sterilized (70% ethanol for 1 min, 3% NaClO for 1 min, three rinses in sterile distilled water for 1 min). Due to the small size of the seedlings at 13 days after sowing, 100–120 mg of plant tissue containing approximately equal amounts of roots, stem and leaves of each sampled seedling was considered as one sample. Each sample was placed in a 1.5 mL Eppendorf tube and kept in a freezer at −20 °C until DNA extraction.

For the day 28 sampling, 100–120 mg of root, stem, and leaf tissue (second true leaf) were sampled for DNA analysis, and an additional 150 mg leaf tissue was sampled for metabolite analysis. Samples for metabolite analysis and for detection of endophytism were collected from the same plants. However, DNA samples were collected from only six plants per control treatment, so four additional plants from the control treatments were randomly chosen for metabolite sampling (Table 1). Samples were collected from different pots than those for the day 13 sampling and each tissue was considered a separate sample. Otherwise, the same protocol was followed for DNA sampling as for the day 13 sampling. Metabolite samples were immediately placed in 1.5 mL Eppendorf tubes and flash frozen. They were kept in −80 °C until extraction of the metabolites. DNA was extracted from each sample separately and presence or absence of *B. bassiana* was confirmed using the protocol described in Section 2.3 and the metabolite analysis in Section 2.4. After the day 28 sampling, any remaining pots with two seedlings were thinned to one seedling per pot. Finally, at 60 days after sowing, biomass from the remaining plants (Table 1) were collected and dried, and the aboveground dry biomass of each plant was weighed.

2.3 PCR-based detection of endophytic *Beauveria bassiana*

Based on the results of a pilot study indicating very slow outgrowth of *B. bassiana* from oilseed rape tissues (data not shown), a two-step nested Polymerase Chain Reaction (PCR)[43] to detect endophytic *B. bassiana* from oilseed rape tissue samples was used. For this, tissue samples were surface sterilized by submerging them first in 70% ethanol for 1 min, then 3% NaClO for 1 min, and afterwards rinsed three times in sterile distilled water for 1 min to ensure that any detected fungi was growing endophytically. After surface sterilization, tissue samples were immediately stored in 1.5 mL Eppendorf tubes at −20 °C until DNA extraction. To assess potential contamination in the surface sterilization procedure, 100 µL of the final rinse water was plated on PDA media.

Invisorb© Spin Plant Mini Kit was used for DNA extraction. The procedure provided by the kit manufacturer was closely followed. Tissue samples were homogenized by loading each sample tube with a grinding ball and glass beads and then shaking them in a homogenizer (QIAGEN TissueLyser II) for 60 s at 24 MHz. After extraction, the concentration of DNA in the sample was measured with a nanodrop spectrophotometer (Thermo Scientific NanoDrop UV/VIS ND-1000 Spectrophotometer). The two-step PCR protocol by Garrido-Jurado *et al.*[43] was used because it can detect concentrations of entomopathogenic *B. bassiana* as low as 10 fg. This was necessary since no amplicons were detected in direct PCR with *B. bassiana* specific primers. For the first step of PCR amplification, ITS (Internal Transcribed Sequence) primers ITS1-F: 5′ CTTGGTCATTTAGAGGAAGTAA-3′ and reverse primer: ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ that amplify its regions of most fungal taxa were used.[44] The PCR contents included 1X PCR buffer, 0.2 mM Deoxynucleosidtriphosphates (dNTPs), 0.3 µM each of ITS1-F and ITS4 primers, 1500 U/mL GoTaq DNA Polymerase (Promega, USA) and 30 ng/µl of extracted DNA. The PCR conditions were 95 °C for 4 min followed by 35 cycles of 95 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 10 min. The presence of amplicons was confirmed by electrophoresis on a 1.0% agarose gel at 120 V for 1 h along with a 100 bp gene ruler (ThermoFisher Scientific). The second step PCR was done using *B. bassiana* specific primers on 1:10 diluted PCR product amplified from the first step PCR. These primers were: forward primer BB.fw

