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Effect of Norwegian entomopathogenic fungal isolates against *Otiorhynchus sulcatus* larvae at low temperatures and persistence in strawberry rhizospheres

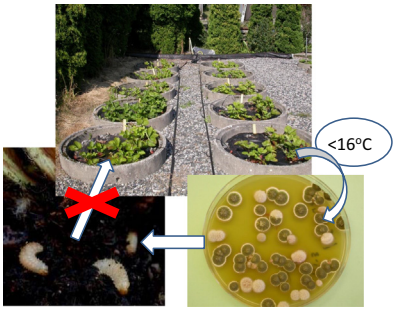
Ingeborg Klingen [a,*], Karin Westrum [a], Nicolai V. Meyling [b]

[a] Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Plant Health and Plant Protection Division, Høgskoleveien 7, N-1430 Ås, Norway
[b] Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

HIGHLIGHTS

- Fungal isolates from Norway killed *Otiorhynchus sulcatus* larvae at low temperatures.
- Two of these fungal isolates increased in abundance in strawberry rhizospheres.
- Norwegian *Metarhizium brunneum* proliferated more in the rhizosphere than an exotic isolate.
- First indication of rhizosphere competence for *Beauveria pseudobassiana*.

GRAPHICAL ABSTRACT



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ABSTRACT

Biological control of belowground stages of the black vine weevil *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae) in strawberries in cool temperate regions using entomopathogens is challenged by low temperatures during the periods when larvae are vulnerable to infections. In a laboratory study we tested six indigenous Norwegian isolates of entomopathogenic fungi (one *Beauveria bassiana*, three *Beauveria pseudobassiana*, and two *Metarhizium brunneum*; Ascomycota: Hypocreales) for their efficacy against *O. sulcatus* larvae at 6, 12, and 18 °C. At the lowest temperature only *Beauveria* spp. affected survival of *O. sulcatus* while all three fungal species reduced larval survival compared to the control treatment at 12 and 18 °C. Two of the Norwegian isolates, one *B. pseudobassiana* and one *M. brunneum*, were then evaluated for long-term persistence (>1 year) in the bulk soil and the rhizosphere soil of strawberries in a semi-field experiment. An exotic isolate of *M. brunneum* sharing origin with a widespread commercial biocontrol agent (F52/Met52 (Novozymes)) was included for comparison. All three isolates showed significantly higher abundances in the rhizosphere soil compared to bulk soil at 153, 366, and 471 days after inoculation, thus indicating rhizosphere competence for *B. pseudobassiana*. Notably, CFU levels for both Norwegian isolates were much higher than for the exotic *M. brunneum* isolate. Selection of locally adapted isolates may therefore be of importance when considering biocontrol strategies of belowground pests in strawberry production.

1. Introduction

The black vine weevil *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae) is an important belowground pest species in soft fruit and ornamental production systems in temperate regions

* Corresponding author.
E-mail address: ingeborg.klingen@bioforsk.no (I. Klingen).

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worldwide (Moorhouse et al., 1992; Cross et al., 2001). The species is polyphagous and is the most important pest among eight root weevil species that can cause damage to strawberries in Norway (Stenseth, 1979; Hesjedal, 1982). In Norway, *O. sulcatus* overwinters predominantly as larvae which become active at 6 °C causing damage in strawberries in late March through May, and the larvae pupate in May–June to emerge as adults in July–August where they oviposit for a couple of months. The larvae of the new generation hatch in September where early instars feed on small roots (Stenseth, 1976).

Biological control of *O. sulcatus* larvae with entomopathogenic fungi (Ascomycota: Hypocreales) has been investigated particularly in the USA (Bruck, 2005; Bruck and Donahue, 2007) and UK (e.g. Moorhouse et al., 1993; Ansari and Butt, 2013) using a specific strain of *Metarhizium brunneum* Petch (until recently *Metarhizium anisopliae*) which has also been commercialized as a formulated product under the trade names F52/Met52 (Novozymes) and GranMet/Bipesco 5 (Samen Schwarzenberger, Austria). Combinations of fungus and other control methods have also been studied (Ansari et al., 2008). Although the same active ingredient/fungal strain has been widely studied and is often among the most virulent isolates when tested against weevils (Moorhouse et al., 1993; Nielsen et al., 2006), its effect at environmental conditions relevant to Norway and other countries, where temperatures are generally lower than in the UK, has received limited attention. Earlier studies have shown that the isolate 275-86, which is the source isolate for F52/Met52 and GranMet/Bipesco 5 (Nielsen et al., 2006), only caused intermediate mortality levels to *O. sulcatus* at 10 and 15 °C compared to other *Metarhizium* isolates (Moorhouse et al., 1994) while it was the most virulent at higher temperatures at greenhouse conditions (Moorhouse et al., 1993). The biocontrol potential of this strain may therefore be limited against *O. sulcatus* in strawberries cultivated in areas where low temperature is a major limiting factor for use of fungi outdoors (Soares et al., 1983). Isolates with activity at low temperatures could therefore be well suited for field conditions in regions where soil temperatures are <12 °C in spring and autumn when control of *O. sulcatus* larvae is possible (Haukeland and Lola-Luz, 2010).

