Virulence of the plant-associated endophytic fungus *Lecanicillium muscarium* to diamondback moth larvae

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Abstract *Plutella xylostella* (diamondback moth) is a prominent pest of brassicas which is now resistant to most insecticides. Despite years of research, the range of available products used in biological control of diamondback moth is still somewhat limited. We isolated putative endophytic fungi from New Zealand cabbage plants to search for unique biological control agents of diamondback moth larvae. The larvae were fed leaf discs from commercially grown cabbage covered in spores from endophytic fungal isolates to test the insecticidal properties of these fungi. Twenty of the 52 fungal isolates tested failed to kill any diamondback moth larvae. However, three isolates of *Lecanicillium muscarium* induced mortality greater than 80%. While these isolates have potential for use in biological control applications, further research into propagation, formulation, and method, rate and timing of application is needed.

Keywords Endophytes, biological control, entomopathogenic fungi

INTRODUCTION

Plutella xylostella (Lepidoptera, Plutellidae) is commonly known as the diamondback moth (DBM) and is a prominent pest of *Brassica* species. It attacks not only vegetables such as cabbages, but also forage brassicas and canola (*Brassica napus*) (Furlong et al. 2013). Worldwide, production of vegetable *Brassica* species is estimated to contribute US$26 billion to the global economy. However, the cost of DBM control combined with crop yield losses can reach US$2.7 billion annually (Furlong et al. 2013).

Control of DBM is currently achieved mostly through the use of broad-spectrum synthetic insecticides. However, indiscriminate use of these chemicals has led to insecticide resistance in various populations of DBM. There are more than 80 compounds falling into all major classes of insecticides for which resistance in DBM has been recorded (Furlong et al. 2013). Insecticide application is increasingly regarded as unsustainable and may be associated with many health and environmental risks (Gilden et al. 2010; Lukowicz et al. 2018). Broad-spectrum insecticides may also be harmful to other invertebrates, which could include natural predators of DBM, thereby reducing the environmental capacity for controlling DBM populations (Furlong et al. 2013).

Diamondback moth has a wide range of natural enemies, including viruses, fungi, bacteria, generalist predators and parasitoids. Parasitic wasps can attack larvae or pupae of DBM. The most commonly promoted species are wasps of the genera *Diadegma, Diadromus* or *Cotesia*, since some can reduce DBM populations by up to 80% (Gurr et al. 2018; Hermansson 2016). Use of the bacterium *Bacillus thuringiensis*

(Bt) has proven to be very effective in controlling DBM in many parts of the world. However, DBM was the first insect to develop field resistance to a Bt toxin, probably due to indiscriminate use, similar to the resistance developed to synthetic insecticides (Furlong et al. 2013).

Diamondback moth is susceptible to several genera of entomopathogenic fungi. Fungal genera such as *Lecanicillium, Isaria* or *Metarhizium* have shown ability to control DBM populations. However, the most widely used fungus in field conditions is *Beauveria bassiana* (Gurr et al. 2018).

Biological control of DBM is a promising tool to tackle the damage caused by this insect, especially because of possible synergistic effects with additional biological control agents. Synergy in DBM control was achieved when using *Beauveria bassiana* and Bt toxins or predators attacking different larval stages (Gurr et al. 2018). Farmers are increasingly looking for environmentally friendly methods to control insect pests. Use of biological control agents is considered less toxic to humans and the environment than chemical pesticides (Brimner & Boland 2003; Heydari & Pessarakli 2010).

One source of new agents to control pests of brassica crops may be organisms already present in brassica plants. Fungal and bacterial endophytes can have beneficial properties, including inducing pest resistance in the plant (Card et al. 2015). As part of a larger study on endophytes of *Brassica* species in New Zealand, we tested 52 isolates from 35 species of fungi obtained from cabbage plants (*Brassica oleracea* var. *capitata*) to determine if any had direct virulence against DBM larvae.

