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Spread and persistence of a rugulosin-producing endophyte in *Picea glauca* seedlings

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ABSTRACT

We have studied *Picea glauca* (white spruce) endophyte colonization and its affect on the growth of *Choristoneura fumiferana* (spruce budworm). Here we examine the spread and persistence of a rugulosin-producing endophyte and rugulosin in needles from trees maintained in the nursery, as well as in trees planted in a test field site. Additionally, we report toxicity of rugulosin against three *P. glauca* needle herbivores: *C. fumiferana*, *Lambdina fiscellaria* (hemlock looper) and *Zeiraphera canadensis* (spruce budmoth). Reduction in body weight for both the *C. fumiferana* and *L. fiscellaria* were observed at 25 and 50 μM, respectively, and head capsules were reduced at 100 and 150 μM. *Z. canadensis* larvae did not perform as well in tests due to an *Aspergillus fumigatus* infection, but were shown to be lighter when tested with 100 and 150 μM compared with controls. The endophyte and its toxin were shown to spread throughout the nursery-grown seedlings. After 3.5 and 4.5 y post-inoculation (one and two years in the test site), the inoculated endophyte and its toxin had remained present with an average rugulosin concentration of 1 $\mu g\,g^{-1}$.

Introduction

Toxic metabolites produced by endophytic fungi (*Epichloë festucae*) in fescue grasses greatly reduce the populations of associated herbivorous insects. This has a significant beneficial effect on plant fitness (Clay & Schardl 2002). This association is evidently a highly specialized adaptation (Takemoto *et al.* 2006). Conifer needles are also infected by systemic fungal endophytes that may fulfil several ecological roles (Carroll 1988; Ganley *et al.* 2004). Here we explore a possible role in limiting conifer needle herbivory.

Choristoneura fumiferana (spruce budworm) is a major cyclical pest of spruce and fir trees, especially in the northeast US and Canada (Royama *et al.* 2005). In 1984, a comprehensive analysis of the population dynamics of *C. fumiferana* was published focusing on the period of 1945 to 1983 in New Brunswick (NB), Canada (Royama 1984). One feature of this analysis is that an unknown 'fifth agent', along with predation, parasites, and various diseases, was required to build models to fit observed population changes. To ascertain whether endophytic metabolites might play a role in limiting herbivory, strains of conifer endophytes were collected from the Acadian forest and studied for their ability to produce compounds toxic to *C. fumiferana* larvae. Bioassay-directed fractionation and other means were used to identify their metabolites (Findlay *et al.* 2003).

Successful experimental inoculation of *Picea glauca* seedlings with anti-insectan toxin producing needle endophytes has been demonstrated in previous studies conducted in growth chambers and under nursery conditions (Miller *et al.*

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2002; Sumarah et al. 2005). In the former studies, occurrence of the fungus and its toxin in needles reduced the growth rate of *C. fumiferana*. These studies have been done with the rugulosin-producing endophyte 5WS22E1 (DAOM 229536, CBS 120377) first reported to produce (+)rugulosin by Calhoun et al. (1992). Based on DNA sequence information, this fungus is a species of *Phialocephala* (unpublished data), related to strains previously reported as endophytic in Norway spruce (Gruenig et al. 2002).

Rugulosin has been isolated from disparate fungi, including many strains of conifer endophytes from our collections, from *Aschersonia samoensis* (Watts et al. 2003), as well as from the organism from which rugulosin was first reported, *Penicillium rugulosum* (Bouhet et al. 1976; Breen et al. 1955). It is toxic to *C. fumiferana* larvae in an artificial diet (Calhoun et al. 1992) and in needles in apparently similar concentrations (Miller et al. 2002). Rugulosin has been reported as toxic to *Drosophila melanogaster* (Dobias et al. 1980) and to ovarian cells of the fall armyworm, *Spodoptera frugiperda* (Watts et al. 2003). Based on limited studies, rugulosin has very low mammalian toxicity (LD_{50} 55 mg kg^{-1} BW ip in mice and 44 mg kg^{-1} BW in rats) (Ueno et al. 1971). Three weeks of dietary exposure of 67 mg kg^{-1} BW resulted in the deaths of 50 % of the male mice in the treatment arm from 'acute liver injury characterized by fatty degeneration and liver cell necrosis' after two years (Ueno et al. 1980). Rugulosin was not cytotoxic to HepG2 cells (human hepatoma cells; Watts et al. 2003). It has been reported by many authors as an antibiotic to both Gram-positive and negative bacteria (e.g. Stark et al. 1978) and is moderately antifungal (Breen et al. 1955).