Although use of entomopathogenic fungi against root feeding insects like *O. sulcatus* usually will rely on an inundative biocontrol strategy, it is important to focus on the habitat in which the arthropod–entomopathogen interaction occurs (Klingen and Haukeland, 2006), such as the interphase between root and soil. Several studies suggest that *Metarhizium* spp. interact with plant roots (e.g. Hu and St. Leger, 2002; Klingen et al., 2002a; Bruck, 2005) and that the interaction occurs in the rhizosphere which is the zone of a few millimeters immediately surrounding the root (Bruck, 2010). Particularly *Metarhizium robertsii* Bishoff, Rehner & Humber has been shown to interact intimately with the plant in the rhizosphere where the fungus is active and proliferates (Fang and St. Leger, 2010; Wang et al., 2011) and this interaction was shown to also occur for other species, including *M. brunneum* and *Beauveria bassiana* (Bals.) Vuillemin (Behie and Bidochka, 2014). The rhizosphere is therefore an important microhabitat in the soil for several entomopathogenic fungi and selection of isolates which persist in the rhizosphere at prevailing environmental conditions could ensure prolonged biocontrol effect of root feeding pests.

In this study we aimed at testing selected indigenous Norwegian entomopathogenic fungal isolates for their efficacy against *O. sulcatus* at low temperatures relevant for the conditions in the soil when the larvae are expected to cause damage to strawberries in southern Norway. In a semi-field experiment, two of the Norwegian isolates were then applied to the soil where strawberry plants were planted to evaluate long-term persistence (>1 year) of these local isolates compared to an exotic fungal isolate in the bulk soil and the rhizosphere soil under the prevailing climatic conditions at a northern temperate location.

2. Materials and methods

2.1. Insect cultures and fungal isolates

Black wine weevil *O. sulcatus* larvae were obtained from a laboratory culture at the Norwegian Crop Research Institute (NCRI), Plant Protection Centre, Ås, Norway, which was initiated from *O. sulcatus* adults collected from the roots of strawberry in a field in Grimstad, Norway. Due to limited supply from this rearing, additional *O. sulcatus* larvae were obtained from UK and reared to third instar for some repetitions of the experiments (see below). To be able to obtain enough larvae for each repetition of the experiment, third instar larvae were, when fully developed, placed in a refrigerator in pots with moist soil at 4 °C for up to 6 weeks prior to infection experiments.

Six Norwegian entomopathogenic fungal isolates used in this study were obtained either by baiting soil samples with insect larvae as described by Klingen et al. (2002b) or as natural infections in host insects (Table 1). All isolates are preserved in the culture collection at NCRI and several are additionally deposited at the USDA-ARS Collection of Entomopathogenic Fungi (ARSEF), Ithaca, NY, USA. The fungi were determined to species by sequencing genomic DNA of the 5′ end of the gene elongation factor 1-α (5′ EF1-α) and comparing these to validated reference sequences following the procedures as described by Meyling et al. (2012a).

2.2. Insect infection at low temperatures

Isolates were grown on Sabouraud dextrose agar (SDA) (DIFCO Laboratories, Detroit, MI, USA) for 19–25 days at 22 °C. Conidia were harvested in 0.05% Tween 20 and concentrations of suspensions were adjusted to 1×10^7 conidia/mL using a Neubauer Improved hemocytometer. Conidia suspensions were kept overnight at 12 °C before used in experiments. The bioassay procedure was adapted from Long et al. (2000). Twelve third instar *O. sulcatus* larvae (mean weight = 0.07 g; mean head capsule width = 1.71 mm) were placed individually on the surface of moist horticultural compost (Evergreen Blomsterjord LOG A/S, mean total moisture = 38% w/v) in wells of a 24 well tissue culture plate (3.4 mL per well; each well received a mean of 0.92 g compost). Fungal isolates were applied by pipetting 25 μL of the conidia suspension onto the back of each larva while in the wells, i.e. 2.5×10^5 conidia per larva. Control larvae were treated with 25 μL 0.05% Tween 20. A sheet of polythene and two medical paper wipes were inserted between base and lid of each well plate and secured with an elastic band to ensure a tight seal. Experimental units were incubated at 6, 12 and 18 ± 1 °C, 70% RH and 16:8 h light:dark regime. Larvae were examined for mortality once a week for four weeks after inoculation. According to Butt et al. (1992) limited growth and/or toxicosis caused by an insect pathogenic fungus may be sufficient to kill the insect, and all dead insects that had been treated with fungus were therefore recorded as killed by the fungus whether there was fungal growth or not. The experiment was repeated four times with 12 larvae for each treatment. First repetition included *O. sulcatus* larvae from the rearing at NCRI while the three other repetitions were conducted using larvae of UK origin.