MATERIALS AND METHODS

Diamondback moth larvae were obtained from a colony maintained for 10 years at Lincoln University, New Zealand. The colony was established in 2008 with DBM collected at fields at Lincoln University, New Zealand. The colony consists of twelve separate populations that are routinely mixed between each other, in addition to wild DBM individuals to prevent loss of genetic diversity. Resistance to insecticides or Bt-based biopesticides has not been observed in the colony. For bioassays, second-instar DBM larvae were fed leaf discs from commercially grown cabbage plants that were covered in spores from selected fungal isolates to test the insecticidal properties of these fungi.

Experimental treatment

Putative endophytic fungi were isolated from cabbage plants in 2015 and 2016, following procedures described in a study by Zhang et al. (2014). Selected fungal isolates were grown for 3–4 weeks on 1/4 strength potato-dextrose agar (PDA; Difco, BD, USA) in a growth chamber at 18°C, with 12h/12h – light/dark period. Conidia of each isolate were harvested from the plates into sterile aqueous 0.01% Triton X-100 (Sigma-Aldrich, New Zealand), which was then filtered through sterile cotton wool. Conidial concentration was adjusted to approximately 10^7 conidia/mL. A disc was cut from the cabbage leaf using a 30-mm cork borer. Leaf discs were washed thoroughly in sterile distilled water and dried using tissue paper prior to treatment. A filter paper disc (30-mm diameter) was placed in a 35-mL clear graduated plastic portion cup and moistened with 100 µL of sterile distilled water. Fifty-microlitre aliquots of each conidial suspension were deposited on each side of the leaf disc and spread over the surface using an inoculation loop so that the whole disc area was evenly covered. Treated leaf discs were allowed to air dry for about 30 minutes before putting them on top of the moist filter paper disc. Five second-instar DBM were placed in each cup with a leaf disc using a fine camel brush. The cup was closed and placed in an incubator at 25°C, with 12h/12h – light/dark period. There were six bioassays in total, each testing a subset of the 52 isolates. All isolates were tested once, except *Lecanicillium muscarium* isolate m2La6, which was tested in two separate bioassays. Each bioassay was arranged in a complete randomised design with 4 replicates.

Measurements

Larval mortality was assessed daily for at least four days after inoculation by observation using a stereo microscope. Unless in a cocoon, live larvae were feeding and moving around the leaf disc and responded to even a slight shake or when touched by a brush. Larvae were considered dead when they did not respond to physical stimuli, and when brown discolouration suggested decomposition of larval tissues (Fig. 1).

Figure 1 A dead *Plutella xylostella* larva, showing brown discolouration of internal tissues.

Statistical analyses

Mean mortalities were compared four days after inoculation in each bioassay using an ANOVA test and subsequent Tukey's honest significant difference test in the R environment (R Core Team 2016).

RESULTS

Twenty of the 52 isolates tested failed to kill any DBM larvae (Fig. 2). However, three isolates of *Lecanicillium muscarium* induced mortality greater than 80% and two other isolates induced mortality just under 50%. Isolate 223S3512 caused the highest DBM mortality of 90% (Tukey HSD P>0.001), while strains a2R14 and 223S352 both killed 81% of the larvae (Tukey HSD P>0.001). In one experiment, *L. muscarium* isolate m2La6 caused 48% mortality, which was significantly different from the control treatment (Tukey HSD P=0.02), but in a repeated experiment the mortality was 43% and the effect was not significant in a post-hoc analysis. *L. muscarium* isolate h2La53 induced 25% mortality, which statistically did not differ from the control. Results are summarised in Figure 2.

