Endophytic potential of entomopathogenic fungi *Beauveria bassiana* on *Brassica napus* for plant protection

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Master's thesis

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Abstract

To ensure food security for the growing human population sustainable agricultural practices are required. Oilseed rape (*Brassica napus* L.) is an important crop cultivated worldwide for its oil. Herbivores and disease can cause major damage to the crop and conventional cultivation is dependent on chemical pesticides. Heavy pesticide use has a negative impact on the environment and is not sustainable and thus alternatives are needed. Potential biological tool for pest management are entomopathogenic endophytic fungi such as *Beauveria bassiana* (Balsamo-Crivelli). These fungi can live asymptomatically inside plants while potentially conferring pest resistance and other beneficial effects. In my thesis I tested whether several strains of an entomopathogenic fungi, *Beauveria bassiana,* can endophytically establish themselves in *B. napus* through inoculation of seeds with fungal conidia and whether the seed treatment affected plant growth. A DNA-based method was developed for identification of endophytic establishment. Additionally, I tested whether longer treatment time of seeds with fungal conidia affects endophytic establishment or plant growth. *B. bassiana* was found to be able to establish itself endophytically in *B. napus* through seed treatment, although at relatively low rates. Additionally, seed treatment did not have any negative or positive effects on the germination or growth of *B. napus*. However, longer treatment time of *B. napus* seeds with *B. bassiana* had some effects on phenology of *B. napus*. The entomopathogenic fungi *B. bassiana* remains a potential candidate for pest management in *B. napus* cultivation, but more research is required for practical application.

Keywords: Entomopathogenic fungi, endophyte, integrated pest management, *Brassicaceae*

Contents

1. Introduction

1.1. Global challenges in pest management

Considering the current global challenges of increasing human population, food security is as important as ever. Global agricultural outputs have more than trebled since the 1961 and food production has thus far been able to increase on par with human population (FAOSTAT 2020, (Wik, Pingali, and Broca 2008). Agricultural intensification of the 20th century has, however, had major environmental downsides, such as excessive pesticide use, soil degradation and greenhouse gas emissions, which raise concerns about the sustainability of conventional practises (Lichtfouse et al. 2009). Even with heavy pesticide use, global losses of major crops to pests and pathogens is estimated to be around 20-40 % annually (Savary et al. 2019). Therefore, one of the current major challenge for sustainable food production is sustainable pest management.

There are several known problems arising from heavy pesticide use, e.g. effects on human health, accumulation of chemicals in soil and animals and resistant pest populations (Cimino et al. 2017; Hatt and Osawa 2019). Additionally, chemical pest management can have harmful effects on beneficial non-target organisms within the agroecosystem, such as natural enemies and pollinators (Pisa et al. 2015). Biological control is an alternative to chemical pesticides where ecological interactions are utilized to minimize crop losses to pests. As regards to herbivores this means introduction or enticement of natural enemies (i.e. predators, parasites, and pathogens) of pests to the crop system (Lichtfouse 2018). Biocontrol can be used as a part of integrated pest management (IPM). IPM is a concept where the environment and population dynamics of the target pest species are considered within socioeconomical context, and all possible techniques are used to keep the pest populations on an acceptable level (Dent 1995). IPM is the global policy decision for pest management, and modern cultivation should largely be based on IPM strategies to minimize chemical pesticide use. For example in EU implementation of IPM is mandatory (Dent 1995; Peshin and Dhawan 2009). However, implementation of IPM has not been perfect and in many countries pesticide use has increased during the 2000s (Peshin and Zhang 2009).

1.2. *Brassica napus* (L.) and its cultivation

The *Brassica* genus is a part of the *Brassicaceae* family, also known as cabbages. The genus contains several cultivated crop plants with great economic and nutritional importance for humans and livestock. *Brassica napus*, its subspecies, cultivars and

varieties are cultivated worldwide for their high-quality oil, as animal fodder and as a cover plant or green manure (Williams 2010). *Brassica napus* is a relatively new species, originating from the allopolyploidy between ancestors of *B. oleracea* (L.) and *B. rapa* (L.) somewhere between 7500 and 12500 years ago (Chalhoub et al. 2014). The cultivation of *B. rapa* likely began 4000 BC in India and had spread to China and Japan by 2000 BC (Snowdon, Lühs, and Friedt 2007). In Europe cultivation of *B. napus* began in the 13th century, mainly for lamp oil production and increased in the 18th century as rapeseed oil was well suited as a lubricant in steam engines (Shahidi 2020; Snowdon, Lühs, and Friedt 2007). Earlier cultivars of *B. napus* contained high amounts of harmful erucic acid, which can cause cardiac damage, and glucosinolates which makes the oil unsuitable for livestock feed. As such, the crop was unsuited for production of cooking oil or as livestock feed and the cultivated area was relatively low (Snowdon, Lühs, and Friedt 2007).

During the 1970 varieties of *B. rapa* low in erucic acid and glucosinolates were identified and bred into a cultivar with zero erucic acid and low glucosinolates content (Snowdon, Lühs, and Friedt 2007). The resulting canola oil has a high nutritional value and is well suited for further processing such as biodiesel production (Shahidi 2020). Consequently, *B. napus* has become one of the most important and widely cultivated oilcrop worldwide, losing only to soybean in cultivation area and plant oil production (FAOSTAT 2020). In the European Union *B. napus* is the most widely cultivated oilcrop and canola oil the most produced plant oil (FAOSTAT 2020). In 2018 the cultivated area in EU was 69000 km^2 which produced nearly 20 million tonnes of seeds (FAOSTAT 2020). In addition to high economic importance, *B. napus* is also an important break crop in many cereal farming systems (Williams 2010). Break crops are crops planted instead of cereals in some years to increase profitability, which they can achieve by disturbing pathogen, herbivore and weed populations, overcoming resistance build-up, and improving soil properties (Finch, Samuel, and Lane 2002)

B. napus is mostly grown in monocultures which are susceptible to a range of pests. In Europe these pests include insects, nematodes, slugs and birds (Williams 2010). The major pest species of *B. napus* differ geographically and between winter and spring cultivation, but are mostly predominated by coleopteran species such as the pollen beetle *Brassicogethes aeneus* (Fabricius) (Coleoptera: Nitidulidae) (Williams 2010). Consequently, large scale cultivation of *B. napus* is dependent on chemical pest management (Dixon 2007; Williams 2010). Negative effects of pesticides on nontarget organisms are especially important to consider in *B. napus* cultivation since insect

pollination increases seed production of *B. napus* crops when compared to just wind pollination (Eisikowitch 1981; Kevan' and Eisikowitch 1988; Stanley, Gunning, and Stout 2013). Therefore, new methods of pest management that minimize the chemical load to the environment and reduce non-target effects are needed for sustainable *B. napus* cultivation.

