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Neem seed cake enhances the efficacy of the insect pathogenic fungus *Metarhizium anisopliae* for the control of black vine weevil, *Otiorhynuchs sulcatus* (Coleoptera: Curculionidae)

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Abstract

Neem seed cake enhanced the efficacy of the insect pathogenic fungus *Metarhizium anisopliae* for control of black vine weevil (BVW), *Otiorhynuchs sulcatus* (Coleoptera: Curculionidae) larvae in out door potted *Euonymus* plants. Both *M. anisopliae* and neem seed cake alone were effective against earlier instar larvae with the degree of control being dose dependant. Significantly higher conidial attachment was observed on larvae recovered from pots treated with combined application of *M. anisopliae* and neem seed cake (5 g/l of peat) suggesting that the neem seed cake increased larval movement and the subsequent acquisition of conidia. At higher concentration of neem seed cake (5 g/l), few BVW larvae survived whereas larvae recovered from neem treated pots (0.5 or 2.5 g/l) were less than half in body size and weight to that of untreated control suggesting that neem acts as growth regulator. Incorporation of neem seed cake or *M. anisopliae* in potting media did not influence adult BVW feeding or oviposition. Since neem seed cake products are considered safer than synthetic insecticides, the interactions we describe could easily be exploited to control insect pests in situations where no synthetic insecticides are permitted. Furthermore, enhanced efficacy of *M. anisopliae* at 100-fold lower doses (1×10^8 conidia/l of peat) in combination with neem seed cake would greatly benefit the grower by reducing the cost of the fungal BCA.

Keywords: *Metarhizium anisopliae*; Neem seed cake; Vine weevil; Conidial attachment; Antifeedant; Enhanced efficacy

1. Introduction

The black vine weevil, *Otiorhynchus sulcatus* Fabricius (Coleoptera: Curculionidae), is a serious economic pest of soft fruit, ornamental, and nursery plants with an extensive geographic range (Lola-Luz and Downes, 2007; Willmott et al., 2002). Adult weevils feed on host leaves; however, the larvae cause the most damage as they feed on plant roots and burrow into stems, tubers and the crown leading to stunting and even death of the plant. Current control consists of entomopathogenic nematodes and chemical pesticides (e.g. fipronil, imidacloprid, chlorpyriphos) which principally target the larval stage (Bruck and Donahue, 2007; Lola-Luz and Downes, 2007). However, these strategies are not optimal since growers discover infested plant material (Bruck and Donahue, 2007); there is clearly scope for the development of more effective, benign control strategies.

Strains of *Metarhizium anisopliae* (Metschnikoff) Sorokin have been identified that show much promise for the control of BVW (Bruck and Donahue, 2007; Moorhouse et al., 1993; Shah et al., 2007). Moorhouse et al. (1993) and Bruck and Donahue (2007) reported its prolonged persistence and efficacy against BVW in potted plants. Few earlier studies have also shown its effectiveness against BVW when incorporated in growing media whereas recent studies clearly demonstrated that this fungus is rhizosphere competent (Bruck, 2005; Hu and

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St. Leger, 2002). The efficacy of this fungus is greatly enhanced when used with sublethal doses of chemical pesticides (Shah et al., 2007). Increasing pressure to reduce the input of chemical pesticides combined with the withdrawal of many pesticides in the EU (Agrochemicals report 2002) increases the pressure to develop chemical free strategies for pest control.

Neem seed cake, a by-product of neem oil production, is used as a fertilizer but is considered to be a benign plant protection product and is known to increase the efficacy of the nematode-pathogenic fungus, *Paecilomyces lilacinus*, for control of plant parasitic nematodes (Nagesh et al., 2003; Tiyagi and Ajaz 2004). This study shows that neem seed cake can enhance the efficacy of *M. anisopliae* for control of BVW larvae, thus offering a chemical pesticide free strategy for the control of media-borne pests.