DISCUSSION

This study found that three of six isolates of the fungus *L. muscarium* (all isolated from cabbage tissues) increased mortality of DBM larvae in a feeding bioassay. The pathogenicity of the *L. muscarium* isolates used in this study is similar to results of other studies which reported the potential of *Lecanicillium* species as a biological control agent of DBM. However, the *Lecanicillium* spp. isolates used in biopesticide formulations have rarely targeted DBM; usually the controlled pests have been aphids or nematodes (Goettel et al. 2008). A review by Brodeur (2012) summarised several commercial products based on *Lecanicillium* species, including products aimed at controlling aphids, mites, thrips and/or whiteflies. A number of studies have reported showing potential for biological control of DBM. In general, the effects of *Lecanicillium* species were consistently better than no treatment, but were usually poorer than the most effective treatments, either a synthetic insecticide or another entomopathogenic fungus. For example, Yamada et al. (2009) found that the strains of hybrid *Lecanicillium* species used in their study at a concentration of 10^6 conidia/mL killed half of the DBM larvae after just two days and resulted in more than 80% mortality in a full screening bioassay, but these isolates were less effective under field conditions even at concentration of 2×10^7 conidia/mL. More recently, Duarte et al. (2016) found that at a concentration of 10^7 conidia/mL a *L. muscarium* isolate caused more than 80% mortality of DBM larvae feeding on cabbage leaf discs, while isolates of *Beauveria bassiana* and *Metarhizium rileyi* caused 98–100% mortality. In the current study, DBM mortality ranged from 25% to 90% after 4 days as a result of contact with the *L. muscarium* conidia. This variability in pathogenicity could be attributed to different virulence strategies among isolates as described by Bye and Charnley (2008), who

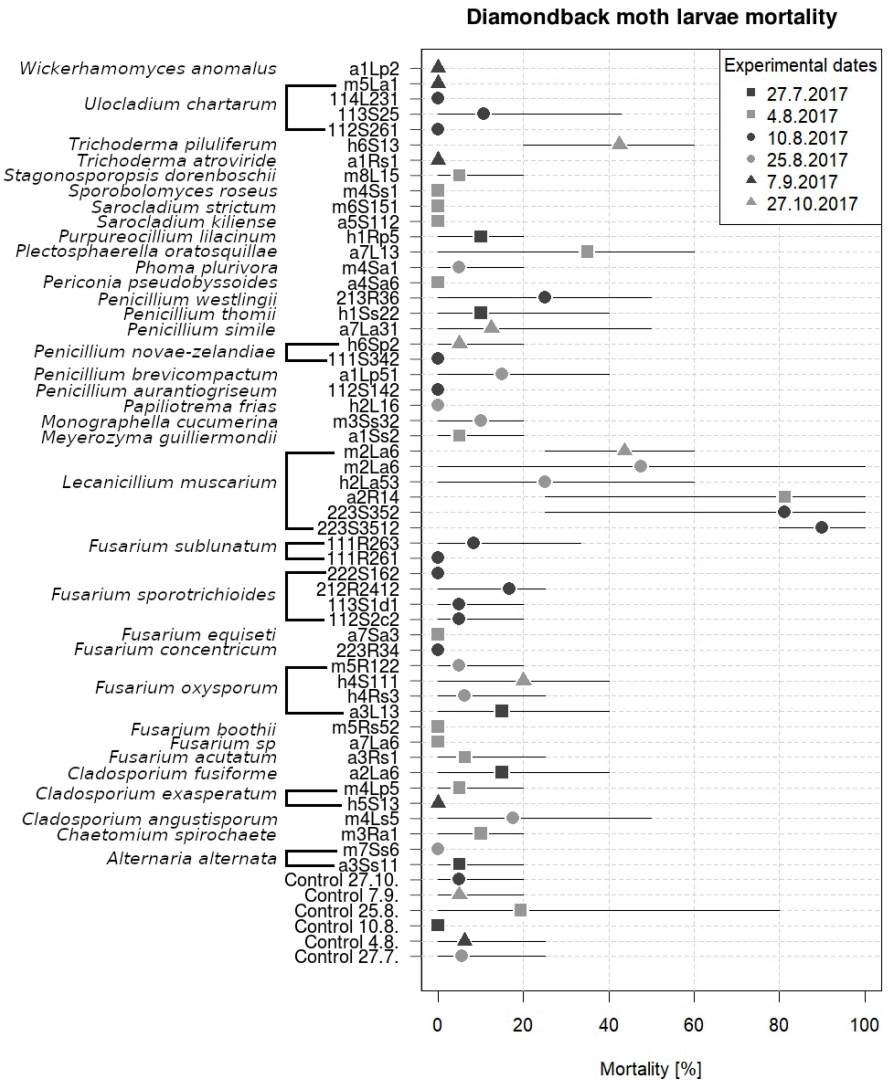

Figure 2 Mortality of *Plutella xylostella* larvae following treatment with a range of fungal isolates. Symbols represent mean mortality (%) in respective bioassays. Whiskers represent the range of observed values.

