The arbuscular mycorrhizal fungus, Glomus irregulare, controls the mycotoxin production of Fusarium sambucinum in the pathogenesis of potato

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Abstract

Trichothecenes are an important family of mycotoxins produced by several species of the genus Fusarium. These fungi cause serious disease on infected plants and postharvest storage of crops, and the toxins can cause health problems for humans and animals. Unfortunately, there are few methods for controlling mycotoxin production by fungal pathogens, and most rely on chemicals, creating therefore subsequent problems of chemical resistance. We tested the impact of the symbiotic arbuscular mycorrhizal fungus Glomus irregulare on a trichothecene-producing strain of Fusarium sambucinum isolated from naturally infected potato plants. Using dual in vitro cultures, we showed that G. irregulare inhibited the growth of F. sambucinum and significantly reduced the production of the trichothecene 4,15-diacetoxyscirpenol (DAS). Furthermore, using G. irregulare-colonized potato plants infected with F. sambucinum, we found that the G. irregulare treatment inhibited the production of DAS in roots and tubers. Thus, in addition to the known beneficial effect of mycorrhizal symbiosis on plant growth, we found that G. irregulare controlled the growth of a virulent fungal pathogen and reduced production of a mycotoxin. This previously undescribed, biological control of Fusarium mycotoxin production by G. irregulare has potential implications for improved potato crop production and food safety.

Introduction

Fusarium sambucinum (teleomorph, Gibberella pulicaris) causes dry rot disease, a major problem worldwide on potato (Solanum tuberosum). In tubers, this disease is characterized by an internal light-to-dark brown or black dry rot of the potato tuber, while in infected plants, F. sambucinum causes wilting and yellowing of leaves that can result in plant death (Ismail et al., 2011). It has been shown that most potato cultivars grown commercially in North America are susceptible to Fusarium dry rot (Leach & Webb, 1981; Secor, 1991; Secor & Salas, 2001). In North America, potato dry rot is one of the most important causes of postharvest potato losses (Boyd, 1972), and its control has relied on the use of chemical treatment of tubers at harvest or before planting. Thiabendazole (TBZ) has been applied postharvest since the early 1970s to control dry rot in storage. TBZ and thiophanate methyl, another benzimidazole fungicide, have been used to prevent decay of seed pieces caused by Fusarium species. These control methods have become problematic because F. sambucinum has become resistant to the benzimidazole fungicides that have been used to control dry rot of potato (Secor, 1991; Staub, 1991). Resistance to TBZ and other benzimidazole fungicides was discovered in Europe in 1973 and in the USA in 1992. Isolates of F. sambucinum have been shown to be the more resistant to benzimidazole than isolates of seven other Fusarium species isolated from dry-rot-infected tubers (Ocamb et al., 2007). Thus, biological control methods have been
proposed as alternative strategies to control *Fusarium* dry rot. For example, the bacteria, *Pseudomonas fluorescens* and *Enterobacter cloacae*, have been tested as biocontrol agents both in the field and under storage conditions (Schisler et al., 2000; Al-Mughrabi, 2010). These bacteria applied as a seed treatment resulted in a significant reduction in *Fusarium* dry rot severity compared with the untreated control inoculated with *F. sambucinum*. The dry rot reduction averaged over the 2 years of the study was 35% for *P. fluorescens* and 26.5% for *E. cloacae* (Al-Mughrabi, 2010). However, the use of fungi as biocontrol agents that prevent the fungal infection and inhibit toxin production by the pathogens remains largely unexplored.

The arbuscular mycorrhizal fungi (AMF) are plant-root-inhabiting fungi that form symbiosis with plants. These fungi are known to improve plant growth by increasing mineral uptake, in particular phosphorus, and therefore, AMF are largely used in agriculture as biofertilizers (Roy-Bolduc & Hijri, 2011). Numerous reports have demonstrated that AMF also impact the populations of pathogenic fungi in root rhizosphere and protect plants against pathogens (Azcoín-Aguilar & Barea, 1997; Bedker et al., 2002; Filion et al., 2009; Roy-Bolduc & Hijri, 2011). AMF interact with soil microorganisms to promote inhibitory or stimulatory reactions, some of which may be competitive, while others may be mutualistic (Filion et al., 1999). Substances released by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* stimulated the growth of *P. chlororaphis* and the germination of *Trichoderma harzianum* (Filion et al., 1999). Besides the direct competitive interactions with other pathogenic fungi, the AMF symbiosis affects the community and diversity of other organisms in the soil. Inoculation with the AMF *G. intraradices* induced changes in the root exudates of canola, which is considered to be a nonmycorrhizal plant species, which impact the bacterial community composition in the rhizosphere (Marschner & Timonen, 2005). Recent research has also shown that AMF release an unidentified diffusion factor, known as the myc factor, which stimulates the activation of plant nodulation by the nitrogen fixing and rhizobial bacteria (Kosuta et al., 2003).