Table 1. Sampling scheme for experiment testing the effect of inoculation time, i.e. 2-h and 6-h seed inoculation either with water (control) or *Beauveria bassiana* isolate ATCC7404 (treatment) on endophytic establishment of *B. bassiana* on oilseed rape (*Brassica napus*). For the detection of endophytic *B. bassiana*, DNA-samples were collected from seed-inoculated and control oilseed rape plants 13 and 28 days after inoculations and sowing. In the same experiment, the effect of endophytic *B. bassiana* on oilseed rape metabolites and growth was studied for which metabolite and biomass samples were taken 28 days and 60 days after inoculations and sowing. Sampling time refers to number of days after seed inoculation and sowing. *n* = number of sampled plants

		n for each sampling			
		Control		Treatment	
Analysis type	Sampling time	2 h	6 h	2 h	6 h
DNA	Day 13	10	10	10	10
DNA	Day 28	6	6	10	10
Metabolites	Day 28	10	10	10	10
Biomass	Day 60	24	18	23	18

–5′-GAACCTACCTATCGTTGCTTC-3′ and reverse primer: BB.rv 5′ ATTCGAGGTCAACGTTCAG-3′.[43] The PCR contents included 1X PCR buffer, 0.2 mM dNTPs, 0.3 μM each of BB.fw and BB.rv primers, 1500 U/mL GoTaq DNA Polymerase (Promega, USA) and 1:10 diluted PCR product from the first-step PCR. The PCR conditions were 95 °C for 2 min followed by 35 cycles of 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 5 min. Each sample was then again analyzed via electrophoresis to determine the presence of *B. bassiana*-specific amplicons. Samples containing *B. bassiana*-specific PCR products were purified with A'SAP PCR clean up kit following the manufacturer's protocol (ArcticZymes). The purified samples were sent to Macrogen Europe for Sanger sequencing for confirmation of *B. bassiana* DNA.

2.4 Metabolite concentrations in oilseed rape with and without endophytic *Beauveria bassiana*

Metabolic profiling by Liquid chromatography–mass spectrometry (LC–MS) was performed from 10 replicates per treatment and time point (2 and 6 h) at the Swedish Metabolomics Center in Umeå, Sweden. PCR-based detection (Section 2.3) revealed that some inoculated plants were negative for *B. bassiana* while some control plants were positive for *B. bassiana*. Therefore, statistical analysis was conducted on the subset of plants that were inoculated and found to be endophyte-positive ($n = 6$) independent of inoculation time and eight control plants confirmed as endophyte-negative ($n = 8$) at the time of metabolite sampling. Sample preparation was performed according to Gullberg *et al.*[45] Information about reagents, solvents, standards, reference and tuning standards, and stable isotopes internal standards can be found as Data S1. In brief, metabolites were extracted from 9 to 12 mg of plant material with 1000 μL of extraction buffer (20/20/60 v/v chloroform:water:methanol). Extracts were analyzed on an Agilent 1290 Infinity UHPLC-system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6546 Q-TOF-MS in both positive and negative ion mode. Both an in-house LC–MS library built up by authentic standards and run on the same system with the same chromatographic and mass-spec settings, as well as an in-house library for *Brassica nigra* were used for the targeted processing.[46]

For statistical analyses of the metabolite dataset, only samples which were initially endophyte inoculated and showed the endophyte DNA during PCR analyses were included. Furthermore, only control samples which did not show *Beauveria* DNA during PCR analyses were included.

2.5 Effect of seed inoculation on germination and seedling survival in semi-field conditions

To test the effect of seed inoculation with *B. bassiana* isolate ATCC74040 on oilseed rape germination and seedling survival in semi-field conditions, a semi-field experiment was conducted in June 2020 at Ruissalo Botanical Garden (Turku, Finland, 60°26′ N, 22°10′ E). Surface sterilized oilseed rape seeds were treated with *B. bassiana* conidial suspension (1×10^8 spores mL^{-1}) and the control seeds were soaked in sterile distilled water containing 0.05% Triton X-100 for 2 h. Four inoculated seeds per treatment were sown in a 1.45 L plastic pot (13 × 13 × 13 cm, Schetelig oy, Vantaa, Finland) containing sterilized potting soil (Kekkilä Viherkasvimulta, Kekkilä-BVB). The potting soil was sterilized by autoclaving at 120 °C for 1 h. Altogether, there were 20 replicates (80 seeds in 20 pots) per treatment for a total of 40 pots including the control. Pots were randomly placed with approximately 25 cm in between them outside on a fabric covered flat surface. Watering was conducted carefully to avoid splashing of water from one pot to another.