IPM strategies suitable for *B. napus* cultivation include the promotion of natural enemies of pests, landscape planning, microbial biocontrol and push-pull strategies i.e. modification of herbivore behaviour by stimuli which repels (push) them from protected crop and attracts (pull) them to a source where they can be removed or where they do not cause economic damage. Large-scale release of reared natural enemies of pests is not economically feasible for field crops such as *B. napus*, but microbial pathogens could potentially be sprayed or distributed through seed and soil treatments.

B. napus as other plants of the family Brassicaceae are considered to be nonmycotrophic i.e. they do not generally host mycorrhizal fungi (however, there are some exceptions, see Regvar et al. 2003) and thus do not have access to improved nutrient uptake facilitated by these mutualists (Smith, Read, and Harley 1997). Nevertheless, it has been shown that *B. napus* can benefit by treatment with arbuscular mycorrhizae when the treatment was combined with biocontrol agent *Trichoderma harzianum* (Poveda et al. 2019). Thus, it seems that fungal interactions can benefit *B. napus* even without the intimate mycorrhizal colonization.

1.3. Plant associated microbes

All plants associate with a wide range of microbial organisms (Hardoim et al. 2015; Rosenberg, Sharon, and Zilber-Rosenberg 2009). These associations can range from antagonists, as in the case of plant pathogens, to mutualists such as mycorrhizal fungi (Hardoim et al. 2015). The association between microbe and its hostplant may also change throughout their life-histories i.e. a fungal species can live in the soil as a saprophyte, then inhabit a plant as an asymptomatic endophyte and finally as a pathogen during the plants senescence (Rodriguez et al. 2009; Saikkonen et al. 1998). Nature of plant-microbe association can also change according to environmental factors. For example, *Diplodia mutila*, a fungi infecting a palm tree seedling was shown to turn from mutualistic to pathogenic according to the light level the host plant received (Álvarez-Loayza et al. 2011). Furthermore, the range of microbes inhabiting a plant can interact with each other

and the host plant resulting in an aggregation of organisms (Hardoim et al. 2015; Saikkonen et al. 2004; Yuan, Zhang, and Lin 2010).

Microbial organisms can be classified according to the part of plant they inhabit. Endophytes are microbial organisms, mainly fungi and bacteria, that live within the plant (Wilson 1995). Although the term endophyte specifies only the location of the organisms, it has been associated especially with microbes living asymptomatically and internally within plant tissues for part or all their life cycle (Wilson 1995). Some endophytic fungi are closely associated with their host plant throughout their life cycle, while some are mostly free living e.g. as soil saprophytes and only associate with plants when an opportunity arises (Rodriguez et al. 2009; Saikkonen 2004). Most endophytes colonize the host plant through its environment, but many can also transfer vertically from the host plant to its progeny (Rodriguez et al. 2009; Saikkonen et al. 1998; Yan et al. 2019). For example, some endophytes of the species *Epichloë* can grow into the developing embryo of the host plant and colonize the developing seedling as it grows (Gagic et al. 2018; Philipson and Christey 1986). Fungi which live a part of their life cycle as an endophyte also occur in the soil and as airborne spores, but they can also be transmitted through herbivorous feeding of insects (Rodriguez et al. 2009).

1.4. Plant-endophyte interactions in plant protection

The biocontrol potential of endophytic fungi against insects and pathogens by the Clavicipitaceae family in the order Hypocreales is well known, especially in agronomic grasses (Clay 1989; Kuldau and Bacon 2008). However, there is considerable evidence that these endophyte-grass interactions can range from antagonist to mutualist in natural ecosystems (Saikkonen, Saari, and Helander 2010; Saikkonen et al. 2006). One of the first publications on the biocontrol potential of endophytes in grass systems was on the avoidance of endophyte-infected ryegrass by argentine stem weevil, reported by Prestidge et al. in 1982. In a review article published seven years later by Clay (1989) 15 species of insects that were negatively affected by endophyte-infected grasses were reported. Currently endophytic fungi are important in perennial grass crops grown for grazing, especially in the United States, New Zealand and Australia where cultivars infected with beneficial endophytes are commercially available (Young, Hume, and McCulley 2013). Furthermore, during recent years endophytic entomopathogens, such as *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales) have been identified as potential biocontrol agents against insect herbivores and pathogens in a wide variety

of crops such as tomato, fava bean and opium poppy (Akello and Sikora 2012; Backman and Sikora 2008; McKinnon et al. 2017; Vega et al. 2012).

Although, pest management potential of endophytic entomopathogenic fungi has been recognized, commercially available mycoinsecticides are usually applied through foliar sprays where the spores of the entomopathogenic fungi occupy the plant surface and can interact directly with the target pest (Skovgaard 2002; Backman and Sikora 2008). During the infection process the target insect is killed either by toxic secondary metabolites produced by the entomopathogen or by disruption of insect homeostasis caused by fungal growth within insect haemolymph (Clarkson and Charnley 1996; Kershaw et al. 1999; Vega et al. 2012). The ability of epiphytic entomopathogenic fungi (i.e. fungi applied or living on the surface of plants) to survive and their potential to infect insects is greatly affected by abiotic factors such as humidity, temperature and ultraviolet radiation (Roy 2010; Roy et al. 2005; Skovgaard 2002; Vega 2018). A humidity of 95% is required for conidia germination and the infection rate and mortality of insects is affected by temperature (Roy et al. 2005). These requirements pose a challenge to agricultural applications as externally applied entomopathogenic fungi and their pest management efficiency is affected by these limitations (Vega 2018). As endophytic fungi live within the plants, they are less affected by abiotic factors outside the host plant and could thus be potentially used in a wider range of agricultural settings (Vega 2018).