We previously showed that the *G. irregulare* inhibited the growth of *F. sambucinum* in vitro and modulated the expression of the trichothecone biosynthetic genes *TRI4*, *TRI5*, *TRI6*, *TRI13*, and *TRI101* (Ismail et al., 2011). The objective of this study was to test the effect of the AMF *G. irregulare* on mycotoxin production of *F. sambucinum*. We tested whether *G. irregulare* reduces 4, 15-diacetoxyisoreniphenol (DAS) production by *F. sambucinum* in vitro- and in *F. sambucinum*-infected potato plants.

### Materials and methods

#### Fungal strain and growth conditions

*Fusarium sambucinum* Fuckel (teleomorph: *Gibberella pulicaris*) strain T5 was grown and maintained on V8 juice agar and on GYEP agar media (2% glucose, 0.1% yeast extract, 0.1% peptone, and 2% agar) (Ismail et al., 2011). Two isolates of the AMF *G. irregulare* (syonym: *Rhizophagus irregularis*) Blaszkowski, DAOM-197198 and DAOM-234328, were grown *in vitro* in co-culture with RIT-DNA-transformed carrot roots (*Daucus carota L.*) on minimal (M) medium. *F. sambucinum* and *G. irregulare* cultures were incubated at 25 °C in the dark.

#### Dual culture assays and quantitative analysis of the trichotheccenes

The confrontation experiments between *G. irregulare* and *F. sambucinum* were performed as described by Ismail et al. (2011). Treatments included *F. sambucinum* confronted with a *G. irregulare* isolate DOAM-197198 or DAOM-234328 and *F. sambucinum* confronted with carrot roots without AMF and *F. sambucinum* alone. Ten replicates were used for each treatment combination. The plates were incubated in a randomized complete design at 25 °C for 7 days. The GYEP agar and *F. sambucinum* biomass from each treatment were chopped into small pieces and extracted in a 250-mL aluminum-foil-sealed beaker on a rotary shaker at 200 r.p.m. for 1 h. Ethyl acetate extracts were transferred in new 250-mL beakers and evaporated overnight in a chemical hood. The residue was dissolved in 2 mL ethyl acetate and transferred into 2 mL GC vials. Extracts were analyzed with gas chromatography and low-resolution mass spectrometry (GC-MS) using a Hewlett Packard 6890 gas chromatograph fitted with a HP-5MS column (30 m × 0.25 mm film thickness) and a 5973 mass detector. The carrier gas was helium with a 20 : 1 split ratio and a 20 mL min⁻¹ split flow. The oven temperature was ramped from 120 °C to 260 °C at 25 °C min⁻¹ and then held at 260 °C for 5.4 min (total run time of 11 min). Under these conditions, DAS elutes at 9.4 min. DAS concentrations were determined using a standard curve with known concentrations.

#### In vitro propagation of potato seedlings and AMF inoculation

Potato seedlings (*S. tuberosum* L.), cultivar Reba, were germinated and maintained *in vitro* using a technique adapted from the study by Suttle (1998). Four-week-old seedlings were individually transplanted under sterile conditions into...
18-cm-diameter pots containing a mixture of loamy soil, peat moss, and expanded perlite (3 : 1 : 1, v/v/v). The soil mixture was sterilized twice by autoclaving at 120 °C for 45 min. Inoculation of seedlings with G. irregulare isolates was achieved by adding approximately 1000 spores in 5 mL of autoclaved water into the soil mixture containing potato seedlings. Non-mycorrhizal plant controls were made by adding 5 mL of autoclaved water to soil mixture. Four weeks after inoculation with AMF, randomized samples were collected from plant roots to check for mycorrhizal colonization using an ink and vinegar root staining method (Vierheilig et al., 1998). Mycorrhizal colonization was also observed by staining roots sampled at the end of the experiment.

