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SHORT NOTE

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Changes in the concentration of trigonelline in a semi-arid leguminous plant (*Prosopis laevigata*) induced by an arbuscular mycorrhizal fungus during the presymbiotic phase

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Abstract An in vitro presymbiotic system between mesquite [*Prosopis laevigata* (Willd.) M.C. Johnst], a semi-arid leguminous plant, and pregerminated spores of *Gigaspora rosea* Nicol. & Schenck was established. After characteristic hyphal branching, high performance liquid chromatographic analyses of methanol extracts from *P. laevigata* roots revealed a concentration change in one ultraviolet-detectable product. This product was identified by nuclear magnetic resonance and mass spectrometry as trigonelline, a pyridine alkaloid. The concentration of trigonelline was constant in the aerial parts of the plant with or without *G. rosea*, but its concentration in the roots increased 1.8-fold when *G. rosea* was present. Trigonelline may be a regulatory factor during early signal events in the establishment of the arbuscular mycorrhizal symbiosis in *P. laevigata*.

Keywords *Gigaspora rosea* · *Prosopis laevigata* · Trigonelline · Semi-arid legume · Mesquite

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Introduction

The symbiosis between land plants and arbuscular mycorrhizal (AM) fungi is one of the plant-fungus interactions most widely distributed in nature. Of the 231,000 known angiosperms, 80–90% can form a symbiosis with 150 AM fungal species (Koide and Schreiner 1992). This widespread distribution is due to the mutual benefits for both the plant and the fungus. The fungus receives photosynthetic products and becomes an extension of the roots, increasing plant capacity to absorb water and nutrients such as phosphorus (Smith and Gianinazzi-Pearson 1988; Brundrett et al. 1996). This close association has increased the geographical distribution of plants, and fossil evidence suggests that it permitted the colonization of land (Phipps and Taylor 1996).

The interaction between plant host and AM fungus begins in the presymbiotic phase and the establishment of mechanisms of signalling/recognition/communication in each partner determines the intraradical colonization of the host. The molecular bi-directional mechanisms involved in the recognition between host plant and the AM fungus in the presymbiotic phase are poorly understood (Nagahashi et al. 1999). The earliest defined response of recognition in the fungus is host-induced hyphal branching, but the trigger compounds released by the plant are still unknown. In the early 1990s, Giovannetti et al. (1993, 1996) used a membrane to separate a host plant (*Ocimum basilicum* L., *Latuca sativa* L. or *Lycopersicum esculentum* Mill.) and an AM fungus (*Glomus mosseae*, *Glomus intaradices* or *Glomus* sp.). When the fungus was physically separated from the host plant, hyphal differentiation occurred in the form of extensive branching. This was not observed with non-host plants (Gianinazzi-Pearson 1996). This morphogenetic response without direct contact between fungus and roots of the host plant was the first evidence that a soluble factor from the host plant (Bonfante-Fasolo and Perotto 1992), also called branching factor (Buee et al. 2000), is involved in hyphal

differentiation. Many authors proposed flavonoids as the most likely candidates for the branching factor in the AM presymbiotic phase (Gianinazzi-Pearson et al. 1989; Bécard et al. 1992), as flavonoids play a key role in presymbiotic communication between leguminous plants and rhizobia (Spaink 1998).

The results of recent studies, however, suggest that flavonoids are not essential as a branching factor in AM presymbiosis. Buee et al. (2000) found hyphal branching in a bioassay with pregerminated spores of *Gigaspora gigantea* and exudates of a maize mutant deficient in chalcone synthase, which is essential in the biosynthesis of flavonoids. These results suggest that other metabolites are involved in the presymbiotic communication between plant host and AM fungus. Secondary metabolites like polyacetates, terpenoids or alkaloids play a key role in plant-microbe interactions and are essential for survival of a plant in its natural environment (Paiva 2000). In mycorrhizal roots of barley colonized by *Glomus intraradices*, Peipp et al. (1997) found fungus-induced accumulation of secondary metabolites such as amides and sesquiterpenoid cyclohexenone derivatives. The accumulation of these compounds was also confirmed in wheat and maize colonized by *Glomus intraradices*, *Glomus mosseae* or *Gigaspora rosea* Nicol. & Schenck (Vierheilig et al. 2000) and in the non-gramineous plant *Nicotiana tabacum* with *Glomus intraradices* (Maier et al. 1999). However, the accumulation of these cyclohexenone derivatives appeared to be related to the establishment of a functional mycorrhiza.