showed that various isolates of *Lecanicillium* species possessed a distinctly expressed battery of cuticle-degrading enzymes. Similar to the cuticle degrading enzymes, other metabolites involved in virulence may have differential expression, such as a recently described bassianolide which suppresses the immune response of DBM (Keppanan et al. 2018).

The current study evaluated only the effect of contact of DBM larvae with conidia already on the surface of cabbage leaves, but there is evidence that endophytic fungi can influence insect feeding and development. For example, *Acremonium alternatum*, a fungal endophyte, reduced DBM feeding and growth rate when applied to the roots of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) (Raps & Vidal 1998). Similarly, endophytic *Metarhizium anisopliae* increased

mortality of DBM larvae feeding on oilseed rape (*Brassica napus*) (Batta 2013). Furthermore, the presence of endophytic *Lecanicillium lecanii* in the leaves of cotton plants slowed reproduction rates of aphids (Gurulingappa et al. 2010). Since isolates in this study originated from internal plant tissues, there is a possibility they are able to endophytically colonise plant tissues and could affect feeding and reproductive patterns or even mortality of DBM. In the current study, potent isolates of *L. muscarium* were obtained from stems (223S3512, 223S352) and roots (a2R14) of cabbage plants, while an isolate recovered from leaf tissue caused lower mortality of DBM. Interestingly, the *L. lecanii* isolate used to control aphids in a study by Gurulingappa et al. (2010) originated from the leaves of cotton plants. However, the tissue preference of the isolates that had the highest mortality of DBM in the current study, or their potential for DBM control when endophytic is not yet known. The effect of an endophytic entomopathogen is usually less substantial compared to direct contact of DBM with conidia (Batta 2013). Nevertheless, research on the extent and nature of the interactions between the fungus and host plant could improve the biological control of DBM, where the reduced effect on mortality could be traded for reduced costs bypassing the need to apply conidia with regards to the presence of susceptible pest stages. However, both approaches would require further research into propagation, formulation, dosage and application manner and timing (Lacey et al. 2001).

Expanding laboratory experiments to field trials is often problematic so other key steps include establishing the practicalities and cost effectiveness of scaling up microorganism production and determining the efficacy of fungal biological control agents under field conditions. The current study evaluated the effect of only a single concentration of conidial suspension on DBM mortality hence a dose response experiment should be one of the first steps in further research, as if the required dose is too high, a product would not be economically viable. Mass production of *Lecanicillium* spp. conidia have been achieved by either solid substrate or submerged liquid fermentation (Jaronski 2014). Potential inundative or endophytic use of *L. muscarium* isolates from current study would define appropriate production method and formulation. Laboratory experiments usually result in higher virulence than those under field conditions (Yamada et al. 2009) so field trials are necessary to assess the efficacy of biological control agent used compared with available commercial products. Also, it is critical to test the interactions of fungal biological control agents with possible non-target species to ensure there is no loss of ecosystem services (Brimner & Boland 2003; Roy & Pell 2000) before these biological control agents could be considered for use in large-scale field applications. In addition, testing for human safety is imperative if the isolates are to be used with crops for human consumption, such as cabbage.

CONCLUSIONS

Of 52 fungal isolates recovered from cabbage tissues, only three showed potential as biological control agents of DBM. All three active isolates were strains of *L. muscarium*. However, this study needs to be followed by further research if the active fungal isolates obtained here are to be developed as biological control agents for field conditions. Mechanisms underlying the observed variability of effects, dose response, temporal dynamics of the induced mortality as well as endophytic properties and interactions with other organisms of these fungal isolates should be further investigated.

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