**Preparation of F. sambucinum and plant infection**

The virulent F. sambucinum strain T5, was maintained on GYEP medium for 7 days. Spores were scraped and collected in autoclaved water. 3 mL of spore suspension was used to inoculate 250-mL flask containing 20 g autoclaved oat (Avena sativa L.) seeds. As a control, oat seeds were treated with 3 mL of autoclaved water. The inoculated or control seeds were incubated at 25 °C for 4 weeks and were then used prior to plant infection. The infected oat seed was used to inoculate roots of 4-week-old transplant potato as described previously (Ismail & Hijri, 2012). Each combination F. sambucinum and/or mycorrhized plants was replicated 10 times. In addition, a combination of an extra 10 plants of F. sambucinum and/or mycorrhized plants was added to the experiment for tuber harvest.

**Trichothecene analysis from shoots, roots, and tubers**

To determine the trichothecene concentration in plant tissues, shoots and roots from each plant were separately collected 2 weeks postinfection with F. sambucinum. Shoots and roots from each plant were immediately rinsed under tap water. Shoots were cut into small pieces, while root tissues were ground with mortar and pestle. Shoots and roots from each plant were extracted in 250-mL beakers containing 100 mL of ethyl acetate on a rotary shaker at 200 r.p.m. for 1 h. Ethyl acetate extracts were transferred to new 250-mL beakers and evaporated overnight in a chemical hood. The residue was dissolved in 2 mL ethyl acetate and transferred into 2-mL GC vials. Extracts were analyzed with gas chromatography as described above. Tubers from each plant were immediately harvested and cut into small pieces and transferred into 250-mL beakers containing 100 mL ethyl acetate. Extracts were analyzed with GC-MS as described above.

**Experimental design and statistical analysis**

Experiments were performed using a factorial arrangement (1 pathogen) × (2 AMF + control) in a randomized complete design with 10 replicates. One-way analysis of variance (ANOVA) was used to examine the significant effect of G. irregulare on DAS production in F. sambucinum. Post hoc comparison between the treatments was made using Tukey’s HSD test using SPSS software, version 17 (SPSS Inc., Chicago, IL).

**Results**

To study the impact of AMF on DAS production by F. sambucinum in vitro, we used confrontation cultures with two isolates of G. irregulare. Fig. 1 shows that the amount of DAS produced by F. sambucinum was significantly lower in the presence of G. irregulare isolates, DAOM-197198 and DAOM-23438, 38.8 µg mL⁻¹ (SD 9.9, n = 10) and 42.1 µg mL⁻¹ (SD 13.3, n = 10), respectively, compared with controls that consisted of F. sambucinum alone, 125.7 µg mL⁻¹ (SD 21.5, n = 10), or F. sambucinum with carrot roots, 144.0 µg mL⁻¹ (SD 40.1, n = 10).

To test whether G. irregulare impact the production of DAS in potato plants, we conducted a growth chamber trial in which clonally propagated potato seedlings were inoculated with G. irregulare and then infected with F. sambucinum (Fig. 2). To allow the establishment of mycorrhizal symbiosis, plants were infected with F. sam-
**bucinum** 4 weeks after inoculation with *G. irregulare*. All plants showed evidence of mycorrhizal colonization by root staining and microscopy observation. After 2 weeks postinfection with *F. sambucinum*, shoots, roots, and tubers were harvested, extracted, and analyzed for trichothecenes using GC-MS. The highest concentration of DAS (mean 115.49 ± 34 SE µg g⁻¹, n = 9) was observed in roots of non-mycorrhized and infected plants with *F. sambucinum* (Fig. 3), while shoots and tubers of these plants showed means of 51.1 ± 15.3 SE µg g⁻¹ (n = 10) and 60.6 ± 7.9 SE µg g⁻¹ (n = 10), respectively. DAS was not detected in the roots of plants mycorrhized and infected with *F. sambucinum*, and the concentration of DAS in tubers of these plants was significantly reduced [mean 8.1 ± 0.01 SE µg g⁻¹ (n = 10)] compared with plants that were not mycorrhized prior to infection with *F. sambucinum*. However, DAS concentration in shoots of plants mycorrhized and infected with *F. sambucinum* [mean 48.3 ± 13.9 SE µg g⁻¹ (n = 9)] was not significantly different from that of plants that were not mycorrhized prior to infection with *F. sambucinum* (Fig. 3). Neither trichodiene- nor other trichothecene-related compounds were detected in extracts of roots, tubers, or leaves.