Results of the pilot study (data not shown) indicated that seed germination takes 5 to 7 days. Thus, pots were monitored for seed germination 6 and 10 days after sowing. Flea beetles, *Phyllotreta sp.*, were observed to damage emerging seedlings. Thus, 10 days after sowing, *i.e.*, upon emergence of the first true leaves, damage by *Phyllotreta* flea beetles was visually assessed by estimating the percentage of leaf area damaged separately for each leaf and a mean calculated for the whole plant. Finally, seedling mortality was monitored after 19 days. As a large proportion of those seedlings that were alive after 19 days stopped growing, or died within a week, the experiment was terminated. Due to high mortality, samples were not collected for detecting endophytism. Instead, the effect of seed inoculation on seedling survival was tested.

2.6 Statistical analyses

2.6.1 *Effect of endophytic* Beauveria bassiana *on plant growth and plant metabolite concentrations*

The effect of the 2 and 6-h seed inoculations with *B. bassiana* isolate ATCC74040 on oilseed rape germination was tested using a generalized linear model for binomial data modeling the probability that a seed would germinate (germinating *vs*. not germinating). In addition, two separate general linear models with the response variables 'number of days from inoculation to germination' and 'dry weight of oilseed rape plants' were conducted to analyze the effect of 2 and 6-h seed inoculation on time until germination and on plant growth. Note that as detection of endophytic *B. bassiana* was conducted only from the subset of experimental plants; these analyses do not test the effect of endophytic *B. bassiana* on germination and plant growth but instead test the effect of seed inoculation on germination and plant growth. Inoculation treatment (2 or 6-h seed inoculation with *B. bassiana* isolate ATCC74040, and 2 or 6-h control), incubator and their interaction were used as fixed explanatory factors in both models. Because only two incubators were used in the experiment, incubator (1 or 2) was included into models and treated as a fixed factor to control for potential variation between the incubators. Additionally, in the analysis testing the effect of inoculation on the probability that a seed would germinate and the number of days from inoculation to germination, pot nested within treatment was initially included as a random factor to control for two

seeds sown per pot. However, it did not have any effect and was removed from the final models. Oilseed rape plant metabolite data was analyzed from $\log_{10}$ transformed peak area values with Student's *t*-test accounting for multiple testing with a false discovery rate correction of the *P*-value.

2.6.2 *Effect of seed inoculation on germination and seedling survival in semi-field conditions*

The effect of seed inoculation with *B. bassiana* isolate ATCC74040 on oilseed rape was tested using a generalized linear model for binomial data to model the probability that a seed would germinate (germinating *vs*. not germinating) and that a seedling would survive (survive *vs*. not survive) in semi-field conditions. In addition, a general linear model with the response variable percentage of leaf area damaged was used to analyze the effect of the seed inoculation on the amount of feeding damage by *Phyllotreta* flea beetles on oilseed rape seedlings. Inoculation treatment (inoculation with *B. bassiana* isolate ATCC74040 or control) was used as a fixed explanatory factor in both models. In this case, pot nested within treatment was used to control for four seeds sown in each pot.

All statistical analyses were performed with Proc GLIMMIX of the SAS/STAT® Software, Version 9.4 (SAS Institute Inc., 2013).

3 RESULTS AND DISCUSSION

3.1 Effect of endophytic *Beauveria bassiana* on plant growth and plant metabolite concentrations

The probability of germination of the *B. bassiana*-inoculated seeds did not differ compared to control seeds ($F_{3,233} = 0.45$, $P = 0.7141$). The mean germination rate of oilseed rape seeds was 80.3% (85 ± 4.6% for 2-h control, 76.7 ± 5.5% for 6-h control, 80 ± 5.2% for 2-h ATCC7404 inoculation and 79.3 ± 5.3% for 6-h inoculation). The number of days from seed inoculation to germination differed significantly between treatments and was shortest for seeds in the 2-h control treatment ($F = 8.77$, $df = 3, 183$, $P < 0.001$; Fig. 1). This suggests that both the longer soaking time (i.e. 6-h control) as well as seed inoculation with *B. bassiana* slowed germination. However, as inoculation treatment did not affect the oilseed rape growth ($F_{3,78} = 0.56$, $P = 0.6445$), slower germination did not affect the overall growth of the oilseed rape plants. Oilseed rape plants harvested after 60 days had a mean weight of 1.077 ± 0.323 g (mean ± SD dry weight); (1.149 ± 0.068 g for 2-h control, 1.056 ± 0.079 g for 6-h control, 1.039 ± 0.07 g for 2-h ATCC7404 inoculation and 1.049 ± 0.079 g for 6-h inoculation). Neither probability of seed germination nor oilseed rape growth differed significantly between the two growth cabinets (seed germination probability: $F_{1,233} = 0.31$, $P = 0.5780$, growth: $F_{1,78} = 0.01$, $P = 0.9206$). However, the number of days from inoculation to germination differed significantly between growth cabinets, with 8.18 ± 0.338 (lsmeans ± SE) days in one cabinet and 10.05 ± 0.348 in the other ($F_{1,183} = 14.78$, $P = 0.0002$). As indicated by the non-significant interaction between treatment and growth cabinet ($F_{3,183} = 1.22$, $P = 0.3029$), the effect of treatment on the time from inoculation to germination did not differ between the cabinets.