2.3. Fungal persistence in strawberry rhizosphere: experimental set up

A semi field experiment was conducted at the Bioforsk experimental field at Kirkejordet in Ås, Akershus, Norway (59°39′N,

Table 1
List of Norwegian fungal isolates indicating host, coordinates for collection site and for which experiments they were used. Species identification was done by sequencing the 5′ end of the gene Elongation Factor 1-α (see Section 2). Sequences were deposited in GenBank (NCBI).

Fungal species	Isolate number[a]	Host	Location	Experiment	GenBank accession
Beauveria pseudobassiana	NCRI 12/96 ARSEF 5510	*Galleria mellonella* (soil baiting)	63°51′N, 11°13′E	Bioassay Rhizosphere	KM456048
B. pseudobassiana	NCRI 11/96 ARSEF 5557	*G. mellonella* (soil baiting)	68°49′ N, 16°31′ E	Bioassay	KM456047
B. pseudobassiana	NCRI 245/02	*Tetropium fuscum*	59°38′N, 10°50′E	Bioassay	KM456049
B. bassiana	NCRI 2/01/Bb ARSEF 6900	*Ips typographus*	59°51′N, 10°25′E	Bioassay	KM456046
Metarhizium brunneum	NCRI 250/02 ARSEF 11661	*G. mellonella* (soil baiting)	58°35′N, 7°48′N	Bioassay Rhizosphere	KM456044
M. brunneum	NCRI 9/96 ARSEF 5513	*G. mellonella* (soil baiting)	67°18′N, 14°26′E	Bioassay	KM456045

[a] Isolates are deposited at −80 °C at Bioforsk, NCRI, Norway, and five are additionally stored at USDA-ARS collection of entomopathogenic fungi (ARSEF), Ithaca, NY, USA.

10°47′E) from 15 May 2007 until 8 October 2009 to evaluate persistence of two Norwegian isolates in the rhizosphere of strawberries (Table 1) compared to a commercially available *M. brunneum* isolate. Twenty concrete rings (internal diameter = 99.5 cm) were each filled with 280 L of soil (Gartnerjord LOG A/S) containing 84% peat, 10% fine sand, 6% clay and added 5.5 kg lime, 1.2 kg Nitrogen (N) Phosphorous (P) Potassium (K) fertilizer (15-4-12) and 0.2 kg Fritted Trace Elements (F.T.E) No. 36 (micronutrients) per m^3 user volume. Bare root strawberry plants (*Fragaria* x *ananassa*), cultivar Corona, were planted at the 2–4 leaves stage in soil inoculated with barley kernels colonized with one of the three fungal isolates (see below) and the plants were watered by drip irrigation. The experiment included four treatments: Two Norwegian fungal isolates *Beauveria pseudobassiana* Rehner & Humber (NCRI 12/96) and *M. brunneum* (NCRI 250/02), one exotic strain (KVL 99-112) of *M. brunneum* from similar origin as those used commercially for Met52/F52 (Novozymes) and GranMet/Bipesco 5 (Samen Schwarzenberger, Austria), and a control treatment with un-inoculated barley kernels. Each treatment was replicated in five individual concrete rings. To enable destructive sampling of plants five strawberry plants with similar treatment were planted in concrete ring, thus 100 plants (4 treatments × 5 plants × 5 replicates) were included in the experiment. Destructive samplings (see below) were conducted at 17 August 2007 (94 days after inoculation), 15 October 2007 (153 days after inoculation), 16 May 2008 (366 days after inoculation), 28 August 2008 (471 days after inoculation) and 8 October 2009 (877 days after inoculation).

Soil temperatures at 10 cm depth at the experimental site were retrieved as daily means for the period from a weather station at Ås (59°66′N, 10°78′E http://lmt.bioforsk.no/weatherstations/).

2.4. Preparation of fungal inoculum on barley

Two-hundred g organic barley without shell (Helios), 165 mL distilled water and 8 mL of commercial sunflower oil were mixed in an autoclave bag (30 × 40 cm) and left at room temperature for 24 h. To allow for air exchange, a cotton plug was placed in the opening of each bag and a cotton string was used to seal the opening of the bag. The bags were then autoclaved twice (121 °C, 20 min) at a 24 h interval. The *M. brunneum* isolate NCRI 250/02 was grown on SDA and the *B. pseudobassiana* isolate NCRI 12/96 was grown on potato dextrose agar (PDA) (DIFCO™) at room temperature for four weeks before conidia were harvested. The exotic *M. brunneum* isolate KVL 99-112, was received shortly before the experiment from University of Copenhagen on SDA and the culture was thus 2 weeks at harvest of conidia. Conidia suspensions were obtained by adding 15 mL sterile 0.05% Tween 80 to sporulating cultures, rubbing with a glass spatula and sieving the resulting suspension through three layers of cheesecloth into a beaker. Ten ml of each suspension was then inoculated into individual bags with autoclaved barley kernels using a sterile syringe. The concentrations of the inoculum were 2.70×10^7 conidia/mL for *M. brunneum* NCRI 250/02, 1.16×10^7 conidia/mL for *B. pseudobassiana* NCRI 12/96, and 2.44×10^7 conidia/mL for *M. brunneum* KVL 99-112. The inoculated barley kernels were mixed in the bags by hand and incubated in darkness at room temperature for 2 weeks, gently shaking the bags every 2–3 days.