**Discussion**

We found that *G. irregulare* blocked the accumulation of DAS in roots and tubers, while it did not affect DAS concentration in shoots of plants mycorrhized and infected with *F. sambucinum*. This could be explained by the requirement of physical proximity of *G. irregulare* and *F. sambucinum*. The habitat of AMF is limited in soil and the cortex of plant roots, and their mycelium never reaches the plant shoots. Although, *G. irregulare* induces PR homologues genes in potato shoots in response to infection by *F. sambucinum* (Ismail & Hijri, 2012), this defense gene induction is not sufficient to affect the production of DAS by the pathogen in the shoots.

In a previous study, using confrontation cultures *in vitro*, we demonstrated that *G. irregulare* inhibited *F. sambucinum* growth and modulated the expression of trichothecene biosynthetic genes (Ismail *et al.*, 2011). Expression of *TRI5*, which controls the terpene cyclase that converts farnesyl diphosphate to trichodiene, was up-regulated, and expression of *TRI4*, which controls...
conversion of trichodiene to oxygenated trichothecene precursors, was down-regulated (Ismail et al., 2011). Although those results suggested that trichodiene or related compounds might accumulate in *F. sambucinum* / *G. irregulare* confrontation cultures, no DAS precursors were detected in this study.

We found that the *G. irregulare* caused a remarkable reduction in *F. sambucinum* DAS production, *in vitro* with confrontation cultures. In addition, *G. irregulare* stopped DAS production in plant roots, while there was only a partial inhibition of DAS production observed in tubers and no change in plant shoots. It has been documented in many reports that AMF can reduce the incidence and severity of root diseases and protect plants against soil-borne pathogens (Azcón-Aguilar & Barea, 1997; Bodker et al., 2002; Filion et al., 2003; Harrier & Watson, 2004; St-Arnaud & Vujanovic, 2007; Lioussanne et al., 2009; Ismail et al., 2011). Possible mechanisms by which AMF act as biological control agents are as follows: impact on soil microbial communities, antagonisms, and stimulation of plant defenses (Azcón-Aguilar & Barea, 1997; Bodker et al., 2002; Filion et al., 2003; Harrier & Watson, 2004; St-Arnaud & Vujanovic, 2007; Lioussanne et al., 2009; Ismail et al., 2011; Ismail & Hijri, 2012). For example, soil bacteria interact with AMF by forming a biofilm on the surface of the hyphae (Lecomte et al., 2011). In a recent study, Cruz & Ishii (2012) isolated three strains of endobacteria from spores of the AMF *Gigaspora margarita* using a hypodermic needle. These bacteria, identified as *Bacillus* sp., *Bacillus thuringiensis*, and *Paenibacillus rhizosphaerae*, were evaluated for their antagonistic effect on the soil-borne plant pathogens *F. oxysporum* f. sp. *lactucae*, *Rosellinia necatrix*, *Rhizoctonia solani*, and *Pythium ultimum*. Interestingly, all of the bacterial strains showed antagonism activities when confronted *in vitro* with the pathogens. The same authors have also shown that the isolated endobacteria can form aggregates, which resemble biofilm structures, on the surface of AMF hyphae (Cruz & Ishii, 2012). These observations along with those reported by Lecomte et al. (2011) support the hypothesis that the AMF impact the soil microbial communities, which could be a plausible mechanism for *G. irregulare* suppression of *F. sambucinum* growth and DAS production in potato. AMF have been shown to reduce the abundance of pathogenic fungi in plant roots by direct competition via interference competition and chemical interactions (Azcón-Aguilar & Barea, 1997; St-Arnaud & Vujanovic, 2007). In another example, AMF extract reduced the germination of *Fusarium oxysporum* f. sp. *chrysanthemi* conidia (Filion et al., 1999). Mycorrhization with *G. irregulare* strongly reduced disease severity on potato plants, which suggested that the biocontrol effect was due to stimulation of plant defense by inducing the expression of defense-related genes ChtA3, gluB, CEV116, OSM-8e, and PR-1 (Ismail & Hijri, 2012). In the present study, we have documented a novel and important effect that AMF have on production of mycotoxins by the soil-borne pathogen *F. sambucinum* in roots and tubers of potato plants.

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