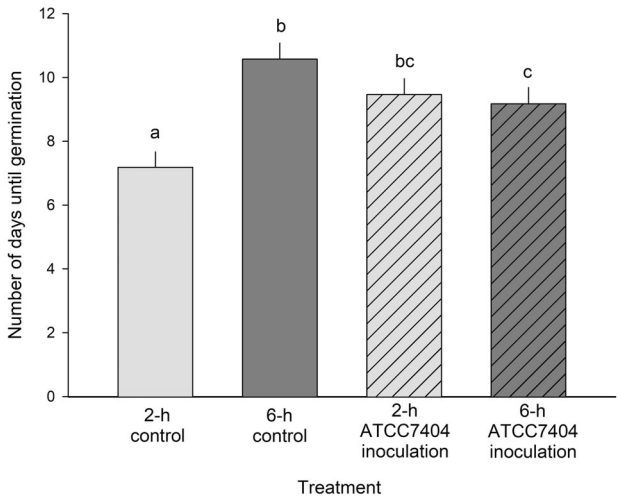

Figure 1. Germination of oilseed rape (*Brassica napus*) following seed inoculation with either water (control) or *Beauveria bassiana*. Mean number of days from seed inoculation to germination (lsmeans + SE) in 2-h ($n = 51$) and 6-h ($n = 46$) control treatment and in 2-h ($n = 48$) and 6-h ($n = 46$) inoculation treatment with *B. bassiana* isolate ATCC7404. Treatments with different letters indicate a significant difference in the number of days from seed inoculation to germination ($P < 0.05$, Tukey's test) among the treatments.

Beauveria bassiana was detected in 10% of the 2-h *B. bassiana* inoculated seedlings and 40% of the 6-h *B. bassiana* inoculated seedlings 13 days after seed inoculation (Fig. 2(a)), but not from control seedlings. This indicates that although the time to germination was prolonged, the 6-h seed inoculation with *B. bassiana* substantially increased the endophytic colonization rate in young seedlings (Fig. 2(a)). At 28 days after seed inoculation, *B. bassiana* was successfully isolated from root, leaf or stem tissues of 50% of the 2-h and 40% of 6-h inoculated oilseed rape plants (Fig. 2(b)). Thus, our findings were not in concordance with those of Jaber and Enkerli[18] which showed that longer inoculation time increased endophytic colonization of *Vicia faba* by *B. bassiana*. Endophytic *B. bassiana* was also detected in the root, leaf or stem tissue of 60% of the 2-h control and 30% of the 6-h control plant samples 28 days after seed inoculation (Fig. 2(b)). Because *B. bassiana* was never detected from the final rinse water from the surface sterilization process and the negative controls in PCR were also always clean, it is unlikely that the detection of endophytic *B. bassiana* from the control plants is due to contamination of samples. Thus, it is more likely that the endophytic colonization of the randomly placed control plants, which shared the same incubators as the inoculated plants, is due to horizontal transmission of *B. bassiana* in the incubator.[40,47,48] In horizontal transmission microorganisms are transmitted outside of a parent-progeny relationship.[49]

Our results confirm that seed inoculation can lead to endophytic establishment of *B. bassiana* in oilseed rape. In addition, our findings demonstrate that *B. bassiana* can also colonize oilseed rape naturally via horizontal transmission. In general, seed inoculation with *B. bassiana* appears to lead to a relatively low endophytic colonization rate in different crop plants varying from 0 to 70% depending on the fungal strain.[50,51] Still, even in cases where endophytic establishment has not been detected at all or only at relatively low rates, seed treatment with *B. bassiana* has been found to have growth-promoting effects[18,50,52] or effects on plant-feeding insects.[53,54] In our study, we did not observe growth promoting effects following seed inoculations. However, as we sampled only a subset of all experimental plants for detection of endophytism and observed also a natural endophytic colonization of control plants by *B. bassiana*, we cannot rule out the possibility that endophytic *B. bassiana* could affect the growth of oilseed rape. Thus, more studies are needed to understand the exact mode-of-action of endophytic *B. bassiana* in oilseed rape.

