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SHORT NOTE

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Effect of mycorrhization on the accumulation of rishitin and solavetivone in potato plantlets challenged with *Rhizoctonia solani*

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Abstract The effect of colonization with the vesicular-arbuscular mycorrhizal fungus *Glomus etunicatum* on the content of rishitin and solavetivone was determined in potato plants cv. Goldrush challenged with *Rhizoctonia solani*. Mycorrhization stimulated significantly the accumulation of both phytoalexins in roots of plantlets challenged with *R. solani* but did not influence phytoalexin levels in non-challenged plantlet roots. No accumulation of solavetivone or rishitin was detected in shoots. In Petri dish bioassays, rishitin and solavetivone inhibited mycelial growth of *R. solani*.

Keywords *Solanum tuberosum* · *Rhizoctonia solani* · *Glomus etunicatum* · Rishitin · Solavetivone

Introduction

Over the last few years, the use of vesicular-arbuscular mycorrhizal (VAM) fungi as biocontrol agents of soil-borne plant pathogens has received increasing attention. Indeed, mycorrhizal inoculation has been reported to reduce the development of various diseases on economically important crops, including *Fusarium* spp. in soybean (Zambolim and Schenck 1983), *Phytophthora nicotianae* var. *parasitica* in tomato (Cordier et al. 1996), and *Verticillium* spp. in alfalfa (Hwang et al. 1992) and cotton (Liu and Liu 1995). In a recent study, Yao et al. (2002) showed that inoculation of potato (*Solanum tuberosum* L.) plantlets cv. Goldrush with the VAM fungus *Glomus etunicatum* Becker & Gerd reduced development of *Rhizoctonia solani* Kühn (AG-3), an important potato pathogen, suggesting that VAM inoculation can improve resistance/tolerance to *R. solani* infection.

Several mechanisms were suggested to explain the protective effect of VAM fungi in different plant-pathogen interactions. These include microbial changes in the mycorrhizosphere, lignification of root endodermal cells, improvement of plant nutrition, competition for photosynthates, and activation of plant defence mechanisms (Singh et al. 2000; Azcón-Aguilar and Barea 1996). With regard to activation of plant defence mechanisms in mycorrhizal plant roots, accumulation of phytoalexins (low-molecular-weight compounds with antimicrobial properties) has been suggested as a mechanism involved in disease reduction (Sundaresan et al. 1993).

Solavetivone and rishitin are well-known phytoalexins produced by potato plants in response to various stresses, such as application of arachidonic acid (Desjardins et al. 1995) and infection with *Erwinia carotovora* (Engström et al. 1999; Lyon and Bayliss 1975). These compounds were reported to reduce the growth of the potato pathogens *Phytophthora infestans* (Engström et al. 1999; Stössel and Hohl 1981) and *E. carotovora* var. *atroseptica* (Lyon and Bayliss 1975). However, antifungal activity of these compounds has not been demonstrated against *R. solani*.

The aim of the present study was (1) to evaluate the effect of potato plantlet inoculation with the VAM fungus *G. etunicatum* on plant content of solavetivone and rishitin, and (2) to determine the effect of these compounds on mycelial growth of *R. solani*.

Materials and methods

Plantlet micropropagation

Potato plantlets (cv. Goldrush) obtained from Agriculture and Agri-Food Canada (La Pocatière, Québec, Canada) were multiplied in vitro by shoot cuttings according to the method of Yao et al. (2002).

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Inoculum of *R. solani*

A virulent strain of *R. solani* (AG-3) isolated from a potato tuber, kindly provided by Service de Recherche en Défense des Cultures (MAPAQ, Québec, Canada), was maintained on potato dextrose agar (PDA; Difco Laboratories, Becton Dickinson, Md.) at 4°C. Sterile oat seeds covered with *R. solani* mycelium, prepared as described by Yao et al. (2002), were used as inoculum.

Experimental design

A completely randomized 2 (VAM-inoculated+non-VAM-inoculated) ×2 (*R. solani*-challenged+non-challenged)×4 (harvest times 1, 3, 5 and 7 days after *R. solani* inoculation) factorial arrangement with two replicates was performed. Each experimental unit consisted of 14 plants.