We investigated secondary metabolites in mesquite (*Prosopis laevigata* (Willd.) M.C. Johnst), a semi-arid leguminous plant, during the presymbiotic phase. *P. laevigata* is known to form symbioses with both rhizobia and AM fungi, such as *Gigaspora rosea* (Stutz et al. 2000). We established an in vitro presymbiotic system in which the roots of *P. laevigata* were placed close to pregerminated spores of *Gigaspora rosea* and a control treatment in which no spores were present. Spectroscopic data, including nuclear magnetic resonance (NMR), mass spectrometry (MS) and ultraviolet (UV) spectroscopy, identified trigonelline as the component in the roots showing the greatest change in concentration when in contact with the spores of *Gigaspora rosea*.

Materials and methods

Plant material

Undamaged and uniform seeds of *P. laevigata* were scarified by submerging in 98% sulphuric acid for 8 min, rinsed five times in distilled water and then submerged in 30% hydrogen peroxide to disinfect the seeds. The scarified and disinfected seeds were germinated on nutritive agar for 3 days. The plantlets free of microorganisms were aseptically transferred to Magenta GA-7 vessels (Sigma, St. Louis, Mo., USA) and cultivated in solid M medium (Bécard and Fortin 1988) until the root and cotyledons developed after 3 days.

AM fungus preparation

Spores of *Gigaspora rosea* (BEG 9; La Banque Européenne des Glomales; International Institute of Biotechnology, UK) multiplied in leek pot cultures (*Allium porrum* L.) were isolated by wet sieving, surface sterilized according to Bécard and Fortin (1988) and stored on magnesium sulphate heptahydrate, 0.1% gelrite upside down at 4°C until used. Healthy cream-coloured spores were selected with the aid of a stereomicroscope and aseptically transferred to Petri dishes containing solid M medium without sucrose for germination.

In vitro presymbiotic system

Plants with 2-cm roots and well-developed cotyledons cultivated in Magenta GA7 vessels were transferred under sterile conditions to upright perforated 90-mm-square plastic sterile Petri dishes with 80 ml of solidified 5% gelrite M medium. The Petri dishes were wrapped in sterile aluminium foil so that the roots developed in the dark, and placed in sterile sun-transparent bags (Sigma). The plants were cultured in a growth cabinet (26°C ± 0.5, relative humidity 30%, 16–8 h light-dark period, light intensity 3000 lux) for 1 week. Individual aseptically germinated spores of *Gigaspora rosea* obtained as described above were placed under sterile conditions close to the roots of *P. laevigata* (ca. 5 mm) and cultivated under the same growth conditions. Pregerminated spores of *Gigaspora rosea* in the in vitro presymbiotic system were monitored daily with a stereomicroscope to determine the effect of the roots on the pattern of hyphal elongation.

The experiments were set up in triplicate and repeated three times. A control treatment without spores was included.

Extraction of roots and aerial parts and high performance liquid chromatography

Plants were harvested from the in vitro presymbiotic system after 1 week when hyphal branching occurred. The roots and aerial parts were submerged separately in liquid nitrogen and mortar crushed. A subsample of 1 g fresh tissue was extracted twice with 5 ml 80% aqueous methanol, first for 15 min and then for 30 min. The two filtrates were mixed and the methanol vacuum evaporated; the aqueous residue was frozen and lyophilized. The lyophilized residue was redissolved in methanol, centrifuged at 6000 rpm and purified over a C18 cartridge (Spe-ed Applied Separations, Allenton, Pa., USA).

