Biological Control 61 (2012) 194–200

Contents lists available at SciVerse ScienceDirect

Biological Control

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

Persistence of *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte following inoculation of radiata pine seed and seedlings

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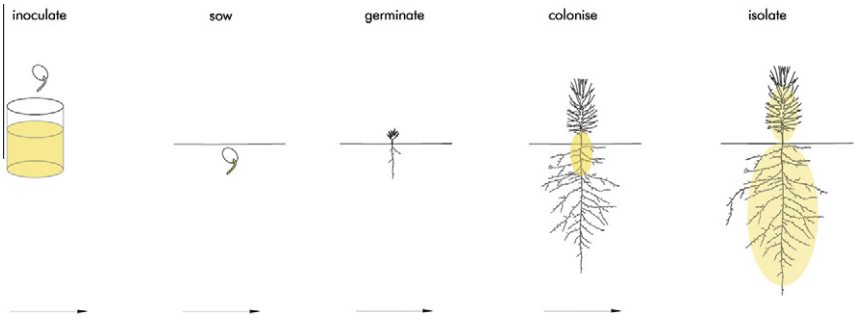
HIGHLIGHTS

- We investigated two methods to establish *Beauveria bassiana* as endophytes of pine seedlings.
- Fungi were applied as seed coating and root dip treatments.
- *B. bassiana* was successfully established in pine seedlings.
- Persistence of established fungi decreased over 9 months.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 April 2011
Accepted 5 January 2012
Available online 14 January 2012

Keywords:
Entomopathogens
Beauveria spp.
Pinus radiata
Endophytes

ABSTRACT

The entomopathogenic fungus *Beauveria bassiana* commonly causes disease on a range of insects, including bark beetle pests of plantation forest trees. However, using broadcast application of the fungus to control pest beetles in large scale plantation forests could be difficult to achieve economically. *B. bassiana* has also been found as an endophyte in plants, including the main commercially planted tree in New Zealand, *Pinus radiata*. In this study we investigated two methods to establish *B. bassiana* as endophytes of pine seedlings, seed coating and root dip. Two isolates previously isolated from within mature pines were used and the seedlings monitored for 9 months. Samples of unwashed, washed and surface sterilised roots, surface sterilised needles and soil were plated on semi-selective agar at 2, 4 and 9 months after inoculation. *B. bassiana* was successfully established in pine seedlings using both root dip and seed coating. The fungus was found in soil, non-sterile and sterilised samples at 2 and 4 months, but only one seedling of 30 was positive for fungus in surface sterilised samples after 9 months.

1. Introduction

The introduced bark beetle *Hylastes ater* (Paykull) (Curculionidae: Scolytinae) is occasionally a significant pest of the predominant plantation forest species (*Pinus radiata* D. Don) grown in New Zealand and can periodically lead to significant mortality of newly planted pine seedlings (Reay and Walsh, 2002). *Hylurgus ligniperda* (F.) (Curculionidae: Scolytinae) is another exotic bark beetle pest that is currently only a minor pest of pines in New Zealand, but causes considerable damage to regenerative forests in Chile (Bain, 1977; Mausel et al., 2006). Feeding damage to seedlings may occur in second (and subsequent) rotation areas when emerging adults feed on seedlings planted in the immediate area (Reay and Walsh, 2002). These bark beetles can vector sapstain and other damaging fungi to live seedlings during sub-lethal feeding events (Reay et al., 2002). Currently, management of the replanting regime is the most effective way to minimize the risk of seedling damage by *H. ater* (Reay and Walsh, 2002). More recently, efforts

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doi:10.1016/j.biocontrol.2012.01.002

have been made to explore the potential for biocontrol agents to mitigate this pest (Glare et al., 2008; Reay et al., 2008, 2010).

While fungi have been shown to be important agents of natural mortality in bark beetle populations, the actual impact of these pathogens on beetle populations is estimated to be relatively low (Balazy, 1968). Fungi from the genus *Beauveria* (Balsamo) Vuillemin have been reported most commonly from bark beetles (Wegensteiner, 2004). The classification of *Beauveria* species is currently in review; using multigene phylogenies, Rehner and colleagues have delimited six well supported clades (Rehner and Buckley, 2005). In addition to the known species (*Beauveria bassiana* s.s., *Beauveria brongniartii, Beauveria vermiconia, Beauveria caledonica* and *Beauveria amorpha*), they have also described *Beauveria malawienisis* (Rehner et al., 2006). All have been shown to be pathogenic to arthropods (Chandler et al., 2000; Glare et al., 2008; Zimmermann, 2007), and are found in a diverse range of habitats (Meyling and Eilenberg, 2007; Quesada-Moraga et al., 2007; Vega et al., 2008). Three species, *B. bassiana B. caledonica* and *B. malawiensis*, have been isolated from soil and infected insects collected in *P. radiata* forests in New Zealand. In these surveys, *B. caledonica* was the prevalent pathogen isolated from bark beetles (Glare et al., 2008; Reay et al., 2008). All three species were confirmed as being pathogenic to both *H. ater* and *H. ligniperda* adults in laboratory assays.

