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Efficiency of commercial entomopathogenic fungal species against different members of the genus *Otiorhynchus* (Coleoptera: Curculionidae) under laboratory and semi-field conditions

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Abstract

The application of entomopathogenic fungi against weevils of the genus *Otiorhynchus* spp. might represent an environmentally friendly management option for biological control of these insects, which have recently increased in their importance as pests in a variety of horticultural plants. As only little information is available on the susceptibility of different weevils of this genus towards various fungal species, laboratory experiments were conducted to evaluate the control potential of three different commercially available entomopathogenic fungi (*Beauveria bassiana, Isaria fumosorosea, Metarhizium anisopliae*) against adults of *O. sulcatus*, *O. raucus*, *O. dieckmanni*, *O. rugosostriatus* and *O. crataegi*. Mortality rates were dependent on fungal and weevil species, respectively. Application of Naturalis® containing *B. bassiana* as the active ingredient caused a significantly higher mortality rate than evident in control groups in four of the five *Otiorhynchus* species assessed. Adults of *O. rugosostriatus* were apparently not susceptible to any of the fungi tested. Efficacy of a single application of Naturalis® against adult *O. sulcatus* was also assessed in three independent semi-field experiments. Weevils got infected during the first 14 days after application, but mortality rates did not substantially increase until 2 months after application. Even 6 months after application, mortality of weevils was caused by the respective applied *B. bassiana* strain, which was confirmed via an amplification of isolate-specific microsatellite markers. Thus, a single application of a *B. bassiana*-based product in the field could have long-term effects on population densities of respective weevils.

Key words: *Beauveria bassiana*, biological control, *Metarhizium anisopliae*, susceptibility, weevils

Introduction

Worldwide, weevils of the genus *Otiorhynchus* (Coleoptera: Curculionidae) are polyphagous and important pests in various horticultural crops as well as in viticulture and forestry (Moorhouse et al. 1992, Wheeler 1999, Lykouressis et al. 2004, Bouchard et al. 2005). In recent years, their significance as pest insects has increased, which is most likely due to changing climatic conditions and/or an enhanced global trade of horticultural plants and products (Wheeler 1999, Majka & MacIvor 2009, Lundmark 2010, Reding & Ranger 2011). Within this genus, the black vine weevil *Otiorhynchus sulcatus* is the most widespread and destructive species, however, other species like *O. raucus*, *O. crateagi* or *O. rugosostriatus* are increasingly recognized as pest insects, causing damage on diverse horticultural crops (Wheeler 1999). Usually, the characteristic crescent shaped notches on flowers and leaves resulting from feeding behaviours of adult *Otiorhynchus* weevils have no life-threatening impact on the plants but may reduce their market value especially for ornamental plants. However, polyphagous *Otiorhynchus* larvae which feed on the roots of their host plants may cause severe damage, with symptoms such as wilting or stunting being frequently observed (Moorhouse et al. 1992). In general, control of *Otiorhynchus* weevils is difficult because of the nature of ground-dwelling larvae and the nocturnal activity of adult weevils, which hide in various shelters or under the soil surface during the day. Larval stages can be controlled by the incorporation of synthetic insecticides into the potting substrate (Cowles 2001, Reding & Persad 2009), by the application of entomopathogenic nematodes, by entomopathogenic fungi or by a combination of these control strategies (Kakouli-Duarte et al. 1997, Ansari et al. 2008, Haukeland & Lola-Luz 2010, Ansari & Butt 2013). For adult weevils, control options include foliar applications of insecticides during the pre-oviposition period of females mainly with pyrethroids or neonicotinoids (Cowles 2001, Reding & Ranger 2011). Yet, due to concerns regarding consumer's health or environmental risks associated with the use of chemical pesticides, non-chemical pest control methods should be preferred in modern integrated pest management strategies.

Besides the use of entomopathogenic nematodes, entomopathogenic fungi are of particular interest as putative biological control agents of coleopteran pest insects such as weevils (Nankinga & Moore 2000, Shah et al. 2007, Sabbahi et al. 2008). Worldwide, more than 130 commercial products based on entomopathogenic fungi have been developed in the past, with around two-thirds based on *Beauveria bassiana* and *Metarhizium anisopliae* (both Ascomycota: Hypocreales) (Jackson et al. 2010). Usually, fungal-based products are either poured as an aqueous suspension onto the soil of potted plants, sprayed as foliar applications, or mixed into the potting substrate at the time of planting (Bruck & Donahue 2007, Hajek & Delalibera 2010, Jackson et al. 2010).