Entomopathogenic fungal endophytes are fungi that can infect insects but also live asymptomatically and internally within plant tissues for part or all their life cycle (Wilson 1995). Many entomopathogenic fungi with endophytic potential inhabit both agricultural and natural soils worldwide (Bidochka, Kasperski, and Wild 1998; Meyling and Eilenberg 2007). Entomopathogenic fungi can be placed on to plant leaves as a result of rain splash or by contaminated insects (Dutta et al. 2014; Munkvold, Hellmich, and Showers, 1997). As a result the fungi can enter the host plant directly through the leaf epidermis or through natural openings such as stomata (Wagner and Lewis 2000). After the conidial germination on the surface of plants the hyphal growth can enter the plant and grow in the airspace between the leaf parenchyma cells but also traverse throughout the plant through xylem potentially colonizing the whole plant (Wagner and Lewis 2000). Experimentally, it has also been shown that entomopathogenic fungi such as *B. bassiana* can endophytically infect plants through seed or soil treatment (Biswas et al. 2013; Lohse et al. 2015; Tefera and Vidal 2009). The negative effects of entomopathogenic fungi are well known for multiple insect species, but the potential for entomopathogenic fungi living endophytically within a plant to actually infect and cause mycosis in insects feeding

on the plant has not been clearly demonstrated (McKinnon et al. 2017; Vega 2018). As actual mycosis by endophytically living entomopathogenic fungi seems to be rare, the negative effects on herbivores are likely the results of fungal secondary metabolites and fungi inducing plant defences (Vega 2018). Fungal secondary metabolites may be directly toxic to insects, decreasing herbivore damage (Gimenez et al. 2007). In addition, fungal endophytes can activate plant hormone-based plant protection pathways. Specifically, the suppression of salicylic acid pathway and upregulation of jasmonic acid pathway in plants by endophytic fungi seems to confer pest resistance (Bastías et al. 2018; Yan et al. 2019). In addition to pest control, some endophytic fungi have been shown to benefit their host plants by disease prevention, carbon sequestration, nutrient intake and improved salt and drought tolerance (Rodriguez et al. 2008; Rodriguez et al. 2009). For example, treatment of tomato and cotton seedlings with *B. bassiana* has shown to add protection against fungal pathogens in the emerging seedlings (Ownley et al. 2008). In an agricultural setting the beneficial effects that endophytic entomopathogenic fungi can provide to the host plant could be used to improve pest control and growth without relying on an increased chemical load.

Evolutionary history of plant-endophyte interactions is complex and involves mutualistic as well as pathogenic and parasitic interactions (Saikkonen et al. 2004). However, in a relatively simple and human-managed agroecosystems endophyte mediated herbivore and disease resistance, as well as improved drought tolerance and nutrient intake have obvious fitness benefits for the plants. As a trade-off the plant will lose some of its photosynthetic products to the fungi and possibly become more susceptible to some fungal pathogens (Christensen, Bennett, and Schmid 2002; Philipson 1989). Additionally, endophytic fungi have been shown to affect the reproductive resource allocation of its host plant by decreasing the trade-off between seed number and weight i.e. number of seeds did not decrease as much in plants infected with endophytic fungi when seeds of higher weight were produced as compared to non-infected plants (Gundel et al. 2012). Thus, human mediated co-operation between plants and entomopathogenic endophytic fungi could have potential to improve crop yields in other systems as well.

Endophytic lifestyle could also help reduce harmful effects on non-target insects of entomopathogenic fungi. *B. bassiana* can infect a very large range of insects and also endophytically colonize a wide range of plants (Devi et al. 2008; McKinnon et al. 2017). Additionally, there are several known strains (i.e. genotypes) of *B. bassiana* with different specialization to a diverse assemblage of host plants and insects (Devi et al. 2008; Maurer et al. 1997). For example, in laboratory experiment by Toledo-Herna (2015) exposure of

four bee species to two different stains of *B. bassiana* showed significantly different mortality rates depending on the bee species and the fungal strain. Thus, each potential strain should be individually assayed for each environment with a consideration to local insect populations when used in a field setting.

1.5. *Beauveria bassiana* in plant protection of *Brassica napus*

The pest management potential of entomopathogenic fungi in *B. napus* cultivation has been studied in the last few decades (Zhang et al. 2014; Hokkanen and Menzler-Hokkanen 2018). Suggested methods of delivery include bioinsecticides extracted from entomopathogenic fungi and then sprayed similarly to conventional pesticides, autodissemination by attracting pests to artificial devices contaminated with entomopathogen, and entomovectoring using pollinators as vectors delivering the entomopathogen and endophytic colonization of the host plant by the entomopathogen (Hokkanen & Menzler-Hokkanen 2017). Furthermore, a direct application of *B. bassiana* conidia mixed in vegetable oil on *Brassica napus* has been shown to effectively increase pollen beetle mortality (Kaiser et al. 2020). In addition to spraying *B. bassiana* conidia on plant surfaces, *B. bassiana* is known to be able to infect *B. napus* endophytically (Lohse et al. 2015; Vidal and Jaber 2015). However, in the work done by Vidal and Jaber (2015) endophytic colonization was achieved through foliar application, which would leave the fungi susceptible to environmental factors in an agricultural setting. In a study by Lohse and colleagues (2015) a seed 1-second dip in *B. bassiana* coating suspension (composed of Na-alginate, Na-pectin, gelatine, agar-agar and *B. bassiana* conidia) resulted in mycelium growth on 100% of non-pesticide coated seeds with no effects on seed germination, whereas fungicide coating greatly degreased the mycelium growth success. However, the endophytic colonization through seed coating was not tested. Further, it has been shown that *B. bassiana* can endophytically colonize plants through seed treatment (Biswas et al. 2013; Jaber 2016; Lopez and Sword 2015). However, to my knowledge this has not been proven on *B. napus* where endophytic establishment through seed treatment could have practical potential. Seed treatment would have the additional benefit of pinpointing the fungal colonization to the crop plant and reducing non-target exposure to the entomopathogen compared to foliar spraying. Based on earlier research *B. bassiana* has great potential as a biocontrol agent in *B. napus* cultivation (McKinnon et al. 2017; Ownley et al. 2008; Vidal and Jaber 2015). However, for practical application the endophytic colonization would likely need to happen through seed treatment.

In my thesis I aim to study potential of several strains of entomopathogenic fungi *B. bassiana* to endophytically establish itself in *B. napus* and thus explore new avenues for development of biocontrol methods for *B. napus*. Specifically, I aim to answer 1) whether *B. bassiana* can endophytically infect *B. napus* through seed inoculation. 2) The potential of nine different *B. bassiana* strains to endophytically infect *B. napus.* 3) To test a DNA based method of detecting endophytic *B. bassiana* from *B. napus* 4) To test the effect of different inoculation times of *B. napus* seeds with *B. bassiana* conidia on sprouting and growth of *B. napus* and to determine the infection rate at 13- and 28-days old plants.