Mycorrhization of acclimatizing potato plantlets

Three-week-old micropropagated potato shoots were excised to obtain uniform size. These were individually rooted and grown in sowing trays half-filled with low-phosphorus peat-based growing substrate (Pro-Mix BX; Premier Tech., Rivière-du-Loup, Québec, Canada) amended with 10 ml of either non-mycorrhizal or mycorrhizal substrate (approximately 2.7 propagules of *G. etunicatum* per ml; Premier Tech). After acclimatization in a greenhouse (2 weeks), the rooted plantlets were then individually transplanted into 18-cm-diameter pots containing a mixture of soil, peat-based growing substrate (Pro-Mix BX) and turface (Turface MVP; Lake Cook Road, Buffalo Grove, Ill.) (3/1/1; v/v/v) previously sterilized by gamma irradiation (Co-60, 15 kGy, 22°C) (Yao et al. 2002). Plants were grown in a glasshouse under the conditions previously reported by Yao et al. (2002).

Infection of potato plantlets with *R. solani*

Seven days after transplantation, soil at the base of the plantlets was gently pushed aside to expose root systems. Non-inoculated or *R. solani*-inoculated oat seeds (five seeds per plant) were then placed directly in contact with roots at five points equidistant from the stem. Roots were covered with soil immediately after inoculum application.

Harvest of plants

Potato plantlets were harvested 1, 3, 5, and 7 days after *R. solani* inoculation. After harvest, roots (including crowns) were separated from shoots and cleaned in water. Both roots and shoots were freeze-dried and stored at –20°C.

Estimation of root colonization by *G. etunicatum*

At each harvest time, a sample of fresh roots (2–3 g fresh weight) randomly selected from each plant was cleared and stained with Trypan blue (Phillips and Hayman 1970). Root colonization by *G. etunicatum* was then estimated using the grid-line intersect method (Giovannetti and Mosse 1980).

Extraction of phytoalexins

Each freeze-dried sample (14 plantlets) was ground with beach sand using a mortar and a pestle. Extraction of solavetivone and rishitin was performed with slight modifications of the methods of Henfling and Kuć (1979) and Charles (1998). Ground sample (corresponding to 3 g fresh weight) was placed in a glass-stoppered flask (125 ml) containing 30 ml of methanol and agitated in a water bath at 50°C for 1 h. The homogenate was filtered under vacuum through Whatman No. 1 filter paper and the residue homogenized in a blender and extracted twice as previously described. The methanolic filtrates were pooled and concentrated to near dryness at 45–50°C using a Rotovapor-R (Büchi, Flawil, Switzerland). The concentrate was suspended in a separating funnel containing 100 ml of a mixture of ethyl acetate/water (1/1, v/v). The organic layer was recovered, completely evaporated under nitrogen flow, and the residue dissolved in ethyl acetate (1 ml) and stored at –20°C.

Determination of solavetivone and rishitin

Solavetivone and rishitin were estimated by gas chromatography (5890 Serie II plus with a mass selective detector 5972; Hewlett-Packard, Mississauga, Ontario, Canada). Typical operating conditions were: helium as carrier gas at 1.0 ml/min, a column head pressure of 13.4 psi at 150°C, a linear velocity of 37.8 cm/s, a split flow of 40 ml/min. The injection volume was 1 μl with splitless mode and a purge time of 1 min. The oven temperature was 150°C isothermal for 5 min, followed by an increase from150 to 250°C at 5°C/min, an increase from 250 to 300°C at 20°C/min and then isothermal for 1.5 min. The total run time was 24 min. The injector and detector ports were held at 290 and 300°C, respectively. Separation was performed with DB-5 (rishitin) and DB-1 (solavetivone) capillary columns (30 m long×0.25 μm film thickness) (Phenomenex, Torrance, Calif.). For rishitin determination, standards and samples were previously derivatized at 60°C for 1 h using N-trimethylsilylimidazole (TMSI) and dimethylformamide as solvent. Presence of solavetivone and rishitin was confirmed by comparing spectral mass with the National Institute Standard and Technology library. Rishitin and solavetivone in each extract was determined in triplicate using standard curves. For standards, rishitin and solavetivone were extracted as described above from UV-irradiated whole tuber slices (Charles 1998) of the potato cv. Goldrush purchased from Ferme Bouchard and Ass. (St-Eloi, Québec, Canada), and purified according to the method of Desjardins et al. (1995).