Previous studies have indicated that entomopathogenic fungi have a good control potential for adults and larvae of the black vine weevil *O. sulcatus* (Kepler & Bruck 2006, Bruck 2007, Shah et al. 2007, Ansari & Butt 2013). How-

ever, few reports are available documenting the susceptibility of other *Otiorhynchus* species to these biological control agents. For instance, Vainio and Hokkanen (1993) showed in laboratory experiments that *B. bassiana* and *M. anisopliae* have varying degrees of efficacy against *O. ovatus* and *O. nodus* (= *O. dubius*) larvae. Moreover, Sabbahi et al. (2008) proved that different *B. bassiana* isolates vary in their virulence against *O. ovatus* adults.

In the present study laboratory and semi-field experiments were conducted to evaluate the control potential of different commercial formulations of entomopathogenic fungi against adults of various *Otiorhynchus* species, in order to identify products for integration into future non-chemical management strategies providing an effective biological control of weevils of this genus.

Materials and methods

Fungal products and strains

The efficacies of two commercially available products, i.e. Naturalis® (Intrachem Bio Italia S.p.A., Grassobbio, Italy) and PreFeRal® WG (Biobest N.V., Westerlo, Belgium), were tested against various *Otiorhynchus* species. Naturalis® contains 69.1 g l^{-1} *Beauveria bassiana* isolate ATCC 74040 and is formulated as an oily suspension concentrate with at least 2.3×10^7 viable conidiospores per millilitre. Naturalis® is registered as a bioinsecticide in several European countries including Germany as well as in the US, Mexico, Korea and other states. PreFeRal® WG contains 2×10^9 CFU g^{-1} (CFU = Colony Forming Units; blastospores) of *Paecilomyces fumosoroseus* (synonym: *Isaria fumosorosea*) Apopka strain 97 and is prepared as a wettable granule (WG) formulation. Both products were diluted in sterile water to produce a 3.75% (~8.6×10^5 conidia ml^{-1}) or 3% suspension of Naturalis® and a 0.1% suspension of PreFeRal® WG (2×10^6 blastospores ml^{-1}). Doses correspond to concentrations recommended for application by the manufacturers of both products for the control of various insects. Moreover, *Metarhizium anisopliae* strain Ma43 (obtained from the Julius-Kühn-Institute, Darmstadt, Germany; active ingredient in products such as GranMet-P® or BIO 1020) was included in the present study. For infection experiments, *M. anisopliae* strain Ma43 was grown on potato dextrose agar until sporulation and an aqueous solution containing 2.23×10^7 conidia ml^{-1} was prepared, according to previous studies by Shah et al. (2007). As Naturalis® is formulated as an oily fluid we were also interested in the physical effects of this type of formulation against weevils. Accordingly, a pure 3.75% oil emulsion of Naturalis® (obtained from Intrachem Bio Italia S.p.A., Grassobbio, Italy) without fungal spores as well as sterile water were included as controls in the infection experiments.

Source of *Otiorhynchus* spp.

Adults of *O. raucus*, *O. dieckmanni* and *O. rugosostriatus* were captured using pitfall traps in two subsequent years (2011 and 2012) in a nursery near Wiesbaden (Germany) (Reineke et al. 2011). Adult *O. sulcatus* were either obtained from laboratory rearings of the Hochschule Osnabrück (Germany) or Geisenheim University (Germany) or were collected in the field. Individuals of *O. crataegi* were collected in the field by members of the Curculio-Institute (Mönchengladbach, Germany). All *Otiorhynchus* individuals were held at least for two weeks in the laboratory prior to the experiments to ensure their vitality and state of health (i.e. no fungal infection). During that period of time, adults were provided with fresh leaves or shoots of *Prunus* sp. or *Taxus* sp. and were kept at room temperature with a 16:8 h light:dark (L:D) photoperiod.