2. Materials and methods

2.1. Fungal strain, BVW, and neem seed cake sources

Details on the source, maintenance and production of *M. anisopliae* strain V275 are given in Shah et al. (2005). Neem seed cake powder (GreeNeem) was obtained from GreeNeem-K. Sivaram Bros, Virudhunagar, India. BVW adults, larvae, and eggs were obtained from a colony maintained at Swansea University as described by Shah et al. (2007).

2.2. Influence of M. anisopliae and neem seed cake on BVW establishment

Rooted cuttings of *Euonymus fortunei* 'Emerald Gold' kindly provided by Johnsons of Whixley (York, UK) were transplanted in 0.5 l pots. Each pot was filled with a peat based medium kindly provided by Bord Na Mona (Ireland). Each plant was infested with 20 melanized BVW eggs 1 week post transplantation.

Both *M. anisopliae* V275 and neem seed cake were premixed into the peat substrate either alone or together. The former was premixed at the recommended and 100-fold lower rates of 1×10^{10} and 1×10^{8} conidia/l of substrate, respectively. Neem seed cake was premixed at the rate of 0.5, 2.5, or 5 g/l of compost alone or in combination with the lower dose (1×10^{8} conidia/l) of *M. anisopliae*. Untreated plants constituted one of the controls.

Trials were conducted outdoors between July and October, 2006 when the average day and night temperatures ranged between 15–25 °C and 10–15 °C, respectively. Plants were destructively assessed 6 weeks post egg infestation to determine the number of live larvae in each pot. Each treatment was replicated 10 times and whole experiment was replicated twice. Treatments were arranged in randomized complete block design with each pot adequately spaced to avoid cross contamination among treatments.

2.3. Larval susceptibility and spore acquisition

Influence of *M. anisopliae* or neem seed cake on larval susceptibility and spore acquisition, was determined in out door potted *Euonymus* plants. Plants were prepared in peat with *M. anisopliae* alone and in combination with 0.5 and 5 g/l of neem seed cake. *M. anisopliae* was applied at 1×10^{10} conidia/l of peat. Neem seed cake was also applied alone at 0.5 and 5 g/l of peat. Both *M. anisopliae* and neem seed cake were premixed in the peat before transplantation of *Euonymus* cuttings. Each cutting was allowed to grow for 2 weeks post transplantation and then infested with two BVW larvae (2nd instar). Each treatment was replicated 10 times in complete randomized design and whole experiment was repeated twice. Untreated plants were used as control.

To determine if neem seed cake influences spore acquisition, half of the pots were destructively assessed 3 days post infestation. All larvae recovered were divided in two groups. Half of the larvae recovered were fixed in 2% formaldehyde and stored at 4 °C until required. The specimens were stained with calcofluor and examined in a Nikon, Eclipse E600 microscope equipped with epifluorescence (Butt, 1997). Spore adhesion to the second larval group was determined by larval maceration and enumeration of conidia. Briefly, the larvae were macerated in 0.5 ml of 0.03% Aq. Tween and conidial numbers counted using an improved Neubaur haemocytometer (Weber Scientific Ltd., UK). The remaining pots from each treatment were destructively assessed 2 weeks post infestation to determine larval mortality.

2.4. Influence of neem seed cake and M. anisopliae on larval development

Influence of neem seed cake or *M. anisopliae* on larval development was determined by examining the body size and weight of larvae recovered from pots treated with different treatments. All treatments and experimental procedure were same as described in Section 2.2. Larvae recovered from different treatments were divided in groups of 5 larvae per group and weighed. A minimum of five groups (5 larvae/group) were weighed for each treatment. Larval size particularly head capsule width was used to determine larval instars. The whole experiment was repeated twice with each treatment replicated 10 times.

2.5. Influence of neem seed cake or M. anisopliae on BVW oviposition

In addition, to the bioassays above, we tried to determine if the neem seed cake acted as an oviposition deterrent. Oviposition assays were conducted in plastic cups (125 ml). Each cup was filled with 100 ml of peat medium and a single *Euonymus* plug provided as a food source for adult BVW. Treatments included neem seed cake premixed at either 2.5 or 5 g/l of peat, *M. anisopliae* premixed