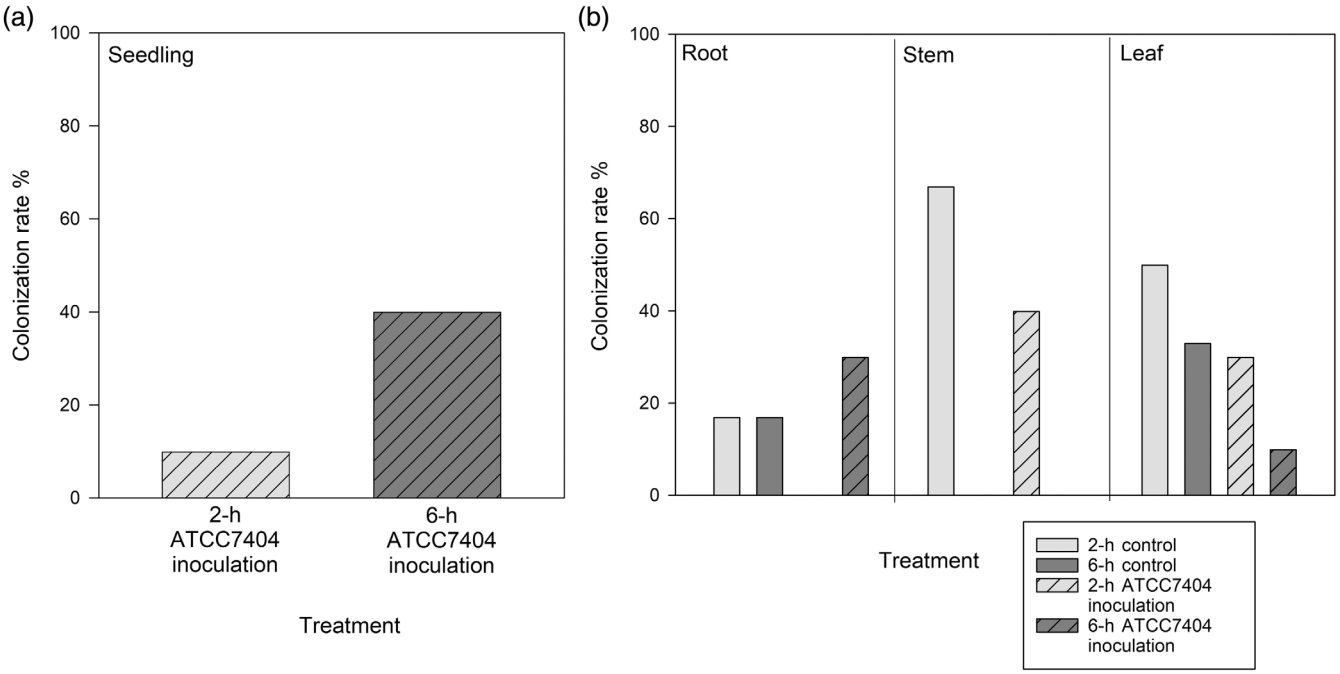

Figure 2. Endophytic colonization of oilseed rape (*Brassica napus*) with *Beauveria bassiana* strain ATCC7404 visualized as colonization rate (%) of (a) seedlings 13 days after seed inoculations and (b) root, stem and leaf tissue 28 days after seed inoculations. Note that in seedlings 13 days after seed inoculations, endophytic *B. bassiana* was not detected from control plants.