Laboratory experiments

The efficacy of different commercially available entomopathogenic fungi (3.75% Naturalis®, 0.1% PreFeRal® WG, 3.75% pure oil emulsion of Naturalis®, Ma43 suspension) was tested in the laboratory against adult *O. sulcatus*, *O. rugosostriatus*, *O. dieckmanni*, *O. raucus* and *O. crataegi*. For each species and fungal suspension or water control, respectively, 30–35 weevils were individually treated by applying 50 µl of each suspension on the weevil's dorsum. Experiments were replicated twice over two consecutive years (2010 and 2011). As the number of *O. rugosostriatus* and *O. dieckmanni* weevils caught in the field was limited in both years, only one replication was possible for both species, except for a treatment of *O. dieckmanni* with 3.75% Naturalis®, which was repeated twice in 2011. After inoculation, adult weevils were held individually in 5.5 cm diameter Petri dishes, 1.5 cm high, lined with moistened filter paper and pieces of *Prunus* sp. leaves or *Taxus* sp. shoots (for *O. crataegi*) as a food source. Dishes with weevils were incubated at room temperature and 12:12 h L:D for 28 d. At the end of the experiment, dead, living and missing adults were counted.

Semi-field trials

The efficacy of product Naturalis® against adult *O. sulcatus* was evaluated in semi-field experiments in 2012. Although the efficacy of Ma43 against adult *O. sulcatus* was higher than Naturalis® in our laboratory tests (Fig. 1A), we performed this assay with Naturalis®, as this product showed good mortality against most of the *Otiorhynchus* species assessed and it is registered as a plant protection product in several European countries including Germany. This test was carried out on a plot of freshly ploughed grassland (bare soil, soil type sandy loam), untreated with any pesticides and located at Geisenheim University, Germany. Test enclosures were made of square (1 m × 1 m) metal frames, which were inserted in the soil approximately 15 cm deep. The enclosures were covered by a mesh to prevent escape of the weevils. A total of 8 frames were inserted as single enclosures, which were spaced about 1 m apart from each other. To provide a food source for adult weevils during the experimental period, either a young grapevine *Vitis vinifera*