Statistical analysis

Analysis of variance (ANOVA) was carried out with the GLM (General Linear Models) procedure of SAS (SAS Institute 1999). When significant ($P<0.05$), treatment means were compared using contrasts.

Effect of solavetivone and rishitin on *R. solani* mycelial growth

The effect of solavetivone (3.2, 6.4, 9.6, 12.9, 16.1 μg/ml) and rishitin (1.6, 3.2, 6.4, 9.6, 12.9 μg/ml) on *R. solani* mycelial growth was determined in 5-cm Petri dishes. Appropriate amounts of purified solavetivone (0, 10, 20, 30, 40 and 50 μg) and rishitin (0, 5, 10, 20, 30 and 40 μg) were dissolved in absolute ethanol (100 μl) and added to 3 ml of molten agar (13 g/l; Difco Laboratories) containing glucose (16 g/l; Sigma, St. Louis, Mo.). After agar solidification, quadruplicate Petri dishes were inoculated with agar plugs (3 mm diameter) covered with actively growing mycelium of *R. solani* and incubated at 27°C for 48 h. The radial mycelial growth was then measured. Mycelial growth inhibition was calculated as [(growth on agar without phytoalexin) – (growth on agar amended with either rishitin or solavetivone)] / (growth on agar without phytoalexin) and expressed as percent.

Results

Root colonization by *G. etunicatum* was not significantly ($P>0.05$) affected by the presence of *R. solani*. For both non-challenged and *R. solani*-challenged plantlets, mean

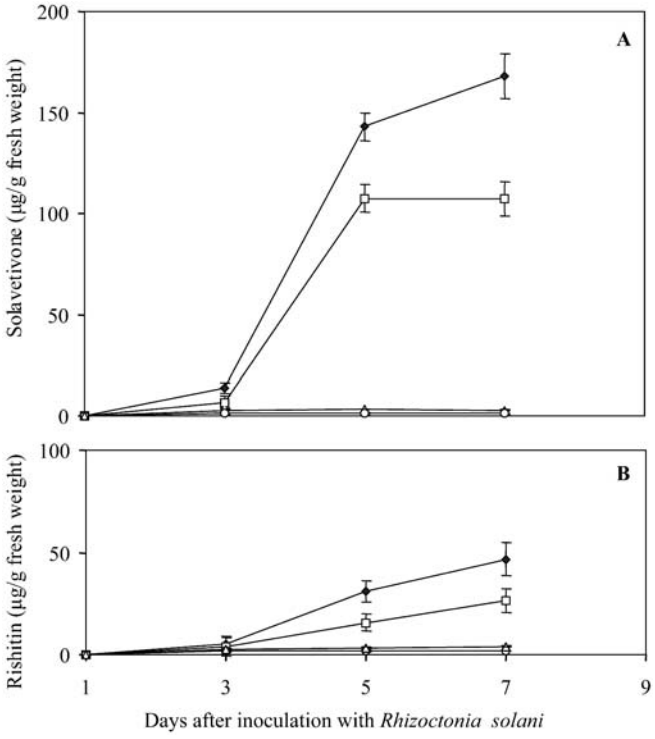

Fig. 1 Time course of solavetivone (**A**) and rishitin (**B**) accumulation in potato roots inoculated or not with *Glomus etunicatum* and challenged or not with *Rhizoctonia solani*. Each value represents the mean of two replicates. *Vertical bars* represent standard errors (◆ Inoculated with *G. etunicatum* and challenged with *R. solani*, ❑ not inoculated with *G. etunicatum* and challenged with *R. solani*, △ inoculated with *G. etunicatum* and not challenged with *R. solani*, ◯ not inoculated with *G. etunicatum* and not challenged with *R. solani*

values of root colonization by *G. etunicatum* rated 22–28 days after VAM-inoculation were about 6%.