2.5. Inoculation of fungal colonized barley kernels

Barley kernels colonized with each of the fungal isolates were mixed in 1 L of soil (specified above) and five bare root strawberry plants were planted in this mix inside each concrete ring on 15 May 2007. For each isolate, the final amount of fungal inoculum was 1×10^9 conidia in 16.6 g barley kernels to 1 L of soil. This was adjusted for each isolate by adding un-inoculated autoclaved barley to the colonized barley kernels. The original concentration per gram of kernels for each isolate was estimated to 1.2×10^9/g barley for NCRI 250/02, 6.1×10^7/g barley for NCRI 12/96, and 1.1×10^9/g barley for KVL 99-112. The concentration was then adjusted 1×10^9 conidia in 16.6 g barley for all. The control consisted of 16.6 g autoclaved barley kernels without fungus mixed with 1 L of soil.

2.6. Destructive sampling for bulk and rhizosphere soil

Plants were sampled by removing a 20 cm soil cylinder around each plant using a garden spade, grasping the top of the plant and gently pulling out the plant and soil cylinder. This soil was considered the bulk soil and was gently shaken into a 2.5 L plastic box. From each of these bulk soil samples, 0.1 L sub samples were placed individually in new plastic bags. Rhizosphere soil samples were then obtained by shaking each of the harvested plants until only soil tightly adhering to the roots remained. Each plant was then cut at the base and the above ground portion discarded. Root systems were placed into separate plastic bags and brought to the laboratory for processing. The 0.1 L sub samples of bulk soil were placed individually in plastic pans (13.5 × 13.5 × 2.5 cm) while root systems and adhering soil from each plant were placed individually in larger plastic pans (14.0 × 19 × 9.5 cm). The samples were left to air dry at room temperature for 4 days to reach 3–5% moisture (w/w).

2.7. Plating soil samples on selective agar

Dry rhizosphere soil was shaken gently off each root system. One g of this soil and one g of dry soil from each bulk soil subsample from each plant was placed separately in 50 mL conical centrifuge tubes (Greiner Bio-One Cellstar®). Ten mL of sterile 0.05% Tween 80 was added to each tube which was vigorously vortexed for 10 s and then sonicated by immersing the centrifuge tubes up to the internal water line in an ultrasonic bath (Bandelin Sonorex RK255H) for 30 s at room temperature. Each suspension was immediately sieved through a 70 μm cell strainer (BD Falcon™) into 30 mL transparent medical plastic vials. A 1 mL subsample of the suspension was serially diluted to 10^{-2}, 10^{-3}, and 10^{-4} with

0.05% Tween 80 in 15 mL conical centrifuge tubes and 0.1 mL of each suspension was plated onto each of three replicate Petri dishes (9 cm) containing selective agar medium as described by Inglis et al. (2012) and adapted after Veen and Ferron (1966) and Liu et al. (1993). This medium contained: 1% glucose, 1% peptone, 1.5% oxgall, 3.5% agar, 10 μg/mL dodine (*N*-dodecylguanidine monoacetate), 250 μg/mL cycloheximide (actidione) and 500 μg/mL chloramphenicol. At the first sampling occasion (94 days after inoculation) dilutions 10^{-2} for bulk soil and 10^{-3} for rhizosphere soil were plated while at second sampling occasion (153 days after inoculation) dilutions 10^{-2} and 10^{-3} were plated for bulk soil suspensions, and 10^{-3} and 10^{-4} were plated for rhizosphere soil suspensions. For each of the remaining three sampling occasions all three dilutions were plated. The selective media plates were incubated in darkness at room temperature, and colony forming units (CFUs) were estimated after 6 days and again after 12 days using the dilution showing a suitable number of CFUs (20–200 CFUs per plate) for counting. The procedures above were conducted for one plant for each treatment in all five replicates at each date of sampling. Although the soil added to the experimental rings were soil purchased and considered clean from microorganisms and soil arthropods a 0.5 L soil sample was collected from all rings in each replicate, treated as described above and plated on the same selective agar medium to investigate whether there were any *Metarhizium* or *Beauveria* propagules in the soil prior to fungal inoculation.

2.8. Data analysis

Effects of treatments of each fungal isolate relative to the control on larval survival were calculated as hazard ratios (HR; relative average risk of death) fitted to the survival data using a mixed Cox Proportional Hazards (PH) regression model (Cox, 1972) including experimental repetition as a random factor. The Cox PH model is based on the assumption that the risk of death is proportional over time, but can be different among treatment groups. A HR value equal to 1 indicates that two treatments have the same risk of death at any given time. Multiple pairwise comparisons between treatments were performed using Tukey contrasts to correct for significance levels by setting each isolate as the reference group in the mixed model and generating pairwise HR values. These analyses were performed in Rstudio using the coxme package.