2. Materials and methods

2.1. Fungal material and experimental plants

To test the potential of different *B. bassiana* strains to infect *B. napus* endophytically I chose nine "wild" strains and one strain isolated from Naturalis®, a commercial plant protection product. The "wild" strains of *B. bassiana* used in this experiment are originally isolated from different insects in Bulgaria by professor Draganova (Plovdiv Agricultural University, Table 1.). These "wild" strains have been kept in stock culture in cool (+8 °C) and total darkness on 90 mm diameter Petri dishes on Potato Dextrose Agar (PDA) media at Department of Biology, University of Turku. In addition, a commercially available *B. bassiana* strain ATCC74040 was isolated from Naturalis® (purchased from Borregaard BioPlant ApS, Denmark) was used. I transferred these strains from stock growths to 6-10 new 90 mm PDA-petri dishes for mass production of conidia. The insertions were made in a sterile laminar flow cabinet using a metal spike, sterilized in a flame between each strain. The transferred strains were then placed in a growth chamber at 25±2 °C and total darkness. I did the initial insertions for strain ATCC74040 from a stock culture maintained at dark and cool (+8 °C) conditions. However, after three weeks these insertions were growing poorly so I made new insertion from a commercially available spore suspension.

Thirty days after the initial inoculation for mass production of conidia all strains showed poor spore production. To ensure adequate spore production visual observation was performed to select the best growing replicates of each strain, which were then divided into several PDA-media containing Petri dishes. After 45 days from the initial insertions, seven of the nine fungal cultures showed signs of spore production and visual observation was used to select the best cultures for production of conidia suspensions.

As study plant in each experiment I used *B. napus* cultivar 'Cleopatra^{BOR}'. Organic seeds were acquired from *Avena* Nordic Grain Oy and stored at University of Turku Department of Biology in cool (+4 °C) and dark place.

2.2. Conidial suspension

I made the conidia suspension for seed inoculation by scraping the fungal conidia with a sterile steel spatula into 50 ml falcon tubes and mixing with 5 ml of sterilized water supplemented with 0.05% triton X-100 (Sigma-Aldrich, Darmstadt, Germany). The mixture was vortexed vigorously until homogenized, which took 2-3 minutes. The conidial suspension was then centrifuged at 3000 rpm for 3 minutes to separate fungal hyphae and bits of agar from the spores. Number of spores were counted under a microscope using a BLAUBRAND® counting chamber "Burker". The suspension was then diluted to contain approximately 1 x 10^8 spores / ml in sterilized water and triton X-100 mixture. Strains 682, 684, 750 (wild strains) and strain ATCC74040 (isolated Naturalis®) had produced enough spores to reach the desired conidia density and were selected for the inoculation of *B. napus* seeds in Experiment 1.

To test the viability of each suspension 250 µl of conidial suspension was transferred to a PDA-media containing Petri dish and set in a dark growth chamber at 25±2 °C for 24 hours. After this period proportion of germinated conidia was counted under a microscope. Germination rate of 95% was set as a threshold for viable suspension. All the strains used reached over 95% germination rate.

2.3. Experiment 1

To test the potential of different *B. bassiana* strains to establish as endophytes in *B. napus* I inoculated *B. napus* seeds with *B. bassiana* strains 682, 684, 750 (wild) and strain ATCC74040 (Naturalis®). *B. napus* seeds were surface sterilized before inoculation in fungal suspension. Surface sterilization was done by submerging the seeds in 1.5% bleach for 3 minutes followed by submersion in sterilized water for 1 minute repeated three times. For inoculation I submerged the sterilized seeds in the fungal suspensions for 2 hours, as well as in control treatment of water and triton X-100 mixture. After the inoculation the seeds were planted in 90 ml plastic pots containing sterilized potting medium (Kekkilä Viherkasvimulta). The soil was sterilized by heating the soil to 120 °C for 1 hour in an autoclave. I placed two seeds of same treatment in each pot containing sterilized growth medium and watered them thoroughly with tap water. The pots were

placed on five trays in a complete block design with 2 pots of each treatment on each tray for a total of ten replicates for each treatment. Altogether 2 x 4 x 10 = 80 seeds were planted. The trays were placed into a growth cabinet with 8 / 16 light-dark period and corresponding 18 / 21 °C temperature. The watering need was assessed by visual examination of the growth medium. If some of the pots were dry tap water was used to water all pots. The germination of *B. napus* seeds were followed daily until 90% of the seeds had germinated.

The cultivation of endophytic *B. bassiana* out of different *B. napus* tissues on PDA plates is time consuming (it often takes more than 30 days to see fungal growth) and further, microscopic identification of fungal species can be unreliable. Thus, polymerase chain reaction-based methods may be more suitable for detection of endophytic fungi in plants (McKinnon et al. 2017).

In order to develop DNA-based method for the detection of endophytic *B. bassiana* from *B. napus* I collected tissue samples from three randomly chosen *B. napus* seedlings for each treatment 10 days after planting. I sampled 100-120 mg of the first true leaf of each replicate. The leaves were surface sterilized by submerging them in 70% ethanol for 1 minute, then 3% chlorine for 1 minute and finally three times in sterilized water for 1 minute. Surface sterilization of leaves ensures that the fungi detected is an endophyte rather than an epiphyte. After sterilization the samples were immediately moved to 5ml Eppendorf tubes and stored in a freezer (-20 °C) until DNA extraction. A 100 µl sample was taken from the last sterilized water rinse and added to a PDA-media containing Petri dish to check for potential contamination in the surface sterilization procedure.

I determined the presence of *B. bassiana* through DNA-extraction and PCR using *B. bassiana* specific primers. All DNA extractions in this study were done using an Invisorb© Spin Plant Mini Kit. The procedure provided by the kit manufacturer was closely followed. The 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 seconds at 24mhz. After extraction the concentration of DNA in the sample was measured with spectrophotometer ND-1000.

For the PCR the extracted DNA samples were diluted to 30 ng/ml. First, fungal specific ITS primers were used to determine whether the sample contained any fungal DNA. The primers used were forward primer: ITS1-F: 5' CTTGGTCATTTAGAGGAAGTAA-3' and reverse primer: ITS4: 5'-TCCTCCGCTTATTGATATGC-3'. The presence of fungal DNA was confirmed by running the samples on a 1.0% agarose gel electrophoresis running at 120V for 1 hour along with a 100bp gene ruler (ThermoFisher Scientific).

Another round of PCR was done on PCR product produced with the ITS primers using *B. bassiana* specific primers. These primers were: forward primer BB.fw -5'-GAACCTACCTATCGTTGCTTC-3' and reverse primer: BB.rv 5' ATTCGAGGTCAACGTTCAG-3'. Each sample was then again run through electrophoresis to determine whether they contained *B. bassiana* specific DNA.

Samples with confirmed *B. bassiana* presence were purified with A'SAP PCR clean up kit following the manufacturer's protocol (ArcticZymes) and the purified samples were sent to Macrogen Europe for Sanger sequencing.