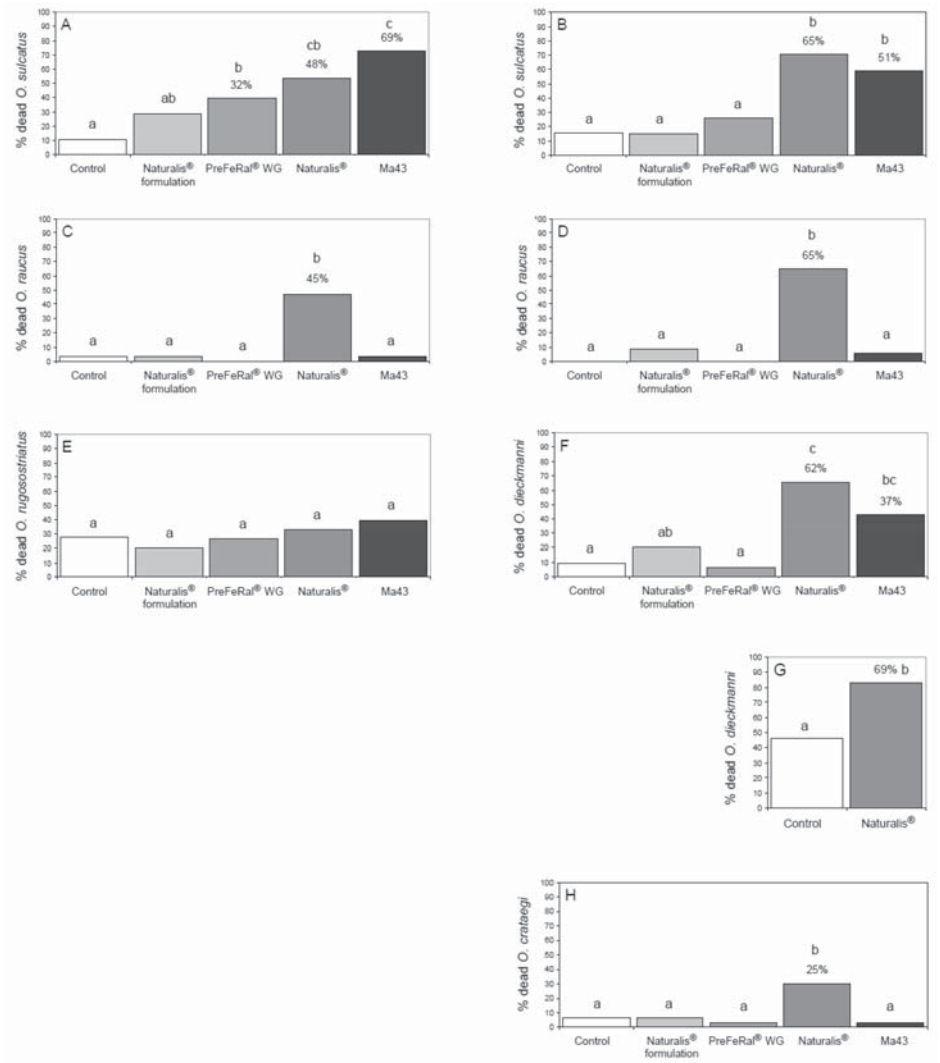

Fig. 1: Percentage of dead *O. sulcatus* (A-B), *O. raucus* (C-D), *O. rugosostriatus* (E), *O. dieckmanni* (F-G) and *O. crataegi* (H) adults after treatment with different entomopathogenic fungi in the laboratory in two independent experiments in 2010 (left) and 2011 (right). Applied test items included the commercial products Naturalis® (a. i. *B. bassiana*) and PreFeRal® WG (a. i. *I. fumosorosea*), a conidial suspension of *M. anisopliae* strain Ma43 as well as the pure oil emulsion of Naturalis® and water as a control. Significant differences between the various test items are indicated with different letters above the bars. In case of significant differences to mortalities in the control group ($p < 0.05$) the degree of efficacy based on corrected mortalities according to Abbot (1925) is indicated on top of

cv. Riesling plant, approximately 20 cm tall, or an *Euonymus fortunei* plant, ca. 15 cm tall, was planted in the middle of the enclosures. Pitfall traps were installed inside the enclosures for 2 days prior to the beginning of the experiment to remove potential predators like carabid beetles. Three independent trials were conducted between May and August 2012 (trial I from May 10 to May 24 2012; trial II from July 26 to August 9 2012; trial III from August 14 to August 28 2012). Air and soil temperatures, amount of precipitation and transpiration, as well as relative humidity (RH) levels were recorded throughout the experimental periods and ranged between 11 and 27°C and 56% to 93% RH in trial I, 18 and 28°C and 69 to 89% RH in trial II, and 20 and 26°C and 64 to 91% RH in trial III, respectively. Soil moisture content was measured during the entire period of the experiments using a tensiometer and plots were watered manually to maintain at least 120 hPa moisture tension. Details on abiotic data during the field trial periods can be provided by the corresponding author on request.

Weevils were released into the enclosures 48 h prior to the application of a 3% Naturalis® emulsion or water as a control to assure that they were adjusted to field conditions and had accepted the field site before treatments. Water instead of the pure Naturalis® formulation was used as a control, to assess the overall effect of product Naturalis® against *O. sulcatus* under practical conditions, which might include a slight physical effect due to the oil-based type of formulation as well as a pathogenic effect due to *B. bassiana*. Each treatment was replicated 4 times in a fully randomized block design. In trials I and II, 15 weevils were placed in each enclosure (replicate), resulting in 60 weevils in total for each treatment, while in trial III 10 weevils were assessed per replicate (40 weevils in total per treatment). In each enclosure, 100 ml of each treatment was applied once on top of the soil with a 1.5-l hand-held sprayer (S1500e EPDM-Handdrucksprühgerät). The test units were inspected 1, 2, 8 and 10 days after spraying by carefully searching for weevils on the soil surface. Dead weevils were removed from the enclosures during this inspection period. After 14 d, numbers of alive, dead and missing weevils were recorded. Living weevils were transferred to the laboratory and were kept in groups according to the respective plots in small

plastic containers (10 × 10 cm, 6 cm high) at 25 °C and 90% RH and were provided with fresh untreated leaves of *V. vinifera* as food. In addition, pitfall traps were maintained inside the enclosures for 3 weeks and weevils remaining in the experimental plots were captured, transferred to the laboratory and held in the same way. Mortality of weevils and possible signs of mycosis were assessed once a week in the laboratory over 6 months. Dead weevils were immediately removed from the plastic containers.