The purpose of this report is to address the spread and persistence of the endophyte and its toxin in trees maintained in the nursery, as well as under field conditions. Experiments have been designed to study persistence of the endophyte in the tree after inoculation and also how it spreads to adjacent trees. Additionally, the extent of spread within a tree is addressed by testing all branches from infected trees. Some data on the dietary toxicity of rugulosin to species of insects herbivorous on *P. glauca* are also discussed.

Materials and methods

Effects of dietary rugulosin on various insect larvae

Choristoneura fumiferana larvae (spruce budworm) were obtained from Insect Production Services, Forestry Canada (Sault Ste. Marie, ON) and stored at 5 °C. For each test, a sufficient number of larvae were hatched and put in creamer cups containing 15 ml artificial diet. The diet was prepared in-house (McMorran 1965). The cups were placed in a growth chamber at 22 °C, 55 % RH with 16 h light/day until the larvae reached second/third instar (McGugan 1954). Previous tests suggested the effective concentration of rugulosin for *C. fumiferana* growth limitation was *ca* 10 μM (Calhoun et al. 1992). A suitable amount of diet was prepared, and four aliquots were dispensed into a flask. Dilutions of pure rugulosin were made in 95 % ethanol to provide the required concentrations (540 μl ethanol solution plus the vehicle control) and added to the flasks. The diet was mixed with a stir bar and two drops of hot liquid diet were each added with a 10 ml sterile pipette (0.1 g D.W.) to a 4 ml tapered plastic sample cup (Fisher Scientific, Ottawa, ON). Vials containing diet and toxin were freeze-dried to eliminate the ethanol and rehydrated with 60 μl sterile water. One larva was placed in each vial, which was returned to the growth chamber to feed for 4 d. After 4 d, all larvae were frozen and weighed on a Mettler 163 analytical balance (±0.02 mg). Previous studies demonstrated that the frozen wet weight was correlated with dry weight ($P < 0.001$). Head capsule widths were determined using a stereo microscope at ×40 with ocular and stage micrometers. *C. fumiferana* larvae were tested in two treatments, the first with concentrations of 5, 10 and 50 μM (75 insects per concentration tested) and the second with rugulosin concentrations of 25, 50 and 100 μM (75 insects per concentration, tested in duplicate).

Lambdina fiscellaria eggs (hemlock looper) were purchased from Forestry Canada in three lots. The eggs were incubated at room temperature for 10 d until larvae emerged. Larvae from each batch emerged within 2 d of each other and were immediately put on diet in cups as above. After they had grown to second instar (*ca* one week), larvae were placed in test vials following the same procedure as for *C. fumiferana* with a concentration range of 5, 10 and 50 μM rugulosin for the first test and 10, 50, 100 and 150 μM for the second test (~67 larvae per concentration, tested in duplicate). After one week on the test diets, the larvae were frozen, weighed, and head capsule width determined.

Zeiraphera canadensis larvae (spruce budmoth) were collected from the wild near Sussex, NB, in mid June (received 13 June 2002 on spruce buds). They were immediately put in test vials and tested at rugulosin concentrations of 10, 50, 100, and 150 μM (75 per concentration, collected in the wild, therefore, tested only once). After 7 d, surviving larvae were frozen and measured.

Preparation of trees and field site

A description of the trees and inoculation methods used in these experiments are given in Sumarah et al. (2005). In early September 2003, 300 (some of the original 330 positives died) of the endophyte/toxin-positive trees from the investigations reported by Sumarah et al. (2005) were planted with greater spacing than normal at a test field site *ca* 30 km from Sussex, NB, (Fig 1A–B). The site has excellent soil characteristics and uniformity. The original forest stand type was mixed wood with red spruce, balsam fir, white birch, yellow birch, and sugar maple. The site was prepared for planting using one pass with a Marden roller and another pass with anchor chains and shark-finned barrels. Half the seedlings were planted on a part of the cut block, which was well drained, and the other half were planted in a wetter area with some seepage.

Endophyte spread at the field site

One year later in July 2004, 250 (15 m old) un-inoculated seedlings were obtained as previously described from the J.D. Irving Ltd genetic improvement programme (Sumarah et al. 2005). Five of these seedlings (20–30 mm tall) were planted around each of 50 randomly selected trees from the