Additionally, 4 weeks after planting I took five tissue samples from both leaves and stems of the *B. napus* plants of each treatment to assess fungal outgrowths. The samples were surface sterilized using the same protocol as before and placed on PDA-media containing Petri dishes. The Petri dishes were placed in a growth chamber at 24±2 °C and total darkness and observed weekly.

Finally, 34 days after planting of seeds I measured the chlorophyll content of 1st and 2nd true leaves as an estimate of photosynthetic activity. I used a handheld optical chlorophyll meter (SPAD-502 Plus, Konica Minolta, Japan). Measurements with SPAD-502 Plus produce relative values that are proportional to the amount of chlorophyll in the leaf (Guler et al. 2006; Ling, Huang, and Jarvis 2011). Since I did not convert the values to absolute units, they only tell the relative difference between the treatments (i.e. plants growing from seeds inoculated with different *B. bassiana* strains). After the measurements I cut the remaining plants to measure their above ground fresh and dry biomass.

2.4. Experiment 2

In Experiment 1. I found that the strain isolated from Naturalis® was the only *B. bassiana* strain that was able to infect *B. napus* endophytically. Thus, I selected this strain for further study. Given the relatively low endophytic establishment in Experiment 1. and previous studies showing that seed inoculation time in conidia suspension can affect the colonization success of endophytes (Jaber 2016), I tested the effect of seed inoculation time on successful establishment of *B. bassiana* as an endophyte. The seeds were inoculated for 2 or 6 hours in conidia suspension and control seeds in sterile milliQ-water. The inoculation protocol was the same as in Experiment 1. with the exception of inoculation time being 2 or 6 hours.

After inoculation the seeds were planted in sterilized growth medium (Kekkilä Viherkasvimulta) in 11cm plastic pots. Two seeds were placed in each pot and treatments were replicated 30 times. Altogether, 4 x 30 x 2 = 240 seeds were planted. The growth medium was watered thoroughly with tap water and kept moist throughout the experiment. The pots were then transferred into two growth cabinets with 8 / 16 light-dark period and corresponding 18 / 21 °C temperature. The pots were observed daily for germination. In addition, to measure the germination percent in different treatments, 10 seeds per treatment were placed on a sterile moistened filter paper and sealed in a 90mm petri dish. These petri dishes were then placed in a dark growth chamber at constant 25 °C temperature and observed daily for germination.

To study the endophytic establishment of *B. bassiana* I took tissue samples 13 and 28 days after planting the seeds. Furthermore, at 28 days the root, stem and leaves were sampled separately to study the spatial location of endophytic infection. At the first sampling 13 days after planting, I took ten samples per treatment and sampled each seedling as a whole (i.e. roots, cotyledon leaves and stem). First, excess growth medium was carefully washed from the roots, then the whole seedling was surface sterilized by submerging in 70% ethanol for 1 minute, 3% chlorine for 1 minute and finally three times in sterile water for 1 minute. Approximately 100-120 mg of plant material containing all parts (roots, stem and leaves) of the seedling were placed in 5 ml Eppendorf tubes and moved to a freezer (-20 °C). DNA was extracted from each sample and presence or absence of *B. bassiana* was confirmed with PCR using the protocol developed in Experiment 1.

28 days after the planting I took a second set of samples for DNA-extraction. I took approximately 100-120 mg tissue samples from the roots, stem and leaves separately to specify the location of *B. bassiana* infection. I sampled six replicates per each control treatment and ten replicates per each Naturalis® treatment. Again, each sample was placed in a separate 5 ml Eppendorf tubes and placed in a freezer (-20 °C) until DNA extraction. Again, a 100 µl sample was taken from the last sterilized water rinse and added to a PDA-media containing Petri dish to check for potential contamination in the surface sterilization procedure.

Two months after initial planting the number of open flowers and fruits was counted from each replicate of each treatment. At this point there were 87 plants left. Finally, the plants were cut and dried, and the aboveground dry biomass was weighed for each plant.

2.5. Statistical analysis

Number of seeds sprouting per pot on experiment 1 were modelled using a multinomial logistic regression with number of seeds sprouting per pot (0, 1 or 2) as a dependent variable and treatment (inoculation with strains 682, 684, 750 or ATCC74040) as an independent variable. Similar model was used for seed sprouting in experiment 2, with the exception of treatments being inoculation time for 2 or 6 hours in control or strain ATCC74040 (total of four treatments) suspension and the growth chamber instead of block as a random variable.

Linear mixed model was used to model the effect of treatment on relative chlorophyll content of 1st or 2nd leaf and on the fresh and dry biomass of aboveground plant parts in experiment 1. The treatment was used as an independent variable on each model with the block as a random variable.

Effect of treatments to days from planting to first sprout was modelled for experiment 2. using a generalized mixed model with number of days from planting to first sprout as dependent value, treatment as an independent value and block as a random value. The model showed no overdispersion or heterogeneity of residuals. For experiment 1. the number of sprouts was first observed 5 days after planting when majority of seeds had already sprouted, thus no model on the effect of different treatments on sprouting time could be made.

Due to the quarantine conditions applied to universities in the spring 2020 because of the nCoV-19 epidemic, I was unable to continue the experiment until all the plants reached flowering stages. Since only 77% of plants reached reproductive stage, it led to an excess of zero values on total number of buds, flowers and fruits. Thus, a logistic regression was used to model whether plants had developed any reproductive structures (i.e. buds, flowers or fruits) 60 days after planting with presence or absence of reproductive structures as a dependent variable, treatment as an independent variable and growth chamber as a random variable.

Zero inflated negative binomial regression was used to model the total number of flowers, buds and fruits between treatments (2- or 6-hour inoculation) in experiment 2 and each of them separately. Treatment was used as the only fixed effect and growth chamber (1 or 2) as a random effect.

All statistical analyses and plots in this study were made using R software version 4.0.3 (R Core Team, 2016)

3. Results

3.1. Experiment 1. Endophytic colonization of *B. napus* by *B. bassiana* and effects on sprouting biomass and leaf chlorophyll levels

Overall, the seeds had a high germination rate with 80% of pots having two sprouting seeds while 20% of the pots had only 1 sprouting seed. No effect was found by different *B. bassiana* strains on the sprouting of seeds compared to control treatment in experiment 1 (df=43, $X^2(4)=4.86$, p=0.30 for treatment).

Out of the four *B. bassiana* strains tested in experiment 1. strain ATCC74040 was found to endophytically colonize and reach the first true leaf tissue of *B. napus.* Colonization was found in 1 out of 3 samples analysed. *B. bassiana* specific amplification was observed in electrophoresis and further sequencing of the sample confirmed that it contained *B. Bassiana* DNA. I found the DNA based identification method for detection of endophytic *B. bassiana* from *B. napus* to be effective.