Assessment of infection by microsatellite markers

To verify that death of *O. sulcatus* weevils was in fact a result of fungal infection by the field-applied *B. bassiana* isolate, isolate-specific microsatellite markers were amplified via a polymerase chain reaction (PCR). From a subset of dead weevils from control and Naturalis® treated plots (with and without signs of mycosis) total genomic DNA was extracted using the MasterPure DNA Purification Kit (Biozym Scientific GmbH, Hessisch Oldendorf) according to the manufacturer's instructions. Three *B. bassiana* isolate ATCC 74040 specific microsatellite primers (Ba01, Ba12, Ba13; Rehner & Buckley 2003) were amplified as described in Reineke et al. (2014). Briefly, PCR amplifications were set up in a total volume of 20 µl consisting of 90 ng DNA, 10x reaction buffer, 10 pmol of each primer of which one was labelled with a fluorescent dye, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.5 U of Phire Hot-Start DNA Polymerase (Biozym Scientific GmbH, Hessisch Oldendorf). PCRs were performed at the following conditions: initial denaturation at 98 °C for 30 sec, followed by 35 cycles of 98 °C for 5 sec, 60 °C for 15 sec and 72 °C for 15 sec, followed by a final extension at 72 °C for 1 min and storage at 8 °C. PCR products were analyzed for SSR sizes by capillary electrophoresis on a Beckman GenomeLab GeXP DNA Genetic Analysis System.

Statistics

For statistical analysis missing weevils from laboratory and semi-field trials were treated as non-available data. Mortality data from both laboratory and semi-field trials were corrected for natural mortality according to Abbott (1925) and efficacy values were rounded to the nearest whole number. Statistical analysis of pair-wise comparisons between mortality data of the different test items was performed using a Chi^2-test.

Results

Laboratory experiments

Otiorhynchus spp. adults showed varying degrees of susceptibility to the different fungal preparations in the laboratory tests. For *O. sulcatus* adults both Naturalis® and *M. anisopliae* strain Ma43 induced significantly higher mortality levels compared to the control in 2010 and 2011, with mortality levels reaching up to 69% for both fungi (Fig. 1A, B). Mortality of adult *O. sulcatus* after application of PreFeRal® WG was significantly lower than mortalities caused by Naturalis® (significant difference only in 2011) or *M. anisopliae*, and was significantly higher than mortality in the control group only in 2010; 32% of the treated insects died (Fig. 1A, B).

Significant differences in mortality of adult *O. raucus* and *O. crataegi* compared to the control were evident only after the application of Naturalis®, with mortality levels of 45% and 65% in 2010 and 2011, respectively, for *O. raucus* (Fig. 1C, D) and 25% for *O. crataegi* (Fig. 1H) in 2011. Treatments of *O. dieckmanni* adults with Naturalis® resulted in a significantly higher mortality than evident in the controls in the two independent experiments, with mortality rates of up to 69% (Fig. 1F, G). Mortality levels caused by *M. anisopliae* strain Ma43 against *O. dieckmanni* were significantly higher than in the control, reaching 37% after 28 days (Fig. 1F). Mortalities caused by Naturalis® or *M. anisopliae* strain Ma43 against *O. dieckmanni* adults were not significantly different (Fig. 1F). No significant effects were observed against adult *O. rugosostriatus* for any of the fungi tested (Fig. 1E). Moreover, no adverse effects were observed form the oil-only Naturalis® formulation (Fig. 1A-F, H).

Semi-field trial

The efficacy of Naturalis® against adult *O. sulcatus* was assessed in three independent semi-field trials. Within the field assessment period (14 d) mortality of *O. sulcatus* adults was similar in both the Naturalis® treated and control plots (Fig. 2). Recovery rates of weevils (dead and alive) from the field plots 14 d after treatments varied between 60 and 98% (Table 1). After 14 d none of the dead weevils recovered from control or treated plots showed any sign of mycosis. However, after weevils were transferred to the laboratory, mortality gradually increased and was significantly higher in the Naturalis® treated group compared to the control group after 2 months (Fig. 2). Over the 6 months assessment period *O. sulcatus* mortality levels progressively increased in weevils treated with Naturalis®, while mortality of adult weevils obtained from control plots was generally low with a maximum of 7% after 6 months (Fig. 2). Overall, mortality levels induced by Naturalis® (corrected according to Abbott (1925)) reached up to 79% after 6 months.