An aliquot of 20 µl was taken from the purified methanol extract, and injected into a Varian chromatograph ProStar 330 with a photodiode array detector (Varian, Walnut Creek, Calif., USA) equipped with a 5-µm silica C18 column (25 × 4.6 mm i.d.; Supelco, Bellefonte, Pa., USA). Trigonelline was separated using a modified linear gradient according to Maier et al. (2000) at a flow rate of 1 ml min^{-1} for 60 min from solvent A (1% aqueous phosphoric acid) to solvent B (80% aqueous acetonitrile). Trigonelline was detected by photometry at 265 nm according to the trigonelline λ maximum, which was selected by using a maxplot between 220 nm and 400 nm.

Identification of trigonelline

The purified methanol extract was concentrated to 500 µl, spotted on silica gel 60 F_{254} thin layer chromatography (TLC) plates of 200 µm (Merck, Darmstadt, Germany) and developed in acetone-water (1:1). The trigonelline band (located by UV light) was compared with an authentic sample of trigonelline (Sigma). The ^{1}H NMR spectrum of trigonelline from the purified methanol extract was registered on a Varian Mercury 300 spectrometer operating at 300 MHz for ^{1}H and using a tetradeuterated methanol solution in a 5-mm tube. The solvent residual peak at δ 3.34 was used as internal reference. This spectrum was compared with the ^{1}H NMR spectrum

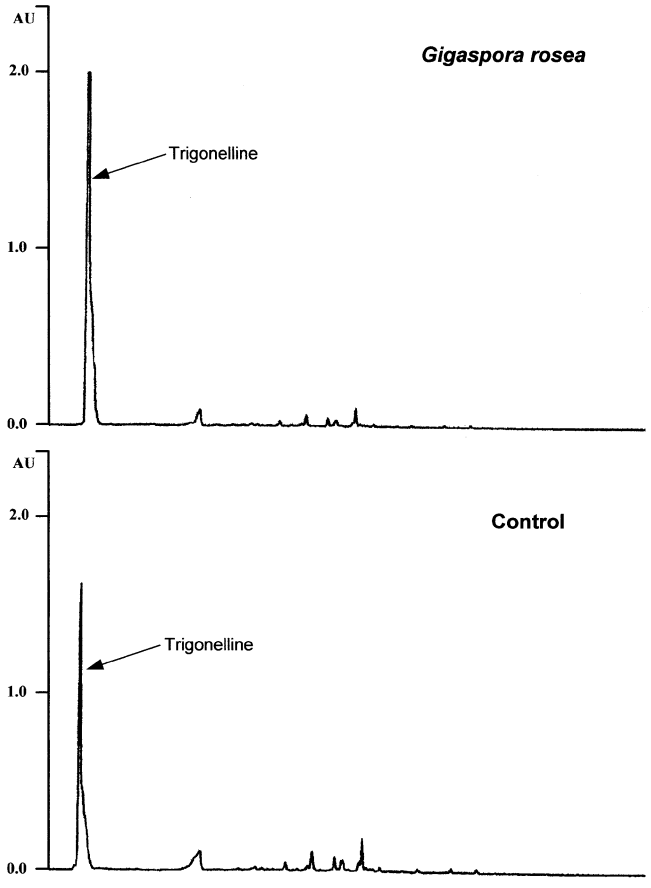

Fig. 1 High performance liquid chromatography (30 min, λ=265 nm) of methanol extracts from roots of *Prosopis laevigata* in contact with *Gigaspora rosea* spores and a control treatment

of an authentic sample of trigonelline. The mass spectra of the trigonelline standard and the trigonelline from the purified methanol extract were registered on a Varian Saturn 2000 spectrometer. Trigonelline concentrations were determined from the high performance liquid chromatography data employing a calibration curve.

Results and discussion

In previous experiments in our laboratory, we established monosporic cultures of *P. laevigata* and pregerminated spores of *Gigaspora rosea* (BEG 9; La Banque Européenne des Glomales; International Institute of Biotechnology, UK) and confirmed their mycorrhizal association (data not shown).