None of the inoculation treatments were found to have an effect on chlorophyll content of 1st or 2nd true leaf (df=32, F=0.55, p =0.70 and df=41, F=0.46, p=0.76 respectively). Neither did the inoculation treatment with any strain affect the fresh or dry biomass of the plants (df=41, F=0.80, p=0.53 and df=41, F=0.56, p=0.69 respectively).

3.2. Experiment 2. Endophytic colonization of *B. napus* by *B. bassiana* and effect on sprouting and inflorescence of *B. napus*

Again, the germination and sprouting rate were high with only 4% of the pots having no sprouts, 31% of pots having 1 sprouted seed and 65% of the pots having two sprouted seeds. None of the treatments had an effect on the sprouting of seeds when compared to control treatments in experiment 2 ($X^2(6)=5.41$, p=0.49). However, treatment had a significant effect on the number of days from planting to sprouting (df=109, $X^2(3)=23,3$; p<0,001). Seeds in the 2-hour control treatment sprouted 37%, 24% and 22% earlier than seeds in 6-hour control, 2-hour strain ATCC74040 and 6-hour strain ATCC74040 treatments respectively. No difference on time from planting to sprouting between any other treatments were found.

The seeds germinated on PDA-dishes also showed a high germination rate. Seeds inoculated in control suspension for two or six hours had a germination rate of 100%

(30/30) and 97% (29/30) respectively and those treated with *B. bassiana* suspension for two or six hours had a germination rate of 90% (27/30) and 100% (30/30) respectively.

B. bassiana colonized endophytically *B. napus* in 5 samples out of 40 in 13-days old plants. Positive endophytic establishment was found in one sample of 2-hour conidia treatment and on four samples 6-hour conidia treatment, but not in any of the control treatment samples. However, in 28-days old *B. napus* plants *B. bassiana* had endophytically colonized plants from all treatments (Table 2.). Surprisingly, even control plants showed endophytic *B. bassiana* infection 28-days after planting. Highest colonization rate was found in 2-hour control treatment, with 4/6 plants sampled and 8/18 total samples showing positive *B. bassiana* colonization. The 6-hour control treatment had 2 positive plants with a total of 3/18 samples showing positive colonization. Plants with 2-hour strain ATCC74040 treatment showed positive colonization on 5/10 plants and 7/30 samples. Finally, 6-hour strain ATCC74040 treated plants showed colonization on 4/10 plants and 4/30 samples. (Table 2.)

No effect by any treatment was found on whether any reproductive structures had developed at 60-days after planting (df=80, $X^2(3)=2.36$, p=0.50, Fig 1.). Neither were any of the treatments found to have an effect on the total number reproductive structures (df=80, F(3)=0.389, p=0.76) or on number of buds (df=80, F(3)=1.48, p=0,23) or fruits (df=81, F=1.40, p=0.25) separately 60 days after the planting of seeds (Fig 1.). However, plants grown from seeds inoculated for 6-hours in strain ATCC74040 suspension did have significantly fewer flowers 60 days after planting than plants in any of the other treatments (df=80, F=3.20, p=0.028, Fig 1.). It seems that the 6-hour treatment in strain ATCC74040 suspension could affect the phenology of *B. napus* i.e. affecting the timing of flowering on the plant.

No effect was found on dry biomass of whole plants (roots included) by any treatment at 60-day old plants (df=77, F(3)=0.68, p=0.64).

As endophytic colonization was not determined from each replicate (due to time constraints) and especially since it was shown that *B. bassiana* had spread to plants in control treatments as well, the results of experiment 2. will have to be considered strictly as effects of the initial treatment of seeds and not as effects of endophytic colonization of the plant.

