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Susceptibility of different developmental stages of large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) to entomopathogenic fungi and effect of fungal infection to adult weevils by formulation and application methods

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ABSTRACT

The large pine weevil, *Hylobius abietis*, is a major pest in European conifer forests causing millions of Euros of damage annually. Larvae develop in the stumps of recently felled trees; the emerging adults feed on the bark of seedlings and may kill them. This study investigated the susceptibility of different developmental stages of *H. abietis* to commercial and commercially viable isolates of entomopathogenic fungi, *Metarhizium* and *Beauveria*. All the developmental stages of *H. abietis* can be killed by *Metarhizium robertsii*, *Metarhizium brunneum*, and *Beauveria bassiana*. The most virulent isolate of *M. robertsii* ARSEF4556 caused 100% mortality of pupae, larvae and adults on day 4, 6 and 12, respectively. This strain was further tested against adult weevils in different concentrations (10^5–10^8 conidia cm^{-2} or ml^{-1}) using two types of fungal formulation: 'dry' conidia and 'wet' conidia (suspended in 0.03% aq. Tween 80) applied on different substrates (tissue paper, peat and Sitka spruce seedlings). 'Dry' conidia were more effective than 'wet' conidia on tissue paper and on spruce or 'dry' conidia premixed in peat. The LC_{50} value for 'dry' conidia of isolate ARSEF4556 was three folds lower than 'wet' conidia on tissue paper. This study showed that 'dry' conidia are more effective than 'wet' conidia, causing 100% adult mortality within 12 days. Possible strategies for fungal applications are discussed in light of the high susceptibility of larvae and pupae to fungal pathogen.

1. Introduction

The large pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae) is a major pest of European coniferous forests (Orlander and Nilsson, 1999; Moore et al., 2004). Adult weevils feed on the bark of conifers and are extremely damaging to saplings used to restock clear felled forest sites with losses being approximately €140 million per annum (Langstrom and Day, 2004). The weevils usually lay eggs at the base of freshly cut conifer stumps and buttress roots and soon after hatching the larvae migrate to and begin their feeding and development under the bark, eventually forming pupae and emerging as adults (Leather et al., 1999).

Current control of *H. abietis* in UK and Europe entails treating the saplings with cypermethrin or α-cypermethrin pre- and post-planting (Willoughby et al., 2004) but the derogation for their use will expire in 15th June 2014 (www.fsc.org; FSC-GUI-30–001a V1–0 EN). New EU legislation is promoting the use of integrated pest management programmes with preference to be given to non-chemical methods of control. Entomopathogenic nematodes offer a more benign alternative strategy for control of *H. abietis* larvae both in the laboratory (Pye and Burman, 1978; Armendáriz et al., 2002) and under field conditions (Brixey et al., 2006; Dillon et al., 2006, 2007) but the nematodes have a relatively short shelf-life (Koppenhöfer, 2000) and can give inconsistent control (Dillon et al., 2006).

The entomopathogenic fungi, *Metarhizium robertsii* (formerly known as *Metarhizium anisopliae* var. *anisopliae*) and *Beauveria bassiana* (Balsamo) Vuillemin are already commercially available (Faria and Wraight, 2007) and show considerable potential for the control of various soil and foliar insects (Butt et al., 2001; Ansari et al., 2004, 2008, 2009; Ansari and Butt, 2012). Previously, few attempts have been made to test *M. robertsii* and *B. bassiana* for the control of adult *H. abietis*, all the tested isolates killed the beetles and sporulated on cadavers. However, the results of early attempts with *B. bassiana* in laboratory and field tests were inconsistent and some were not very promising (Samsinakpva and Novák, 1967; Waldenfels, 1975), whereas, significant isolate-dependent differences were found in *M. robertsii* against adult *H. abietis* (Markova, 2000).

The current study aims to find better strains of fungi and focuses on evaluating the virulence of commercial and commercially promising strains of *M. robertsii*, *Metarhizium brunneum* and *B. bassiana* against larvae, pupae and adults of *H. abietis*. The larvae

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and pupae were treated with conidia suspended in 0.03% aqueous Tween 80 (hereafter referred to as 'wet' conidia), whereas adult weevils were exposed to different substrates treated with 'dry' and 'wet' conidia of the fungal isolates to determine to what extent the formulation and substrate influence fungal efficacy. The findings of this study will help in selecting the most virulent isolate of fungus but also influence formulation and application strategies for the control of the different developmental stages of *H. abietis*.

2. Materials and methods

2.1. Insects

Late-instar (larval stage before pupa) and pupae of *H. abietis* were collected from Sitka spruce, *Picea sitchensis* (Bong.) tree stumps at Cwm Berwyn, South Wales, UK. The collection sites had not been treated with any insecticides or nematodes during the previous year. Larvae and pupae were stored individually in moist peat in 30-ml plastic containers (surface area: 15.9 cm^2) and fed slices of a carrot. The insects were incubated at 15 °C for 1 week to ensure only healthy insects were used in the studies. Adults were collected using freshly cut Sitka spruce billets (27–33 cm in length; 7.5–15 cm in diameter) at Cum Berwyn plantation, South Wales. Fifty weevils were kept in each plastic container (25 × 25 cm; 15 cm in depth) (Wilkinsons Ltd, Swansea, UK) with ten freshly cut Sitka spruce twigs (15 cm in length; 1.5–2 cm in diameter) as a food source and replaced once all the bark had been consumed. These containers were kept at 15 °C for 2 weeks to recognize unhealthy adult before use in experiments.