PCR verification of infection in the field

A subset of dead weevils obtained from Naturalis® treated and control plots of semi-field trials I – III were subjected to PCR analysis with *B. bassiana* isolate ATCC 74040 specific microsatellite primers. For most of the weevils (71 – 94%) showing obvious signs of mycosis, an isolate-specific fragment was amplified with at least one of the three microsatellite primers (Table 2). In dead weevils obtained from the treated plots but not showing any mycosis, amplification was successful in 40 – 88% of the cases. This indicates that

Table 1: Total number of *O. sulcatus* weevils (dead and alive) recovered from the field plots after 14 d and number of dead weevils obtained from three separate field trials (I-III) at different time points after treatment with water (Co) or with product Naturalis® (Nat). For trials I and II treatments were replicated four times with 15 weevils per replicate (60 weevils in total), for trial III 10 weevils were included per replicate (40 weevils in total).

No. of weevils	Co I	Nat I	Co II	Nat II	Co III	Nat III
Total no. of weevils recovered after 14 d (% recovery)	36 (60%)	50 (83%)	37 (62%)	59 (98%)	31 (78%)	30 (75%)
No. of dead weevils after						
14 d	0	0	4	7	4	5
1 month	0	0	4	12	4	6
2 months	0	16	4	21	4	11
3 months	0	32	4	40	4	18
4 months	0	40	4	44	4	23
5 months	0	46	5	46	4	30
6 months	0	47	7	49	6	31

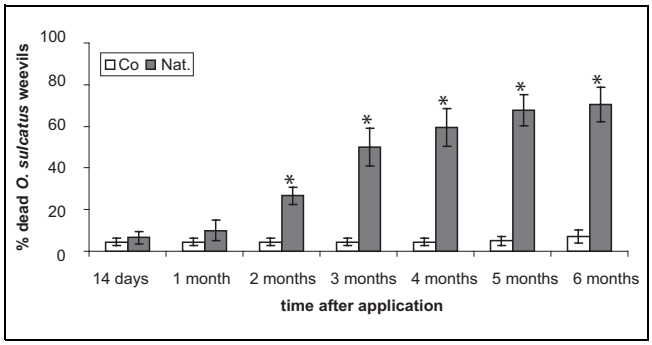

Fig. 2: Cumulative mean percent mortality of *O. sulcatus* weevils at different time points after application of 3.75% Naturalis® (Nat.) or water control (Co) to field plots. For the first 14 days after application weevils were kept in separate plots under semi-field conditions, before they were transferred to the laboratory for further mortality assessment. Bars represent standard error of three independent trials. Significant differences between mortality of weevils in control and treated plots are marked with an asterisk ($p < 0.05$).

these weevils were indeed infected by *B. bassiana* isolate ATCC 74040 and that the fungal isolate applied in the field was the probable cause of observed mortality. No positive reads were obtained from weevils recovered from control plots (data not shown).

Discussion

Results from the present study demonstrate the general potential of various entomopathogenic fungi for management of different adult *Otiorhynchus* spp. For the purpose of our study, we were particularly focusing on the potential of commercial fungal-based products, which are available for growers on the European market or are currently in the process of registration. In laboratory experiments, product Naturalis® caused significantly higher mortality than the control treatment in four of the five *Otiorhynchus* species assessed. Adult *O. rugosostriatus* were apparently not susceptible to any of the fungi tested; however, due to a shortage of weevils of this species trials were not repeated in year 2 and so results should be treated with caution. In addition, of the three fungal species tested, *B. bassiana* was the only one causing significant mortality in *O. raucus* adults. Different insect species of the same genus or even insect populations are known to vary in their response to entomopathogenic fungi in part due to genetic variations in immune defence reactions (Tinsley et al. 2006, Enriquez-Vara et al. 2012, Wang et al. 2013). Recent laboratory studies have shown that insects can develop resistance to entomopathogenic fungi, albeit at the cost of reduced fecundity in females (Kraaijeveld & Godfray 2008, Dubovskiy et al. 2013). Moreover, protection of insects against certain fungal pathogens can be mediated by endosymbionts as has been demonstrated in particular for aphids and *Drosophila* spp. and various endosymbiotic bacteria (Łukasik et al. 2013, Parker et al. 2013). For two of the five *Otiorhynchus* species assessed for susceptibility against entomopathogenic fungi in the present study (*O. sulcatus* and *O. rugosostriatus*) differences in their community composition of endosymbiotic bacteria were recently demonstrated (Hirsch et al. 2012). Whether this difference contributes to the different responses to fungal challenges observed here remains to be determined. Symbiont-mediated protection against fungal pathogens has been recently shown for pea aphids (Parker et al. 2013).