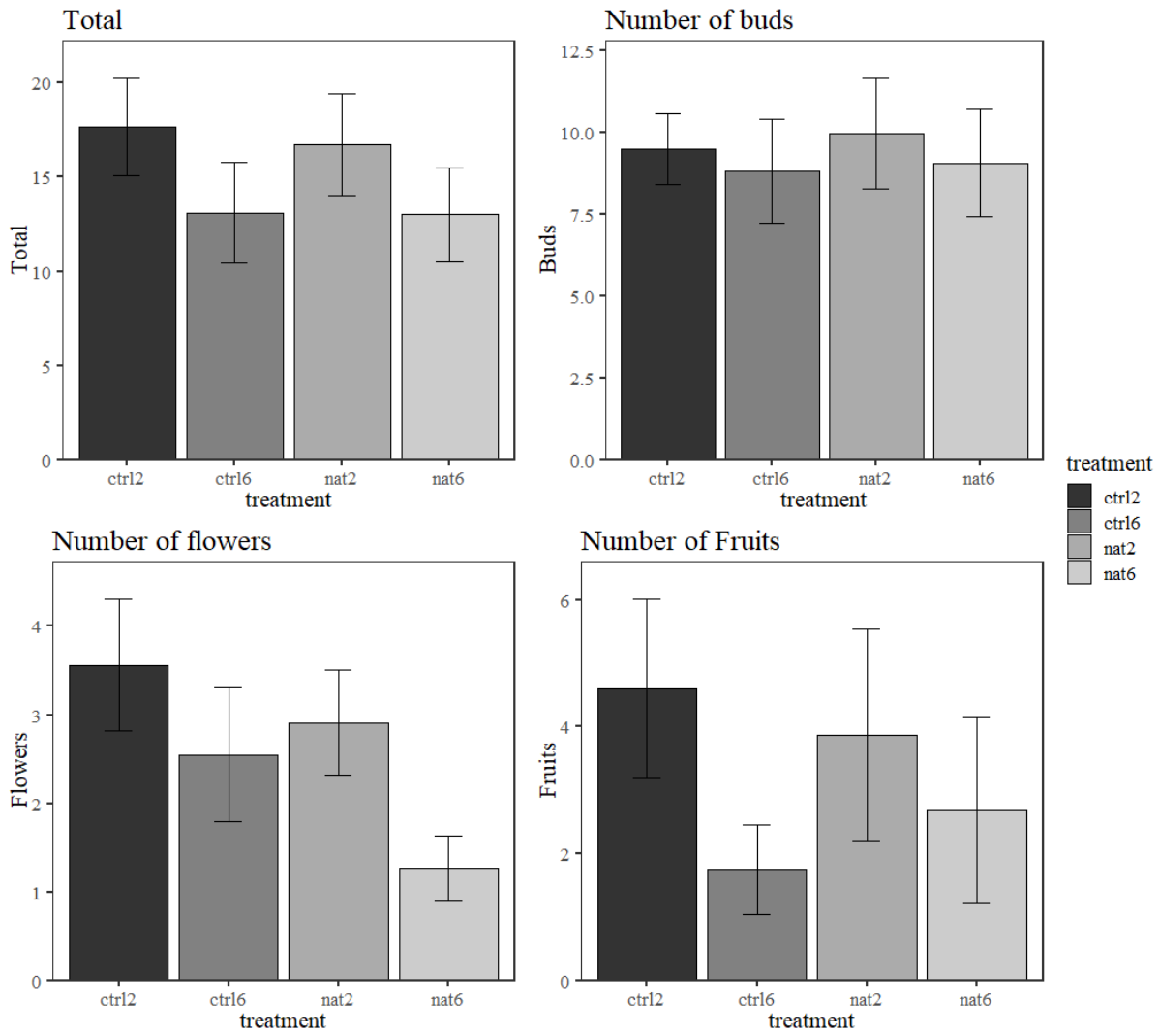

Figure 1. Effect of different *Beauveria bassiana* treatments (ctrl2 = in control suspension for 2-hours, ctrl6 = in control suspension for 6-hours, nat2 = in ATCC74040 (Naturalis) suspension for 2-hours, nat6 = in ATCC74040 (Naturalis) suspension for 6-hours) on the mean ± standard error for number of buds, flowers, and fruits and the total number of reproductive structures on 60-day old *Brassica napus*.

Table 1. Beauveria bassiana wild strains used in Experiment 1 and the insects they were originally isolated from.

Strain	Isolated from
716	*Tuta absoluta* (Povolny) (Lepidoptera: Gelechiidae)
684	*Cydia pomonella* L. (Lepidoptera: Tortricidae)
672	*Malacosoma neustria* L. (Lepidoptera: Lasiocampidae)
682	*Agriopis marginaria* F. (Lepidoptera: Geometridae)
688	*Tanymecus dilaticollis* Gyll. (Coleoptera: Curculionidae)
709	*Larinus latus* Herbst (Coleoptera: Curculionidae)
750	*Grapholita funebrana* Tr. (Lepidoptera: Tortricidae)
762	*Orthosia cerasi* Fabr. (Lepidoptera: Noctuidae)
strain ATCC74040	Commercial strain, isolated from Naturalis®

Table 2. Number of positive *Beauveria bassiana* detections in *Brassica napus* samples taken from different treatments (in control suspension for 2- or 6-hours or in strain ATCC74040 (Naturalis) conidia suspension for 2- or 6-hours) and different plant tissues using the DNA-based detection method developed in Experiment 1.

	Number of positive *B. bassiana* detection / all samples			Number of plants with positive
treatment	root	stem	leaf	detection
control 2h	1/6	4/6	3/6	4/6
control 6h	1/6	0/6	2/6	2/6
Naturalis 2h	0/10	4/10	3/10	5/10
Naturalis 6h	3/10	0/10	1/10	4/10
total	5	8	9	15

4. Conclusions

In my thesis I showed that *Beauveria bassiana* can establish endophytically in *Brassica napus* through seed treatment, although the establishment rate was low. I developed PCR-based method and showed it to be relatively quick and practical for endophytic detection of *B. bassiana* in *B. napus*. I tested nine *B. bassiana* strains for their endophytic potential but found only one strain of *B. bassiana* (ATCC74040, isolated from Naturalis®) to be able to establish endophytically in *Brassica napus*. The inoculation time of *B. napus* seeds (2- or 4-hours in suspension) seemed to have little effect on endophytic establishment of *B. bassiana*. Furthermore, inoculation of *B. napus* seeds in *B. bassiana* suspension for 2- or 6-hours had no positive or negative effects on germination, growth, or number of reproductive structures of *B. napus*. However, inoculation time possibly affected the phenology of *B. napus* since sprouting was significantly earlier for 2-hour control plants and additionally, total number of flowers was found to be smaller on 60-day old plants inoculated in strain ATCC74040 suspension for 6-hours than in any other treatments.

The results of this work confirm the ability of *B. bassiana* to endophytically infect *B. napus* through seed treatment with conidia suspension. In earlier studies with *B. napus*, the successful endophytic infection has been achieved through application of liquid conidia formula on leaves of the plant and encapsulated conidia placed between the roots (Lohse et al. 2015; Vidal and Jaber 2015). Especially application of conidia on leaves seems to result in relatively high endophytic establishment rate (Lohse et al. 2015), but practical application of this method would leave the conidia vulnerable to UV-radiation and desiccation. However, I found that the infection rate following seed treatment was rather low (5.5 % of strain ATCC74040 treated samples positive in experiment 1. and 18 % of strain ATCC74040 treated samples positive on experiment 2.). Endophytic infection, although relatively low (40% of plants showed positive *B. bassiana*

establishment in some part of the plant for both 2-hour and 6-hour strain ATCC74040 treatments), was confirmed for seeds inoculated in *B. bassiana* conidia suspension for two and six hours. Since there was no significant difference in the infection rate, and especially since the results of this study point to the direction that longer inoculation with strain ATCC74040 was likely to delay flowering of *B. napus*, I would suggest that seed inoculation in conidia suspension for two hours is adequate for potential infection.

Out of the four *B. bassiana* strains only strain ATCC74040 was able to endophytically infect *B. napus*. There are known to be considerable differences in endophytic potential of *B. bassiana* strains, and it is possible that the strains tested were simply not adapted to infect *B. napus* (Barra-Bucarei et al. 2020; Vidal and Jaber 2015). Whereas traits that have contributed to commercialization of strain ATCC74040 as Naturalis® potentially include the ability to infect a wide range of insects and plants. Strain ATCC74040 has previously been successfully established as an endophyte in at least in *B. napus*, fava bean (*Vicia faba*), squash (*Cucurbita pepo*), wheat (*Triticum aestivum*), tomato (*Solanum lycopersicon*) and grapevine (*Vitis vinifera*) (Jaber 2016; Jaber 2018; 2015; Jaber and Salem 2014; Klieber and Reineke 2016; Rondot and Reineke 2018; Vidal and Jaber 2015). Out of these species endophytic establishment has been successfully achieved through seed treatment for fava bean and wheat (Jaber 2016; Jaber 2018). Inoculation method plays an important role in the successful endophytic establishment of *B. bassiana* and at least for *B. napus* it seems that foliar application leads to higher levels of establishment (Lohse et al. 2015; Vidal and Jaber 2015). However, as I found the infection rate to be low even for strain ATCC74040, it is likely that by testing more strains it could be possible to find strains with greater endophytic potential and ability to infect *B. napus* through seed treatment. The observed relatively low infection rate would likely have limited usefulness in agricultural application and thus *B. bassiana* strains with higher endophytic establishment rates are required. Potential fungal strains could be screened and tested from field or deliberately bred for this purpose.