2.2. Fungus

The details of the fungal strains used in this study are summarized in Table 1. The *M. robertsii* isolates were selected because of their high virulence against various insect pests of crops (Ansari et al., 2007, 2008, 2009) and livestock (Ansari et al., 2010, 2011). All isolates were passed through *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae to ensure the cultures were not attenuated and re-isolated on oatmeal dodine agar medium. Single spore colonies were transferred to Sabouraud Dextrose Agar (SDA) and incubated at 25 ± 1 °C for 15 days. Conidia obtained from the first subculture were used for the mass production of inoculum.

Aerial conidia of fungi were produced on broken Basmati rice (East End Foods plc, West Midlands, UK) as previously described (Ansari et al., 2011). Briefly, the harvested conidia were dried at room temperature until the moisture content was < 10%. To determine the number of conidia g^{-1} of dry powder, 0.1 g conidia was suspended in 100 ml of 0.03% (v/v) aqueous Tween-80 (Fisher Scientific, Leicestershire, UK) and counted using a haemocytometer (Weber Scientific, Teddington, UK) under a light microscope (400 × magnification). Conidial viability was assessed using the plate count technique on SDA and viability was > 95% for all isolates (Goettel and Inglis, 1997). Prior to use, the air dried conidia were stored in air-tight plastic containers in the dark at 4 °C.

2.3. Fungal susceptibility test

2.3.1. Late-instar and pupae

These experiments were designed to identify fungal isolates that were highly virulent against pine weevil larvae and pupae. Since pupae are immobile, to see if they are alive, individual pupa was touched with a plastic forceps and considered to be alive if they compress their body. Insects were dipped in 10 ml of the fungal suspension containing 1×10^8 conidia ml^{-1} for 20 s. Control insects were dipped in 0.03% Aqueous Tween only. Ten treated larvae or pupae were placed individually in a 250-ml plastic cup (8 cm in diameter, 7 cm in height, and 50 cm^2 in surface area; Tesco, Swansea, UK) filled with 100 ml soil with tree bark (collected from the same stumps where larvae and pupae were collected). A slice of carrot was placed in each container as a food source and was replaced at each observation time. Cups were kept in a constant temperature room (20 ± 1 °C; 60–70% RH, and L16:D8) and insect mortality was recorded after 2 days post-inoculation for 6 days. Dead insects were placed on moist filter paper in Petri dishes (9 cm in diameter) and incubated at 25 °C under saturated conditions for 3–5 days. The cause of death was confirmed by examination of the fungal sporulation of the cadaver. Each treatment was replicated three times (10 insect/replicate) and the whole experiment was conducted twice.

2.3.2. Adults

This experiment was designed to evaluate the virulence of fungal species/isolates to adult *H. abietis*. Adult weevils were immersed in fungal suspension in the same way as larvae except that treated they were placed in white opaque plastic containers (25 cm in width × 25 cm in length; 15 cm in depth; 625 cm^2 in surface area) (Wilkinsons Ltd, Swansea, UK). One ventilation hole (10 × 10 cm) was made in each lid and covered with metal sieve (2 × 2 mm pore size). A double layer of moist tissue paper (Kruger Ltd, UK) was placed in each container so that it covered the bottom. Ten freshly cut Sitka spruce twigs were placed in each container as a food source and replaced when all the bark had been consumed. Insect mortality was assessed 3 days post-inoculation for 24 days. Dead adults were collected and placed in the Petri dish as described above. Each treatment was replicated three times and the whole experiment was conducted twice.

2.3.3. Effect of concentrations, formulations, and application methods on susceptibility of adult weevils to *M. robertsii* ARSEF4556

Metarhizium robertsii isolate ARSEF4556 was identified as highly virulent against larvae, pupae and adults (Figs. 1 and 2) and was

Table 1
Origin of entomopathogenic fungi used in this study.

Fungal species/strains	Host or source of origin	Geographic origin
Metarhizium robertsii		
V275 (=F52) [*]	*Cydia pomonella* (Lepidoptera: Torticidae)	Austria
ARSEF 4556[*]	*Boophilus* sp. (Acari: Ixodidae)	USA
V1004	*Hylobius abietis* (Coleoptera, Curculionidae)	UK
Metarhizium brunneum		
ARSEF 3297[*]	*Boophilus* sp. (Acari: Ixoididae)	Mexico
Beauveria bassiana		
SU12	*Hylobius abietis* (Coleoptera, Curculionidae)	UK
BotaniGard®	Laverlam International Corporation	USA

[*] ARSEF, US Department of Agriculture. Agricultural Research Service, Collection of Entomopathogenic fungus culture, Ithaca, NY, USA.