The numbers (+1) of CFUs on plates for 10^{-3} dilutions were log10-transformed and analyzed by mixed models in PROC MIXED in SAS with random effects of experimental ring adjusting degrees of freedom by Satterthwaite formulae (Littell et al., 1996). No CFUs of target fungi were ever observed in the controls, thus these data were omitted for further analyses and only fungal treatments were analyzed. Data for each collection day were analyzed separately for fixed effects of treatment (fungal isolate) and soil sample position (bulk or rhizosphere soil) and their interaction. Significant differences were compared by lsmeans and adjusted by the Tukey–Kramer adjustment to identify pair-wise differences ($P < 0.05$).

3. Results

3.1. Effect of fungal exposure at low temperatures

Survivorship of *O. sulcatus* larvae in bioassays incubated at the lowest temperature (6 °C) was generally high (>80%) and significantly affected by treatment ($\chi^2 = 17.458$; d.f. = 6; $P = 0.0077$; Fig. 1a). However, the three isolates *B. bassiana* NCRI 2/01, *B. pseudobassiana* NCRI 12/96 and NCRI 11/96 had HR values not significantly different from the control treatment after multiple comparisons with Tukey contrasts (HR = 1.41, $z = 2.211$, $P = 0.281$; HR = 1.34, $z = 2.36$, $P = 0.208$; HR = 1.51, $z = 2.482$, $P = 0.16$, respectively).

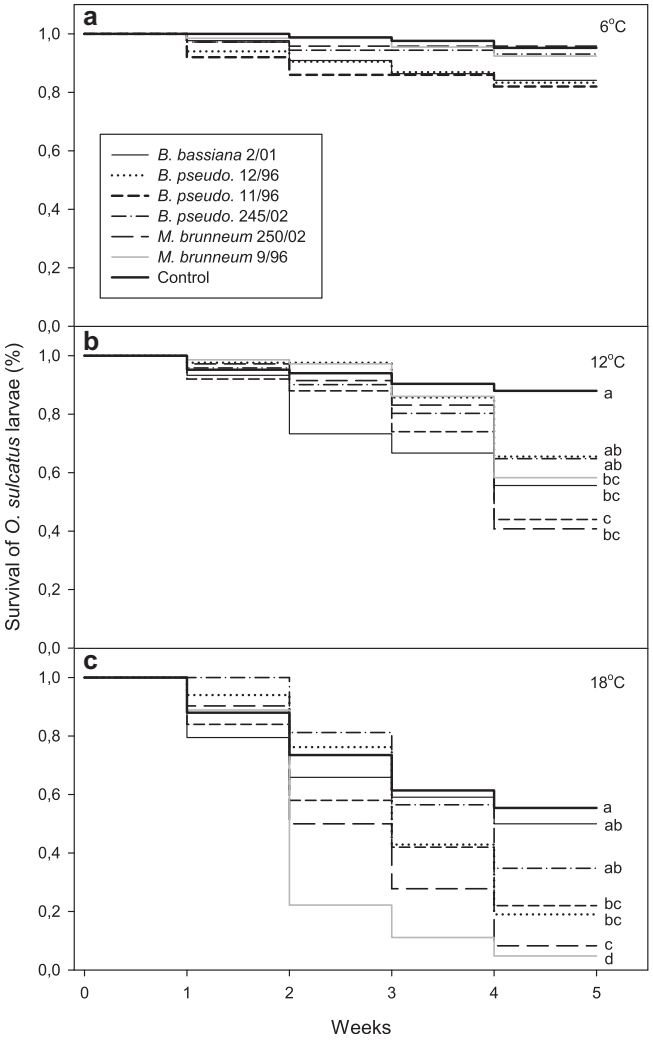

Fig. 1. Survivorship curves of *Otiorhynchus sulcatus* larvae after exposure to six Norwegian entomopathogenic fungal isolates or control incubated at 6 °C (a), 12 °C (b) and 18 °C (c) for 4 weeks. For each temperature, survivorship curves with different letters are significantly different (Cox Proportional Hazards regression model with Tukey contrasts).

When inoculated *O. sulcatus* larvae were incubated at 12 °C, a significant effect of treatment was observed on survival ($\chi^2 = 66.006$; d.f. = 6; $P < 0.0001$; Fig. 1b). All fungal isolates reduced larval survival compared to the control treatment (Tukey contrasts: $P < 0.05$) except the *B. pseudobassiana* isolates NCRI 245/02 and NCRI 12/96. Larvae exposed to these two *B. pseudobassiana* isolates had higher survivorship than the third conspecific isolate NCRI 11/96 (HR = 0.92, $z = 3.263$, $P = 0.0181$ and HR = 0.85, $z = 3.118$, $P = 0.0291$, respectively) which together with *M. brunneum* NCRI 250/02 were the only treatments exhibiting more than 50% mortality, each with median LT50 of 4.0 weeks.

At 18 °C, survivorships among treatments were different ($\chi^2 = 151.67$; d.f. = 6; $P < 0.0001$; Fig. 1c). All fungal isolates imposed more than 50% larval mortality with the two *M. brunneum* isolates NCRI 250/02 and NCRI 9/96 showing median LT50 of 2.5 and 2.0 weeks, respectively. The treatments with *B. pseudobassiana* isolates NCRI 2/01 and NCRI 245/02 showed survival of *O. sulcatus* larvae that were not significantly different from the control treatment (HR = 0.32, $z = 1.138$, $P = 0.9137$ and HR = 0.33, $z = 1.466$, $P = 0.7593$, respectively).