Only traces of solavetivone and rishitin were detected in mycorrhizal and non-mycorrhizal potato roots not challenged with *R. solani* (Fig. 1). The solavetivone and rishitin contents of roots colonized with *G. etunicatum* did not differ significantly ($P>0.05$) from non-mycorrhizal roots, but the values for both rishitin and solavetivone rated 5 and 7 days after inoculation with *R. solani* were significantly ($P<0.05$) higher in *R. solani*-challenged than in non-challenged plantlets. In contrast, *G. etunicatum* stimulated significantly ($P<0.05$) rishitin and solavetivone accumulation in roots of *R. solani*-challenged plantlets. There was no accumulation of solavetivone or rishitin in shoots.

The effect of solavetivone and rishitin on *R. solani* mycelial growth is presented in Table 1. Mycelial growth of *R. solani* was progressively inhibited by increasing concentrations of solavetivone or rishitin incorporated into the medium. For each concentration tested, rishitin showed a stronger inhibitory effect than solavetivone.

Discussion

Previous work has shown that, even at low level (5–7%), mycorrhization of potato plantlets with *G. etunicatum* provides resistance/tolerance against *R. solani* infection (Yao et al. 2002). As part of ongoing research aimed at elucidating the mechanism(s) by which mycorrhization reduced *R. solani* infection of potato plantlets, the present study now shows that mycorrhization at a low level (6%) stimulated the accumulation of both rishitin and solavetivone in *R. solani*-challenged plantlets only. Thus, it appears that the pathogen was essential for phytoalexin stimulation. Only traces of rishitin and solavetivone were detected in non-infected plantlets inoculated or not with *G. etunicatum*. These results are in agreement with previous studies with other plant pathogen-VAM fungus systems showing that mycorrhization results in accumulation of phytoalexins at a level lower than pathogenic interactions (Morandi 1996; Wyss et al. 1991). However, our results clearly show that mycorrhization can amplify phytoalexin production in *R. solani*-infected potato plantlets.

In the present study, rishitin and solavetivone were detected only in roots, suggesting that phytoalexins were either not translocated (Kuć 1995) to the shoot or were rapidly degraded. On the other hand, rishitin accumulation was previously reported to be very localized in potato tubers (Lulai 2000).

Rishitin and/or solavetivone were reported to inhibit the growth of different microorganisms, including *Botrytis cinerea* (Glazener and Wouters 1981), *E. carotovora* var. *atroseptica* (Lyon and Bayliss 1975) and *P. infestans* (Engström et al. 1999; Stössel and Hohl 1981). Rishitin

Table 1 Effect of different concentrations of the phytoalexins rishitin and solavetivone on mycelial growth of *Rhizoctonia solani*. Each value represents the mean±standard error of four replicates. Mycelial growth inhibition was calculated as follows: [(growth on agar without phytoalexin) – (growth on agar amended with either rishitin or solavetivone)] / (growth on agar without phytoalexin) and expressed as percent (ND Not determined)

Phytoalexin concentration µg/ml	Rishitin		Solavetivone	
	Growth (cm)	Inhibition (%)	Growth (cm)	Inhibition (%)
0	4.7±0.00	–	4.7±0.00	–
1.6	4.4±0.03	6.4	ND	ND
3.2	4.1±0.04	12.8	4.7±0.03	0.0
6.4	3.8±0.02	19.1	4.2±0.05	10.6
9.6	3.7±0.06	21.3	4.2±0.05	10.6
12.9	3.0±0.16	36.2	3.9±0.05	17.0
16.1	ND	ND	3.4±0.05	27.6

was shown to inhibit *B. cinerea* mycelial growth in both solid and liquid media (Glazener and Wouters 1981). In solid media, a concentration of 30 μg/ml reduced the growth of *B. cinerea* by 41%. Rishitin and solavetivone at a concentration of 50 μg/ml caused a 6 and 2% inhibition of *P. infestans* growth on agar, respectively (Engström et al. 1999). Thus *R. solani* seems more sensitive to rishitin and solavetivone than *P. infestans*.

The results presented show that mycorrhization of *R. solani*-infected potato plantlets amplified the accumulation of rishitin and solavetivone and that these phytoalexins inhibited in vitro the growth of *R. solani*. However, it remains to be investigated whether the amplified accumulation of these compounds contributes to effective protection of potato plantlets against *R. solani*.

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