Although the relatively low endophytic colonization rate observed here and in other studies may reflect the overall colonization success of *B. bassiana*, it might also be related to the method used to detect endophytic colonization.[40] Except in the case of tiny seedlings, normally only a small part of the whole plant is sampled, and this may reduce the probability of fungal detection in plant tissue. In addition, colonization success of endophytic *B. bassiana* also depends on what part of the plant is inoculated.[18,55] Further, the endophytic colonization of *B. bassiana* has been shown to decline over time, for instance in maize, broad bean, and other crop plants,[18,51] and this might be due to either competition between *B. bassiana* and other faster growing endophytes or plant immune responses rejecting the fungus as a systemic endophyte.[18] Here, endophytic *B. bassiana* was still detected in oilseed rape tissue 28 days after seed inoculation. In fact, because the results showed a clear increase in endophytic colonization over time following 2-h inoculation with *B. bassiana*, and a slight decrease following 6-h inoculation, they did not point to decline of endophytic colonization over time. However, as we also detected endophytic *B. bassiana* from control plants, we cannot rule out the possibility that the endophytism detected in *B. bassiana*-inoculated plants could also be due to horizontal transmission. Although the high endophytic colonization rate of control plants in the growth chamber experiment complicated the interpretation of the results, it showed that *B. bassiana* can naturally colonize oilseed rape as an endophyte.[47] In fact, it has been reported that seeds of a common cultivar of oilseed rape regularly contain *B. bassiana*,[23] which indicates a high compatibility in nature and a promising basis for further investigations on their pest control potential in oilseed rape cultivation.

3.2 Metabolite concentrations in oilseed rape with and without endophytic *Beauveria bassiana*

The mean cumulative peak area of all compound features was approximately 1.3 times higher in endophyte positive plants (mean ± SE: $2.08 \times 10^8 \pm 6.5 \times 10^6$) compared to endophyte-free plants (mean ± SE: $1.59 \times 10^8 \pm 6.0 \times 10^6$, student's *t*-test: $t = -5.48$, $P < 0.01$), indicating that endophytism with *B. bassiana* induced the biosynthesis of plant metabolites.[56,57] Out of the putatively identified 134 metabolites, 29 metabolites showed increased concentration in oilseed rape with *B. bassiana* as endophyte, whereas none of the metabolites was higher in control samples (Table 2). Induced metabolites were grouped in flavonols (flavonoids) (16), hydroxycinnamic acids and derivatives (6), phospholipids (3), organic acids (2) and nucleosides (1). A comparison between control samples which were endophyte-free and control samples showing endophyte-infection revealed five flavonoids that were induced in endophyte-positive plants. This result indicates that 'natural' infection shows a similar induction as inoculation, but lower fungal density may explain why the response appears less pronounced (Table S1). Flavonoids are plant metabolites which are linked to numerous functions, such as antioxidant activity, flower coloration and plant resistance against insect herbivores and pathogenic microbes.[58–60] Endophytic *B. bassiana* has been shown to enhance flavonoid levels in cucumber plants, which altered the plant interaction to herbivorous insects.[42] Among the *B. bassiana* induced flavonoids in our study were exclusively *O*-glycosylated metabolites, in particular kaempferol and quercetin derivatives (Table 2). *B. bassiana* can catalyze the formation of glycosides by addition of sugar molecules to flavonoid skeletons, which may have explained the increased concentrations of *O*-glycosylated flavonoids.[61] Higher concentrations of flavonoids are generally linked to better plant resilience against biotic and abiotic stressors.[62] Thus, glycosylated kaempferol and quercetin derivatives may contribute to a better defense of oilseed rape against herbivores,[63] but this remains to be tested for oilseed rape plants with endophytic *B. bassiana*. In addition, the exact mechanism of endophyte-induced biosynthesis of flavonoid derivatives should be elucidated to understand

Table 2. Effect of endophytic *Beauveria bassiana* (isolate ATCC7404) on oilseed rape (*Brassica napus*) metabolomics. Metabolomics of the leaf extract of endophyte-positive ($n = 6$) and endophyte-negative ($n = 8$) oilseed plants revealed that 29 compounds were induced by endophytic *Beauveria bassiana*. Metabolites were putatively annotated with comparison to standard compounds (ID level 1), mass and retention time (Rt) comparison to library compounds (ID level 2) which sometimes resulted in several possible structures (ID level 3). Mode refers to positive (pos) and negative (neg) ionization mode during Mass Spectrometry