The fungal response was observed 1 week after contact between the roots of *P. laevigata* and the *Gigaspora* spores and was characterized by stimulated hyphal growth and an intense hyphal branching with the formation of many clusters of short and curled hyphae. HPLC of the methanol extract of the roots in contact with *Gigaspora rosea* spores showed a significant 1.8-fold increase (P<0.05) in trigonelline concentration relative to the control treatment (Fig. 1, Table 1). In contrast, there was no change in trigonelline concentration in the aerial parts

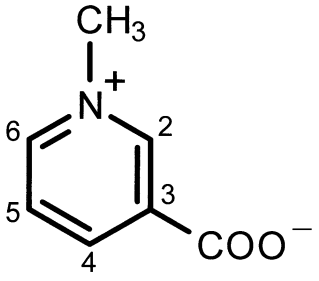

Fig. 2 Structure of trigonelline

Table 1 Trigonelline concentration (mg g^{-1} fresh wt.) in roots and aerial parts of *Prosopis laevigata* after 1 week in an in vitro presymbiotic system (*n.s.* not significant according to the Student test at P<0.05)

Presymbiotic system	Trigonelline	
	Root	Aerial part
In contact with spore	2.112	1.500
Control	1.169	1.510
	**	n.s.

**Significantly different according to the Student test at P<0.05

of *P. laevigata* (Table 1). Our system did not allow investigation of trigonelline in root exudates of *P. laevigata*. Trigonelline (1-methylpiridinium 3-carboxylate) was identified by comparison of its retention factor (R_f) in TLC, retention time (R_t) in HPLC, UV spectrum, ^{1}H NMR spectrum and electron impact mass spectrometry (EIMS) data with those of an authentic sample (Fig. 2). The two substances showed identical R_t and R_f values (R_t = 3.33 min, R_f = 0.54) and UV spectrum (λ =265 nm) when processed by HPLC using a photodiode array-UV detector. Similarly, the ^{1}H NMR signals of trigonelline from the purified methanol extract showed a signal pattern identical to that found in the ^{1}H NMR spectrum of the standard [δ 9.25 (1H, br s, H-2), 8.95 (1H, br d, J = 8.0 Hz, H-4), 8.93 (1H, br d, J = 6.0 Hz, H-6), 8.11 (1H, dd, J = 8.0, 6.0 Hz, H-5) and 4.40 (3H, s, Me)]. The mass spectra of the two trigonelline samples were similar [m/z (rel. int.) 153 $[M+O]^+$(5), 152(10), 138$[M+1]^+$(18), 137$[M]^+$(14), 136(15), 124(13), 123(24), 106(63), 105(40), 94(10), 80(100), 79(44), 65(3)].

Trigonelline has been found in a wide range of plant species and is particularly abundant in seeds and roots of leguminous plants (Tramontano et al. 1986), but it has not been reported in *P. laevigata* (Bisby et al. 1994). Trigonelline is present in legume root exudates and induces nodulation gene (*nod*) transcription in *Rhizobium meliloti* by activating the regulatory protein NodD2 (Phillips et al. 1992). Trigonelline is also known to regulate the cell cycle in the roots of legumes (Tramontano et al. 1982). Thus it appears that trigonelline, like other plant secondary metabolites (e.g. phenolic compounds), has multiple functions, both in the plant and associated microorganisms. Goldmann et al. (1991)

suggested that trigonelline accumulation in the legume host only occurs during the first stages of seed germination and plays a role in early plant-microbe interaction. Additionally, the simultaneous presence of trigonelline in the legume host plant and its corresponding catabolic genes in the pSym megaplasmid of *Rhizobium meliloti* suggested that trigonelline is used as nutrient by rhizobia strains (Boivin et al. 1991). This characteristic might be beneficial for rhizosphere survival, increase efficiency of plant infection, and perhaps serve as a signal in the early stages of seedling colonization.

Communication signals in the presymbiotic phase may depend on the presence of multiple compounds in the exudates of the host plant, e.g. flavonoids, terpenoids and alkaloids, or possible synergistic effects between them and/or changes in their concentrations. In the *P. laevigata* – *Gigaspora rosea* in vitro presymbiotic system, changes in the concentration of trigonelline, a pyridine alkaloid, may be an important regulatory mechanism during early signalling events.

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