Biological Control 89 (2015) 53–60

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

The endophytic fungal entomopathogens *Beauveria bassiana* and *Purpureocillium lilacinum* enhance the growth of cultivated cotton (*Gossypium hirsutum*) and negatively affect survival of the cotton bollworm (*Helicoverpa zea*)

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HIGHLIGHTS

- Many fungal insect pathogens can also colonize plants as endophytes.
- Endophytic *Beauveria bassiana* and *Purpureocillium lilacinum* enhanced cotton growth.
- Endophytic *B. bassiana* and *P. lilacinum* reduced survival of *Helicoverpa zea* larvae.
- Manipulating fungal endophytes can play a role in sustainable IPM strategies.

ARTICLE INFO

Article history:
Received 20 February 2015
Accepted 12 March 2015
Available online 21 May 2015

Keywords:
Endophyte
Entomopathogen
Beauveria bassiana
Purpureocillium lilacinum
Helicoverpa zea

ABSTRACT

The effects of two entomopathogenic fungal endophytes, *Beauveria bassiana* and *Purpureocillium lilacinum*, were assessed on the growth of cultivated cotton (*Gossypium hirsutum*) and development of the cotton bollworm (*Helicoverpa zea*). In two replicate greenhouse trials, cotton plants were inoculated as seed treatments with two concentrations of *B. bassiana* or *P. lilacinum* conidia and evaluated for effects on both plant dry biomass, number of nodes and number of developing flowers (squares). We similarly treated cotton plants and evaluated *H. zea* performance using no-choice *in planta* assays starting at the 2nd larval instar. Treatment with both fungal endophytes resulted in a significant increases in plant dry biomass (ANOVA, $P = 0.024$). Plant developmental stage and number of squares were also significantly enhanced in the endophyte treated plants (ANOVA, $P = 0.005$ and $P = 0.027$, respectively). The survivorship of *H. zea* was significantly different among the endophyte treatment groups (Kaplan–Meier, $P = 0.02$), where insects feeding on control plants exhibited higher survival than insects on the endophyte treated plants. There were no significant endophyte treatment effects on larval or pupal weights of *H. zea* individuals. There was no endophyte effect on days to pupation among treatments, but there was a marginal effect on days to eclosion (Kaplan–Meier, $P = 0.07$). Overall, our results demonstrate (i) the positive plant growth enhancing effects of the target endophyes on cultivated cotton under greenhouse conditions and (ii) the negative effects of endophytic *P. lilacinum* and *B. bassiana* on *H. zea* survivorship and development using whole plant assays.

1. Introduction

Fungal endophytes can protect plants from a wide range of stressors including insect pests (Porras-Alfaro and Bayman, 2011). In this study, we refer to endophytes as defined by Schulz and Boyle (2005), as microorganisms (fungi or bacteria) found in asymptomatic plant tissues for all or part of their life cycle without causing detectable damage to the host. Here we focus on entomopathogenic fungal endophytes (Vega et al., 2009) and the ecological roles these fungi can play in agricultural systems. Entomopathogenic fungal endophytes have been isolated from a variety of plant species and tissues, and single isolates can be inoculated to establish as an endophyte across a range of

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http://dx.doi.org/10.1016/j.biocontrol.2015.03.010
1049-9644/

phylogenetically divergent plants (Vega et al., 2009; Rodriguez et al., 2009; Gurulingappa et al., 2010; Porras-Alfaro and Bayman, 2011). These entomopathogenic fungal endophytes are classified as non-clavicipitaceous (Rodriguez et al., 2009), referring to fungal endophytes that are usually horizontally transmitted. Several non-clavicipitaceous entomopathogens including *Beauveria bassiana*, *Lecanicillium lecanii*, *Metarhizium anisopliae* and *Isaria* (*Paecilomyces*) spp. can have negative effects on insect pests when *in planta*, may antagonize plant pathogens, and also promote plant growth (Ownley et al., 2004, 2008; Vega et al., 2009). For example, the application *B. bassiana* as an endophyte to tomato and cotton seedlings increased plant stand counts and height of the plants when infected by damping off disease caused by the fungal plant pathogen, *Rhizoctonia solani* (Ownley et al., 2004, 2008; Griffin et al., 2005). The mechanisms by which *B. bassiana* had a positive effect on plant growth may have been due to its antagonistic activity to *R. solani* either due to direct competition or by a systemic induced resistance in the plants (Ownley et al., 2008). A similar study using *M. anisopliae* conidia applications to seedlings for control of wireworms increased the stand count of corn and increased the yield at the end of the field season (Kabaluk and Ericsson, 2007). The mechanism underlying the increase in yield was suggested to be due to the reduction in wireworms attacking roots, thereby allowing plants to better obtain soil nutrients and water (Kabaluk and Ericsson, 2007).