This study was the first to confirm the ability of *B. bassiana* to endophytically establish in *B. napus* through seed treatment in sterilized soil. However, an important factor for large scale field applications are abiotic and biotic qualities of soil (e.g. soil moisture, chemical properties, pesticide residues and soil microbiota) as these factors might inhibit or prevent successful growth and endophytic establishment of *B. bassiana*. While I used sterilized growth medium in my study, earlier studies have indicated that establishment of endophytic *B. bassiana* in non-sterilized soils seems to limit endophytic establishment of *B. bassiana* in *B. napus* and other plant species (Lohse et al. 2015; Tefera and Vidal

2009). Seed coating, which could be used to protect the fungi on the surface of the seed, and other methods should be further studied to improve the endophytic infection rate of crop plants through seed treatment in field conditions. Additionally, *B. bassiana* has been shown to germinate on *B. napus* seeds coated with certain fungicides (Lohse et al. 2015). Theoretically entomopathogenic fungi introduced through seed treatments could thus be also used in conventional *B. napus* crop systems. However, it is also known that many pesticides such as certain glyphosate formulations have fungicidal properties on *B. bassiana* (Mietkiewski, Pell, and Clark 1997; Morjan, Pedigo, and Lewis 2002).

Furthermore, endophytic fungi are often more beneficial for plants in situations where the plant is under abiotic or biotic stress, such as drought, salinity or disease (Rodriguez and Redman 2008). My study was performed under optimal growing conditions so it is likely that different results could have been achieved if factors such as drought or low nutrients were included in the experimental design. These are also important factors to be considered and studied in the future for potential field applications of endophytic fungi in *B. napus* cultivation as optimal growing conditions are seldom achieved in field conditions.

It is, promising that no major negative effects were found on germination, growth, or development of reproductive structures of *B. napus* when seeds were treated with conidia suspension. However, the results of this study suggest that longer suspension time has some effect on phenology of *B. napus* which need to be considered in development of potential applications. Overall, the method of seed inoculation described in this study does seem to have potential for mass inoculation of *B. napus* seeds without interfering with the plant growth. Similar results of high germination rates were reported by Lohse et al. (2015) for *B. napus* seeds dipped in *B. bassiana* conidia suspension. However, in their study the seeds were not allowed to grow to confirm endophytic infection as they had found *B. bassiana* germination rates to be low in non-sterilized soil (Lohse et al. 2015).

In my study, the positive *B. bassiana* establishment was surprisingly found not only in plants with fungal seed treatment but in control plants as well. There are few possibilities that might have caused the observed infections of the control plants: 1) the samples were mixed or incorrectly marked during sampling or PCR procedures, 2) the control samples were contaminated during surface sterilization or PCR procedures or 3) *B. bassiana* spread from the infected plants to the control plants during their growth in the growing chambers. The first option is unlikely, since all the samples were systematically taken and processed during the sampling occasions. Neither was there any confusion with the

markings during the PCR procedures. There is a possibility for contamination during surface sterilization or PCR procedures. However, since the last rinsing water in surface sterilization procedure was always tested for fungal contaminants and was always negative and negative controls in PCR were also always clean, contamination seems unlikely. The third option seems most likely since to prevent potential differences between different growth cabinets, I randomly placed control and treated plants into same cabinets. When this was done, I considered that keeping control and treated plants in different cabinets might have caused more bias to the results than there was potential risk for *B. bassiana* to spread through air or irrigation water from treated plants to control plants. However, this proved to be a mistake since I found endophytic *B. bassiana* in multiple control treatment *B. napus* plants after 28 days of growth in growing cabinets. Since all control samples taken during the first sampling occasion 13 days after sprouting were clean, the most likely option is that *B. bassiana* had spread from treated plants to the control plants during their growth. Spreading of *B. bassiana* could have happened through air flow when cabinets were opened and closed, and plants moved. Additionally, as *B. bassiana* is a soil born fungi, it could have spread from inoculated seeds to the sterilized soil around it and then spread through excess irrigation water to soil of control pots and endophytically infecting non-treated plants from there.

Although contamination of control plants in my study was unfortunate, it did give interesting insight into spreading and endophytic establishment of *B. bassiana.* First of all, it seems that endophytic establishment of *B. bassiana* in *B. napus* was at least as successful through spontaneous spreading than that of intentional seed inoculation. Thus, inoculation through soil treatments should be further studies. Although contamination happened in an enclosed environment and sterilized soil where factors are likely to be optimal for spontaneous spreading it is interesting to see that *B. bassiana* can establish itself as an endophyte in *B. napus* quite readily. The beneficial effects of *B. bassiana* for host plants' herbivore and disease resistance are well known (e.g. McKinnon et al. 2017; Ownley et al. 2008; Yan et al. 2019). It would be optimal if the fungi could establish itself endophytically through soil or air on neighbouring plants which do not have the fungal endophyte. However, based on results of *B. bassiana* it seems unlikely that such endophytic spreading would occur in field conditions (Lohse et al. 2015).

Developing our understanding of endophytic fungi establishment in crop plants is important, as it is a potential way to use ecological interactions to reduce pest damage and decrease dependence on chemical pesticides in cropping systems. Additionally, endophytic fungi could potentially increase plant resistance to stress such as drought or

disease. Methods to successfully establish endophytic fungi in crop plants are needed. Seed treatment with fungal conidia is a promising method since it could be used to establish beneficial fungi in cropping systems without leaving the fungal spores exposed to abiotic and biotic factors such as UV-radiation, drought, and pesticides. Furthermore, endophytic fungi could be pinpointed to the plants themselves which would limit their contact with non-target organisms in the environment. Although, the strain tested in this study was not able to infect *B. napus* endophytically at high rates through seed treatment, infection did occur, which proves the potential of incorporating endophytic fungi to seeds of *B. napus*. Additionally, seed treatment with fungal conidia did not seem to have a negative (or positive) effect on the plant even when submerged for six hours. Further studies are required to determine if high endophytic establishment rates can be achieved through different fungal strains or inoculation methods and if they can function in field conditions. For example, potential methods for inoculation resistant to competition with other microbes in soils, such as seed coatings with incorporated fungal conidia or other means to protect the conidia until endophytic colonization of host plant happes, require further study for field applications. Incorporation of entomopathogenic endophytic fungi into *B. napus* cropping systems remains a potential method of increasing pest resistance without additional chemical pesticides, but further study, especially in endophytic establishment and overcoming rhizosphere competition, are required.

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