Compound	T stat	*P*-value	Rt (min)	Mass	Mode	Metabolite group	ID level
2-Hydroxyadipic acid/3-Hydroxyadipic acid	4.093	0.001	1.3	162.0526	Neg	Fatty acid	3
Isorhamnetin-3-*O*-sophoroside	3.049	0.010	3.01	640.1636	Neg	Flavonoid	2
Kaempferol-3-4′-diglucoside-7-(feruloyl)-glucoside	3.355	0.006	3.16	948.2533	Neg	Flavonoid	2
Kaempferol-3-*O*-(caffeoyl)sophoroside	3.087	0.009	3.37	772.1849	Neg	Flavonoid	2
Kaempferol-3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside	4.311	0.001	3.01	918.243	Neg	Flavonoid	2
Kaempferol-3-*O*-(methoxycaffeoyl)-sophoroside	3.259	0.007	3.36	802.1954	Neg	Flavonoid	2
Kaempferol-3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside	4.011	0.002	2.94	978.2651	Neg	Flavonoid	2
Kaempferol-3-*O*-(sinapoyl)-sophorotrioside	3.449	0.005	3.12	978.2636	Neg	Flavonoid	2
Kaempferol-3-*O*-(sinapoyl)-sophorotrioside-7-*O*-glucoside	3.625	0.003	2.9	1140.3167	Neg	Flavonoid	2
Kaempferol-3-*O*-sophoroside-7-*O*-(feruloyl)-glucoside	3.693	0.003	2.98	948.2544	Neg	Flavonoid	2
Kaempferol-3-*O*-sophoroside-7-*O*-glucoside	4.005	0.002	2.62	772.2063	Neg	Flavonoid	2
Kaempferol-7-*O*-(sinapoyl)-sophoroside	3.618	0.004	3.45	816.211	Neg	Flavonoid	2
Quercetin-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside	3.272	0.007	2.66	950.233	Neg	Flavonoid	2
Quercetin-3-*O*-(feruloyl)sophoroside-7-*O*-sophoroside	3.145	0.008	2.73	1126.3009	Neg	Flavonoid	2
Quercetin-3-*O*-(feruoyl)sophoroside-7-*O*-glucoside	4.033	0.002	2.75	964.2494	Neg	Flavonoid	2
Quercetin-3-*O*-(feruoyl)sophorotrioside	3.488	0.004	3.01	964.2482	Neg	Flavonoid	2
Quercetin-3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside	3.069	0.010	2.6	980.2436	Neg	Flavonoid	2
Chlorogenic acid	3.235	0.007	2.13	354.0953	Neg	Hydroxycinnamic acid	2
1,2′-diferuloylgentiobiose	3.461	0.005	3.7	694.2104	Neg	Hydroxycinnamic acid gly	2
1–2-2′-trisinapoylgentiobiose	3.153	0.008	3.66	754.2314	Neg	Hydroxycinnamic acid gly	2
1-sinapoyl-2-feruloylgentiobiose	3.762	0.003	3.64	724.2213	Neg	Hydroxycinnamic acid gly	2
2-Feruloyl-1,2′-disinapoylgentiobiose	3.210	0.007	3.93	930.2789	Neg	Hydroxycinnamic acid gly	2
1,2-Disinapoylgentiobiose	3.188	0.008	3.77	754.2314	Neg	Hydroxycinnamic acid gly	2
Succinyladenosine	3.108	0.009	1.94	383.1077	Pos	Nucleoside	1
Glucaric acid	3.658	0.003	0.35	210.0381	Neg	Organic acid	2
Gluconic acid	3.800	0.003	0.35	196.0591	Neg	Organic acid	1
LysoPC (0:0/18:2(9Z-12Z))	3.193	0.008	6.08	519.3327	Pos	Phospholipid	1
LysoPC (0:0/18:3)	3.183	0.008	5.8	517.3171	Pos	Phospholipid	1
LysoPE (0:0/18:3(6Z-9Z-12Z))	3.402	0.005	5.84	475.2701	Pos	Phospholipid	1

whether it is a defense response of the plant to its endophytic invader or possibly even biosynthesized by the endophytic *B. bassiana*. As we did not record any disadvantages in terms of plant performance, the extra biosynthetic effort did not appear to result in reduced plant performance.