3.2. Fungal persistence in the rhizosphere of strawberries: semi-field experiment

No CFUs of *Beauveria* or *Metarhizium* were observed in the control treatment at any sampling date. Generally, CFU counts from rhizosphere soil samples were higher than corresponding bulk soil samples for each of the three fungal isolates (Fig. 2), but CFU levels varied over time with peaks observed in rhizosphere soil at the third sampling occasion (366 days after inoculation) and then decreasing towards the fourth and fifth sampling occasions (471 and 877 days after inoculation, respectively). At 94 days after inoculation a significant interaction was found between fungal isolate and soil sample position ($F_{2,\ 68.8} = 35.96$; $P < 0.0001$), with *B. pseudobassiana* NCRI 12/96 exhibiting more CFUs in the rhizosphere than in the bulk soil (Fig. 2a) while *M. brunneum* isolates NCRI 250/01 and KVL 99-112 exhibited CFU levels which were not different between respective rhizosphere and bulk soil samples at this date (Fig. 2b and c).

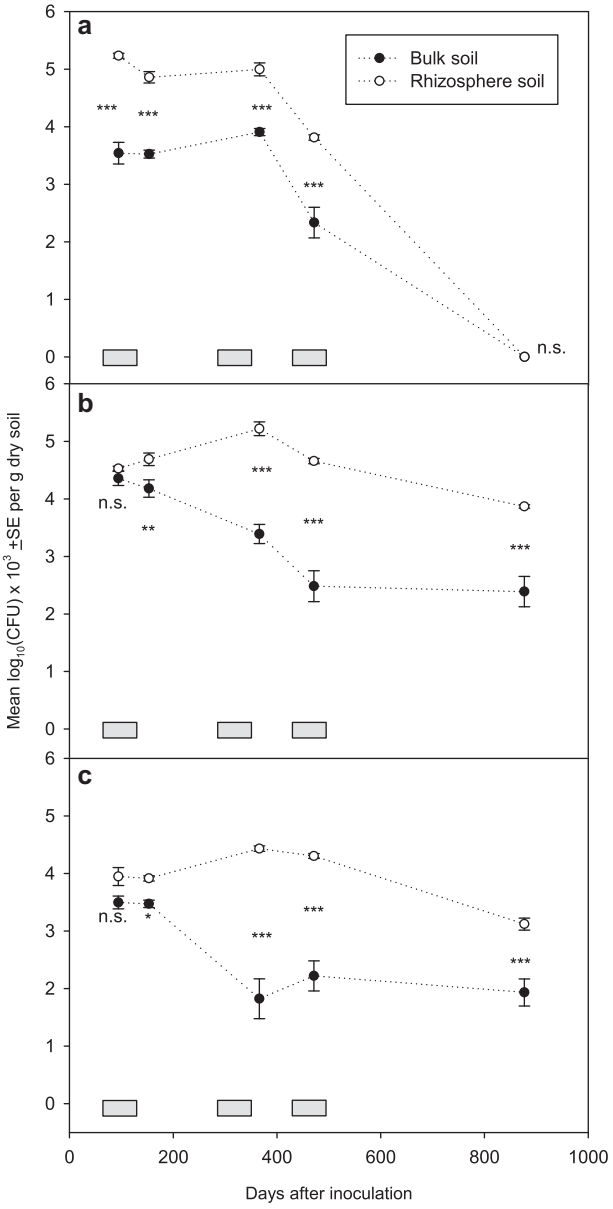

Fig. 2. Variation over >1 year in mean numbers ($\log_{10}$) of colony forming units (CFU) ± SE per g of dry soil either close to but not adhering to roots (bulk soil) or adhering to roots (rhizosphere soil) of three different isolates of entomopathogenic fungi inoculated to the soil substrate of strawberry plants. The plants were grown at field conditions in a semi-field experiment in southern Norway. The treatments included two indigenous Norwegian isolates; *Beauveria pseudobassiana* NCRI 12/96 (a) and *Metarhizium brunneum* NCRI 250/02 (b) and an exotic *M. brunneum* isolate KVL 99–112 (c). For each isolate and sampling time point, significant differences in $\log_{10}$CFUs between bulk and rhizosphere soil are indicated (n.s. = not significantly different; * = $0.01 < P < 0.05$; ** = $0.001 < P < 0.01$; *** = $P < 0.001$; Tukey–Kramer adjustment). Gray shading indicates approximate periods where biocontrol of *Otiorhynchus sulcatus* larvae is most feasible.