B. bassiana is capable of endophytic colonization of a range of plant species. These include maize (Bing and Lewis, 1991; Wagner and Lewis, 2000; Cherry et al., 2004), tomato (Ownley et al., 2004), cocoa (Posada and Vega, 2005; Vega et al., 2008), coffee (Posada et al., 2007; Vega et al., 2008), bananas (Akello et al., 2007), date palm (Gómez-Vidal et al., 2006) and opium poppy (Quesada-Moraga et al., 2006). In a recent survey of 33 sites from four geographically distinct areas in New Zealand, *B. bassiana* was found to be present as an endophyte in *P. radiata* (Reay et al., 2010). Reay et al. (2010) recovered 18 isolates from *P. radiata* needles collected from 125 trees, a single isolate from four *P. radiata* seedling root samples and a single isolate from seeds taken from four cones removed from a single mature tree. All of these *B. bassiana* isolates were confirmed as entomopathogens in laboratory assays against *H. ligniperda* adults and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae. *B. bassiana* was previously isolated from *Pinus monticola* by Ganley and Newcombe (2006), but to our knowledge the fungus has not otherwise been documented from pines. The colonization of plant tissues by *Beauveria* has been demonstrated to provide protection against insect damage, or has inhibited insect development and establishment (Bing and Lewis, 1991; Cherry et al., 2004; Ownley et al., 2004; Vega et al., 2008). Some protection against phytopathogens has also been documented (Ownley et al., 2004, 2010). However, in most endophytic occurrences by *B. bassiana*, the effect on insects is unknown.

Using *Beauveria* spp. to control bark beetles by conventional spray application techniques would likely be ineffective due to the behavior of bark beetles and associated high costs of treating large forested areas. Consequently, alternative methods of delivery are required to use these pathogens in an effective biocontrol strategy. As part of a study exploring the potential use of *B. bassiana* to regulate bark beetles through endophytic colonization, we investigated the ability of some *B. bassiana* isolates to establish as endophytes in pine seedlings after root and seed inoculation.

2. Material and methods

2.1. Production of inoculum

Isolates of *B. bassiana* originally recovered from mature pine trees in New Zealand were used in the experiments. Isolates F647 (Genbank GU237004) and F668 (Genbank GU237005) were previously isolated from needles of mature *P. radiata* trees from two distinct geographical locations (North and South Islands) in New Zealand (Reay et al., 2010). Both isolates belong to Clade A (*B. bassiana sensu stricto*) of Rehner and Buckley (2005).

Conidia were harvested from 12-day old cultures grown at 20 °C on potato dextrose agar (PDA) (Merck, NJ) overlain with clear cellophane; this system allows easy removal of conidia by peeling the cellophane off the surface of the medium. Suspensions containing ca. 10^8 conidia/mL 0.01% Triton X-100 were prepared for incorporation into the coating matrix.

2.2. Inoculation of Pinus radiata

P. radiata seeds were obtained from a commercial supplier (ex New Zealand, strict origin undeterminable). Two methods of inoculation were subsequently used: a seed treatment and root dip. Prior to inoculation, seeds were surface sterilised by soaking in 99% ethanol for 1 min before being sterilised in 10% NaOCl for 5 mins. Subsequently, they were rinsed twice in sterile distilled water for 1 min and then soaked in sterile distilled water for 24 h at 4 °C.

2.3. Seed coating

Prior to seed coating treatments being applied, seeds were placed on sterile filter paper in 55 mm Petri dishes (10 seeds/dish) and air dried in a laminar flow hood. Seeds were coated with either a preparation of xanthan gum (Xan) (0.2%) or methylcellulose (MC) (2%). Coatings were prepared by adding 5 mL of a 2×10^8 conidia/mL 0.01% sterile aqueous Triton X-100 suspension to 20 mL of coating to give a final concentration of 5×10^7 conidia per mL. Viability of the conidia was assessed by spreading 100 μL of inoculum over the surface of a Sabouraud dextrose agar (SDA) (Difco, NJ) plate and incubating at 20 °C. Germination was assessed after 18 h by putting one drop of lactophenol cotton blue (Merck, NJ) onto the plate, overlaying with a coverslip and examining under a microscope; three assessments were made on 100 randomly selected conidia for each germination assessment (Goettel and Inglis, 1997). Viability was >90% for all batches of conidia used.

Control treatments were prepared for each fungus using the same method but 5 mL of 0.01% Triton only was added to the xanthan or methylcellulose coating. In total, six treatments were prepared; 1. F668 Xan, 2. F668 MC, 3. F647 Xan, 4. F647 MC, 5. Xan Control (no fungi), 6. MC Control (no fungi).

Fifty gram batches of seed were placed in a rotating Erweka (Heusenstamm, Germany) coating pan (45° angle, 200 rpm) and 25 mL of coating material applied via a spray gun at 4 bar pressure. Where seeds started clumping, unheated forced air was applied using a hairdryer to break clumps. After coating, seeds were removed from the pan and placed onto trays until dry in appearance, prior to packaging into TGT bags (Convex Plastic Limited, Hamilton, NZ) (six bags per 50 g treatment). Bags were held at 20 °C.

Spore loadings on the seeds were determined after preparation. For each coating and fungus, 10 seeds were placed into 10 mL sterile 0.01% Triton X-100 in a sterile 20 mL plastic tube. Two replicate tubes were prepared for each coating. Seeds were soaked for 30 mins to allow coatings to rehydrate before shaking the tubes on a wrist shaker (Lab-Line, India) set at maximum for 10 mins. Serial dilutions were then prepared and 100 μL aliquots plated onto a semi selective agar medium, BSM (*Beauveria* selective medium: quarter strength PDA containing 350 mg/L streptomycin sulfate, 50 mg/L tetracycline hydrochloride and 125 mg/L cycloheximide (Sigma)). Two replicate plates were prepared for each dilution. Plates were incubated at 20 °C and colony forming units (cfu's) counted after 10 days.