Different species of adult weevils of the genus *Otiorhynchus* spp. cause the same visual damage symptoms when feeding on a host plant and are morphologically difficult to distinguish. At the same time, results of our laboratory tests stress the importance of correctly identifying the respective weevil species causing damage on the relevant crop plant, so that a suitable control strategy can be selected. This is particularly important if a fungus-based product is to be used. Identification of adults or larvae of the genus *Otiorhynchus* is possible either using a digital key on the basis of morphological

Table 2: Number of weevil cadavers with or without signs of mycosis showing *B. bassiana* isolate ATCC 74040 specific amplification products with three different microsatellite primers. Weevils were collected from Naturalis® treated plots in three independent replicates of semi-field trials and were assessed for signs of mycosis up to 6 months in the laboratory after field-application of the product.

Semi-field trial	Total no. of weevils assessed	No. of weevils with successful amplification of isolate specific markers		
		Ba01	Ba12	Ba13
Trial I				
with mycosis	16	15	14	14
without mycosis	10	4	4	4
Trial II				
with mycosis	14	7	12	12
without mycosis	8	2	7	7
Trial III				
with mycosis	7	4	4	5
without mycosis	14	6	6	8

characteristics (Sprick & Stüben 2012) or a recently developed molecular fingerprint (Hirsch et al. 2010).

Semi-field trials using Naturalis® against *O. sulcatus* weevils demonstrated a significant, but prolonged effect of *B. bassiana* on mortality of adult weevils. Insects were infected during the first 14 days after application of this product in the field, but mortality levels did not substantially increase until 2 months after application. After transfer to the laboratory infections continued to develop in the weevils up to 6 months after application of Naturalis®. However, it has to be taken into account that these data result from constant conditions in the laboratory, which might be more advantageous for the progression of fungal pathogenesis than field conditions with varying abiotic conditions. Overall, this indicates that a single application of a *B. bassiana*-based product like Naturalis® can have long-term effects on population densities of weevils, but might not be suitable to achieve an immediate high control level. However, for parthenogenetic adult *O. sulcatus* females a pre-oviposition period for maturation feeding of 21 to 50 d is necessary, depending on the host plant species (Cowles 1995). After that time egg-laying will continue until temperatures drop and adults start to overwinter. Overwintering adults, though, may resume egg laying in the following spring and substantial quantities of eggs (up to 700) may be laid (Cowles 1995). Both the long pre-oviposition period and life-span of adult *O. sulcatus* females offers an extended window of time for biological control agents like entomopathogenic fungi to infect and cause mortality before the majority of eggs are actually laid.

In the past, several studies have shown the potential of different formulations of various strains of *M. anisopliae* against the larval or adult stage of *O. sulcatus*, either applied solo (Bruck 2007) or in combination with entomopathogenic nematodes (Ansari et al. 2008, Ansari & Butt 2013) or synthetic insecticides (Shah et al. 2007). Control levels obtained in this study based on a single application of a *B. bassiana*-based product against adult weevils were lower and mortality took longer to occur compared e.g. to the study by Bruck (2007), where a 100% efficacy against *O. sulcatus* larvae was evident 28 d after application of *M. anisopliae* to container-grown plant material maintained outdoors. Overall, it remains to be tested if two or several applications of a bioinsecticide like Naturalis®, different modes of applications than the one used here or a combination of a *B. bassiana*-based bioinsecticide, entomopathogenic nematodes and/or a systemic insecticide would increase efficacy levels of this entomopathogenic fungus against *O. sulcatus* in the field.

Entomopathogenic fungi like *B. bassiana* can also spread within an insect population and can become established in the soil environment for an extended period of time (Scheepmaker & Butt 2010) at levels that will cause mortality of target insects (Vanninen et al. 2000, Enkerli et al. 2004, Bruck & Donahue 2007). The natural spread and persistence of an entomopathogenic fungus like *B. bassiana* is in agreement with the concept of classical biological control (Hajek & Delalibera 2010) and may have a long-lasting suppressive effect on insect pests like weevils. For growers, a single application of a biological control agent like *B. bassiana* which might provide a season-long but however prolonged control effect and has a dual activity against both larval and adult stages of *Otiorhynchus* species is therefore likely to be an acceptable management option.

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