Also at 153 days after inoculation, a significant interaction was seen between fungal isolate and soil sample position ($F_{2,\ 77.5} = 15.42$; $P < 0.0001$). All three fungal isolates showed significantly higher CFU levels from rhizosphere soil compared to respective bulk soil samples (Fig. 2) but while CFUs of *B. pseudobassiana* NCRI 12/96 and *M. brunneum* NCRI 250/01 from rhizosphere soil samples were equal (Tukey–Kramer adjustment $P = 0.7039$) both were higher than CFUs of *M. brunneum* KVL 99-112 CFU levels from rhizosphere soil samples (Tukey–Kramer adjustment $P < 0.0001$).

At the third sampling occasion, a year after inoculation (366 days after inoculation), the interaction between fungal isolate and soil sample position was significant ($F_{2,\ 80} = 5.81$; $P = 0.0044$). CFU levels in the rhizosphere soil samples were higher than corresponding bulk soil samples for all three isolates (Fig. 2) but rhizosphere CFU levels of isolates *B. pseudobassiana* NCRI 12/96 and *M. brunneum* NCRI 250/01 were significantly higher than for *M. brunneum* KVL 99-112 (Tukey–Kramer adjustment $P = 0.0002$ and $P < 0.0001$, respectively) while they were not different from each other (Tukey–Kramer adjustment $P = 0.4409$).

CFU levels were higher for all three isolates in rhizosphere soil samples than corresponding bulk soil samples at 471 days after inoculation (Tukey–Kramer adjustment $P < 0.0001$; Fig. 2) but to variable degrees due to significant isolate and soil sample position interaction ($F_{2,\ 74.2} = 39.51$; $P < 0.0001$). At this sampling date, CFU levels of bulk soil samples were similarly low for all isolates (Tukey–Kramer adjustment $P > 0.9$) while *B. pseudobassiana* NCRI 12/96 showed lowest CFU levels in rhizosphere samples compared to the two *M. brunneum* isolates (Tukey–Kramer adjustment $P < 0.0001$). Of *M. brunneum*, CFUs of NCRI 250/01 were recovered at higher levels than of KVL 99-112 in rhizosphere samples (Tukey–Kramer adjustment $P < 0.0001$). At the last sampling occasion (877 days after inoculation) no CFUs of *B. pseudobassiana* NCRI 12/96 were detected in neither bulk nor rhizophere soil samples while the two *M. brunneum* isolates could be found at low CFU levels in both types of soil, but still at significantly higher levels in the rhizosphere soil than in the bulk soil (Tukey–Kramer adjustment $P < 0.0001$). Comparing the two *M. brunneum* isolates revealed most CFUs in rhizosphere soil of NCRI 250/01 to KVL 99-112 (Tukey–Kramer adjustment $P < 0.0001$).

Mean monthly soil temperatures at 10 cm depth at the weather station varied through the year between −1.6 and 17.0 °C (Fig. 3). During the period in the spring where biocontrol of *O. sulcatus* larvae is most feasible, mean monthly temperatures ranged between 1.1 and 10.9 °C in 2008, and 0.0 and 10.5 °C in 2009, while mean monthly temperatures in August–September ranged between 11.0 and 16.3 °C.

4. Discussion

The present study revealed that indigenous Norwegian entomopathogenic fungal isolates collected between 58 and 68°N decreased *O. sulcatus* survival in bioassays at 12 and 18 °C while no isolate reduced larval survival significantly at 6 °C. Effects of entomopathogens at temperatures at or below 12 °C are important for biocontrol of *O. sulcatus* larvae in northern temperate regions because the larval stages that are most relevant to target are active in autumn and early spring (Fitters et al., 2001) and low soil

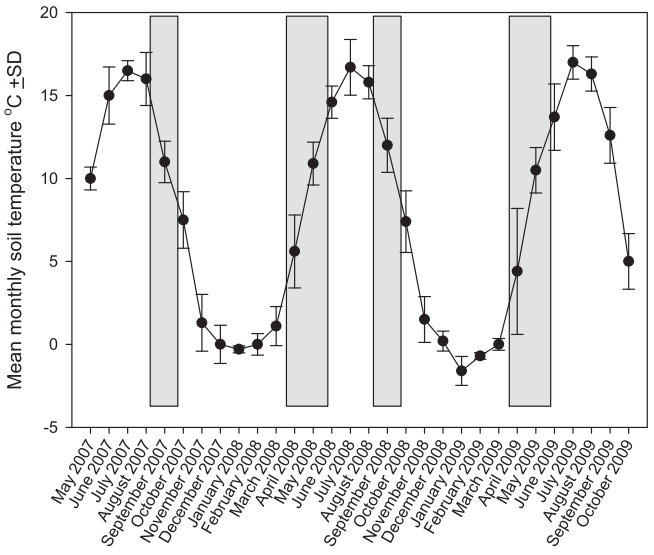

Fig. 3. Mean monthly soil temperatures °C ± SD at 10 cm depth at weather station located next to the experimental field site. Gray shading indicates approximate periods where biocontrol of *Otiorhynchus sulcatus* larvae is most feasible.