The Heliothinae is a subfamily of about 365 species of noctuid moths that includes a number of the world's most economically important crop pests such as the Old World bollworm (*Helicoverpa armigera*) (Fitt, 1989; Matthews, 1999). In North and South America, the New World bollworm (*Helicoverpa zea*) is most commonly known as the corn earworm or cotton bollworm, and has been reported to feed on over 100 plant species including important economic crops in the United States such as corn, soybean, cotton and peanuts (Cho et al., 2008). Management of this insect has relied mostly on chemical control either by insecticidal sprays or by the use of genetically modified crops expressing transgenic insecticidal proteins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) (Jackson et al., 2008). The endophytic activity of *B. bassiana* has received particular attention due to its negative effects on a variety of insect herbivores including the cotton bollworm (Bing and Lewis, 1991; McGee, 2002; Cherry et al., 2004; Powell et al., 2009; Leckie et al., 2014). The fungus, *Purpureocillium lilacinum*, more widely known by its former name, *Paecilomyces lilacinus* (Luangsa-ard et al., 2011), has been mainly considered a nematophagous, egg-parasitizing fungus, specifically against the root-knot nematode, *Meloidogyne incognita*, and several other plant-parasitic nematode species including *Radopholus similis*, *Heterodera* spp., *Globodera* spp. (Carrion and Desgarennes, 2012; Kannan, 2012; Khan, 2012; Sharma and Trivedi, 2012). However, *P. lilacinum* can also be pathogenic to insects (Castillo-Lopez et al., 2014). To our knowledge, the only study to date demonstrating negative endophytic effects of *P. lilacinum* on insect herbivores is Castillo-Lopez et al. (2014) who showed negative effects when present as an endophyte in cotton on reproduction of the cotton aphid, *Aphis gossypium* Glover, under both greenhouse and field conditions.

Several studies using fungal endophytes in *in planta* feeding assays or utilizing fungal extracts from endophytes have tested for negative effects on lepidopteran fitness (Bing and Lewis, 1991; Cherry et al., 2004; Powell et al., 2009; Reddy et al., 2009; Jaber and Vidal, 2010; Mantzoukas et al., 2014; Leckie et al., 2014). Most of these studies have evaluated the survivorship and developmental rate of lepidopteran species, and mainly through the duration of the larval stage only. In contrast, Jaber and Vidal (2010) showed negative effects on adult life history parameters (i.e., fecundity) of the lepidopteran *H. armigera* feeding on endophyte inoculated plants versus control. The same significant negative effects were also observed in the F2 generation. The effects of *B. bassiana* as an entomopathogenic endophyte on *H. zea* have not been tested in *in planta* feeding assays utilizing cultivated cotton. Similarly, there are no published studies to date testing for effects of the entomopathogenic endophyte *P. lilacinum* on any lepidopteran species. Here we, (i) examined the plant growth enhancing effects of endophytic *B. bassiana* and *P. lilacinum* in cotton when inoculated as seed treatments using two different conidial concentrations, and (ii) tested the same endophytic entomopathogens against *H. zea* in cotton for effects on survivorship, larval weight, pupal weight, days to pupation and days to eclosion using whole plant *in planta* feeding assays.

2. Materials and methods

2.1. Plants and endophytic fungi strains

The cotton seeds used for all experiments were variety LA122 (All-Tex Seed, Inc.). The *P. lilacinum* strain was isolated from a field survey of naturally-occurring fungal endophytes in cotton (Ek-Ramos et al., 2013). This strain was confirmed to be *P. lilacinum* by diagnostic PCR and subsequent sequencing of the ribosomal ITS region using specific species primers (Atkins et al., 2004). The *B. bassiana* was cultured from a commercially obtained strain (Botanigard, BioWorks Inc., Victor, NY). Stock spore solutions of each fungus were made by adding 10 ml of sterile water to the fungi cultured on potato dextrose agar (PDA) in 10 cm diameter petri dish plates and scraping them with a sterile scalpel. The resulting mycelia and spores were then filtered through cheese cloth into a sterile beaker. A haemocytometer was used to calculate the conidia concentrations of the resulting stock solutions. Final treatment concentrations were reached by dilution using sterile water.

2.2. Cotton seed inoculation

Seeds were surface sterilized by immersion in 70% ethanol for 3 min with constant shaking, then 3 min in 2% sodium hypochlorite (NaOCl), followed by three washes in sterile water, based on Posada et al. (2007). The third wash was plated on PDA media to confirm surface sterilization efficiency. Seeds were then soaked for 24 h in two different conidia concentrations of the two fungi and sterile water was used as the control. Spore concentrations for each fungus were zero (control), 1×10^6 spores/ml (treatment 1) and 1×10^7 spores/ml (treatment 2) based on inoculum concentrations used in previous studies of endophytic entomopathogens (Posada and Vega, 2005; Posada et al., 2007; Vega et al., 2008; Gurulingappa et al., 2010, 2011) including one of our own using the same protocol in which positive endophytic colonization frequencies of at least 50% were conservatively estimated for both fungi using the same variety of cotton (Castillo-Lopez et al., 2014). Beakers containing the soaking seeds were placed in a dark environment chamber at 28 °C until the next day for planting. Soaked seeds were planted in individual pots (15 cm diameter) containing unsterilized Metro mix 900 soil consisting of 40–50% composted pine bark, peat moss, vermiculite, perlite and dolomitic limestone. All plants were grown in a greenhouse at ~25 °C with natural photoperiod for the duration of the experiment. Pots were placed in a complete randomized design, watered as needed, and not fertilized throughout the experiments.

2.3. Cotton plant performance test

A factorial design was used to evaluate performance of plants inoculated as seeds with two different *B. bassiana* concentrations (1×10^6 and 1×10^7 spores/ml), two different *P. lilacinum*