3.3 Effect of seed inoculation on germination and seedling survival in semi-field conditions

A mean of 43.4 ± 7.2% (lsmeans ± SE) of *B. bassiana*-inoculated and 55.2 ± 7.2% of control oilseed rape seeds germinated in the semi-field conditions, but the germination probability did not differ between the treatments ($F_{1,38}$= 1.32, $P = 0.2577$). Survival probability for *B. bassiana*-inoculated seedlings was 29.7 ± 5.8% (lsmeans ± SE) and for control seedlings 44.9 ± 6.4%. However, the difference in the survival probability was only marginally significant ($F_{1,38} = 2.98$, $P = 0.0922$; Fig. 3). Previous studies have suggested that inoculation may have a temporary cost to young plants.[52,64] Although the germination and survival probabilities were not significantly lower for *B. bassiana*-inoculated seedlings, this kind of cost could explain the observed trend. The amount of leaf area damaged by *Phyllotreta sp.* beetles was 50.7 ± 4.3% (lsmeans ± SE) for *B. bassiana*-inoculated seedlings and 48.7 ± 3.8% of control seedlings and it did not differ between the treatments ($F_{1,29} = 0.12$, $P = 0.7288$). *Phyllotreta sp.* beetles are known to be

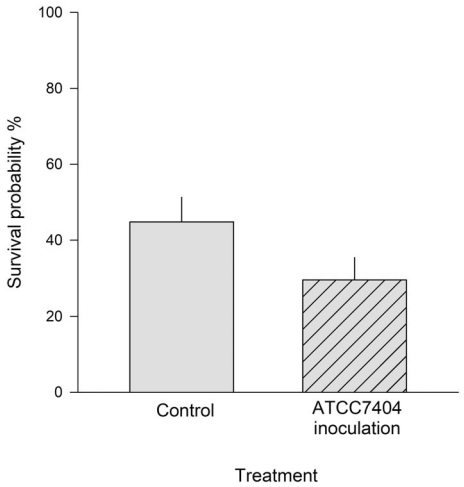

Figure 3. Survival probability (%) of oilseed rape (*Brassica napus*) seedlings following seed inoculation with *Beauveria bassiana* strain ATCC7404 or water controls in a semi-field experiment under natural herbivore (*Phyllotreta spp.*) pressure (lsmeans + SE).

able to lethally damage small oilseed rape seedlings through feeding damage.[8] Given the high amount of damage by *Phyllotreta sp.* beetles, it seems probable that they contributed to high mortality of oilseed rape seedlings in this experiment. Furthermore, these results suggest that seed inoculation with *B. bassiana* did not protect oilseed rape seedlings against *Phyllotreta sp.* beetle damage as the amount of leaf area damaged did not differ between *B. bassiana* inoculated and control seedlings. It is worth noting that the endophytic colonization of oilseed rape plants by *B. bassiana* was not tested in this semi-field experiment due to high mortality of the study plants. High mortality made it impossible to compare the potential endophyte-mediated impacts on mortality. Thus, the results were interpreted as differences between *B. bassiana* inoculated and non-inoculated seedlings.

There is a growing body of evidence that endophytic entomopathogenic fungi including *B. bassiana* can protect a range of different host plants against insect pests.[40,42] However, there are also studies that have reported that entomopathogenic fungi as endophyte or seed inoculations with entomopathogenic fungi have neutral and even positive effects on insect pests.[40,53,65] For instance, Jensen *et al.*[53] showed increased fecundity of aphid *Aphis fabae* aphids on broad bean (*Vicia faba*) following both seed and leaf inoculation with *B. bassiana*. In this study, seed inoculation with *B. bassiana* was found to induce biosynthesis of flavonoids in controlled conditions, but it did not seem to affect *Phyllotreta sp.* feeding preference on inoculated or non-inoculated oilseed rape seedlings. However, more studies are needed to reveal whether seed inoculation and endophytic *B. bassiana* mediated changes in oilseed rape quality affect pest insect preference and/or performance.

4 CONCLUSION

Although our results show that *B. bassiana* has endophytic potential following seed inoculations and natural colonization on oilseed rape, seed inoculation did not protect seedlings from *Phyllotreta sp.* feeding damage. Based on these results, plant protection potential of endophytic *B. bassiana* on oilseed rape remains to be demonstrated. Some of the plant metabolites induced by endophytic *B. bassiana* have previously been linked to plant protective features but whether they could really contribute to increased plant protection against some other insect pests or pathogens needs to be validated in future studies. In addition, future studies need to address whether 40% colonization rate is enough to protect an entire field. As both the endophytic and biocontrol potential of different *B. bassiana* strains has been shown to differ substantially, screening more fungal strains could help to identify candidates with more biocontrol potential on oilseed rape. Furthermore, elucidating the host plant metabolic activity induced by endophytic *B. bassiana* and understanding the importance of different abiotic environmental factors for the outcome of plant-fungus-interactions is likely to help overcome the challenges related to the use of entomopathogenic *B. bassiana* as biological control agent.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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