temperatures are generally limiting the efficacy of entomopathogenic nematodes against *O. sulcatus* larvae (Long et al., 2000; Haukeland and Lola-Luz, 2010). Few fungal isolates are able to infect and kill insect hosts at low temperatures. Doberski (1981) found isolates of *B. bassiana* and *Isaria farinosa* (syn. *Paecilomyces farinosus*) to infect larvae of elm bark beetles *Scolytus scolytus* at 2 and 6 °C while isolates of *Metarhizium anisopliae sensu lato* were ineffective at these temperatures. Both *B. bassiana* and *B. pseudobassiana* had seemingly some effect at 6 °C in the present study, and these species have been recovered even in soils of Artic Greenland from 69 to 76°N (Meyling et al., 2012b) suggesting that at least some genotypes of these entomopathogenic species are well adapted to cold climates. In addition, Oddsdottir et al. (2010) obtained infections in *O. sulcatus* larvae in an Icelandic nursery soil at 64°N inoculated with a local isolate of *M. anisopliae sensu lato* (KVL 00-37) thus locally adapted genotypes of *Metarhizium* spp. are likely to be candidates for biocontrol of soil-borne pests in cool temperate regions.

In our study at environmental conditions in south eastern Norway two of the Norwegian isolates, belonging to *B. pseudobassiana* and *M. brunneum*, showed higher increase in CFU levels in strawberry rhizosphere soils than the exotic *M. brunneum* isolate KVL 99-112 originating from Austria. Despite this observation, all three isolates were recovered at higher levels in rhizosphere soil than in bulk soil for more than 1 year indicating that propagation of fungal biomass occurs close to the plant root over time. Hu and St. Leger (2002) showed that CFU levels of *Metarhizium anisopliae sensu stricto* (ARSEF 1080) were higher in soil within 1 cm of cabbage roots than at 4–5 cm distances. Similar effects were observed for *M. robertsii* (Fang and St. Leger, 2010), and Bruck (2005, 2010) reported of higher levels of *M. brunneum* CFUs in rhizosphere samples than in surrounding bulk growth media. Persistence and even increase of fungal propagules in the rhizosphere are desirable traits of a biocontrol agent applied against root feeding pests. The commercially available *M. brunneum* isolate F52/Met52 has proved to be effective in biocontrol of *O. sulcatus* larvae using root inoculations (Bruck, 2005; Bruck and Donahue, 2007; Ansari and Butt, 2013). However, these experiments were conducted at higher temperatures than in the present study. Bruck (2005) tested mortality effects at greenhouse conditions (>25 °C) while Bruck and Donahue (2007) incubated bioassays at 24 °C. Ansari and Butt (2013) tested efficacy of Met52 in strawberries in two field trials in UK, but soil temperatures in April and May were 17 and 16 °C, respectively, while soil temperatures at the conclusion of the field trials in October were 13–14 °C. Soil temperatures during the summer between these time points are expected to have been higher. In the present study, spring and autumn soil temperatures were considerably lower than in Ansari and Butt (2013), but they were within the range tested in the bioassays when *O. sulcatus* larvae are expected to cause damage to strawberry roots and are exposed to fungal infections in Norway (Haukeland and Lola-Luz, 2010). The exotic *M. brunneum* isolate KVL 99-112 has the same origin as F52/Met52 (Ansari and Butt, 2013) and this strain (as 275-86) was found by Moorhouse et al. (1994) to be inferior compared to other *Metarhizium* spp. isolates in infection assays against *O. sulcatus* larvae at 10 °C. Although Moorhouse et al. (1994) did not include *Beauveria* spp. isolates in their experiments, their results and the data of the rhizosphere persistence study reported here suggest that this commercial isolate is not most suitable for use in soil applications in southern Norway. Indigenous entomopathogenic fungal isolates, both of *M. brunneum* and *B. pseudobassiana*, were better in proliferating under the environmental conditions and may therefore hold more potential as future biocontrol agents against *O. sulcatus* in strawberry production in cool temperate regions.

This is the first study to indicate that *B. pseudobassiana* is also rhizosphere competent. In a survey of naturally occurring entomopathogenic fungi conducted in Oregon, USA, Fisher et al. (2011) isolated *M. brunneum* from more than 35% of strawberry rhizosphere soil samples while *B. pseudobassiana* was only isolated once, occurring in a blueberry rhizosphere soil sample. Thus *B. pseudobassiana* was not found to be associated with plant roots, let alone strawberries, in the survey reported by Fisher et al. (2011). Behie and Bidochka (2014) did not include *B. pseudobassiana* in their experiments, but our results suggest that this entomopathogenic fungus should also be considered among the species being active in and on plant roots.

Based on the present study there are still challenges for successful biocontrol of *O. sulcatus* larvae with entomopathogenic fungi in strawberries at the environmental conditions in cool temperate regions which may be compromised by low temperatures during the window of control. In this study, indigenous Norwegian fungal isolates appeared to be more promising candidates than the exotic isolate tested. The rhizosphere competence indicated by *B. pseudobassiana* and the significance of this in relation to root feeding pests should be investigated in more detail. However, our strains would need to be registered and authorized before they could be used legally by growers in areas where low temperature is a major limiting factor for use of fungi for biocontrol outdoors.

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