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Separated components of root exudate and cytosol stimulate different morphologically identifiable types of branching responses by arbuscular mycorrhizal fungi

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ABSTRACT

Two morphologically distinct hyphal branching responses by the AM fungus, *Glomus intraradices*, were stimulated by separated components of carrot root exudate. Complex branching up to the sixth order was induced by compounds most soluble in 35 % methanol, whereas the formation of more lateral branches (second order) was stimulated by compounds most soluble in 70 % methanol. This same 70 % alcohol soluble fraction also stimulated a completely different type of branching pattern in another fungus, *Gigaspora gigantea*. This pattern consisted of a very periodic distribution of dense clusters of hyphal branches that had a very high degree of complexity. In contrast to exudate components, separated cytosolic components of carrot roots did not stimulate any of the observed hyphal branching patterns. Alcohol-soluble fractions actually inhibited hyphal tip growth of *G. gigantea* and induced the formation of ''recovery'' branches that were identical to those induced by an inhibitor found in the exudate of Chard (*Beta vulgaris* ssp. cicla), a non-host plant.

Introduction

The first necessary interaction between an AM fungus and a host root is an increase in hyphal growth/branching by the fungus in response to chemical components found in the host root exudates (Elias & Safir 1987; Garriock *et al.* 1989; Giovannetti *et al.* 1993). A previous experimental system demonstrated that hyphal branching and growth were stimulated, although the fungus was physically separated from the host root by a membrane filter (Giovannetti *et al.* 1993). This result clearly indicated that a branching stimulator or factor (BF) could readily diffuse from the root surface and was confirmed by an *in vitro* system in which partially purified exudate components induced branching of germinated spores of *Glomus rosea*, *G. margarita* (Buee *et al.* 2000), *G. intraradices*, and *Gigaspora gigantea* (Nagahashi & Douds 2000).

A recent report has identified strigolactone (5-deoxystrigol) as a BF that stimulates hyphal branching of *G. margarita* (Akiyama *et al.* 2005). Strigolactone is a type of sesquiterpene, which was found in the exudates of *Lotus japonicus* and some of these compounds were previously identified as seed-germination stimulants for the parasitic weeds *Striga* and *Orobanche* (Bouwmeester *et al.* 2003). Strigolactone is unlikely to be the only BF that stimulates AM fungal growth because a previous report showed that multiple active compounds from a root exudate could be separated by t.l.c. (Nagahashi & Douds Jr

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2000). We report here that separated chemical components from a carrot root exudate can induce morphologically distinct hyphal branching patterns when applied to germinated spores of AM fungi. In addition, a morphologically distinct hyphal tip inhibition/recovery pattern is induced by components of both carrot cytosol and Chard root (non-host) exudate.

Materials and methods

Spore production and germination

Azygospores of *Gigaspora gigantea* were produced in pot cultures in a greenhouse using *Paspalum notatum* as a host. The pot culture soil was air-dried and stored at 4 °C for four months to relieve dormancy of the spores. Spores were isolated by wet sieving and centrifugation (Gerdemann & Nicolson 1963; Jenkins 1964), sterilized as described by Bécard & Fortin (1988), and stored at 4 °C until use. The spores were then transferred to Petri dishes containing M medium (Bécard & Fortin 1988) solidified with 0.4 % gellan and placed in an incubator at 32 °C in a 2 % CO_2 atmosphere. After germination, individual spores were transferred in a plug of medium to a Petri dish of fresh M medium and allowed to grow for 3–6 d before testing for hyphal branching stimulators. Spores from *Glomus intraradices* (DAOM 181602) were obtained from a split culture plate technique described previously (St-Arnaud *et al.* 1996).

Intact plants, root cultures and exudates

Transformed (Ri T-DNA) carrot roots (*Daucus carota*) grown in liquid culture were used to produce the exudates as described (Nagahashi & Douds 2000). Roots were grown for two weeks in liquid M medium at 24 °C in a shaking water-bath. The medium was then harvested and either replaced with normal liquid medium with 35 μM phosphorus (+Pi) or M medium without Pi (−Pi) and the roots were allowed to grow an additional week. At this time the exudates were harvested and concentrated in a C18 Sepak cartridge (0.5 g), eluted first with 3 ml of 35 % acetonitrile (v/v) to remove the yellow brownish colour (this was more effective than using 35 % methanol), followed by 3 ml 70 % acetonitrile and finally 3 ml 100 % acetonitrile. These fractions contained the compounds of interest and were dried under a stream of nitrogen and stored frozen until used. The frozen dried samples were dissolved in 1 ml of the appropriate concentration of methanol, rather than acetonitrile, which could not be used in the bioassays. The original percent acetonitrile fractions were replaced by the same percent in alcohol. Therefore, the fractions will be identified as the 35 % alcohol soluble fraction (ASF), 70 % ASF, and 100 % ASF.

Chard (*Beta vulgaris* ssp. *cicla*), a non-host plant, was grown in a greenhouse. Twenty-five rainbow chard seeds were planted in 4″ pots filled with vermiculite. The pots were watered daily and given full-strength Hoagland's solution once a week (Hoagland & Arnon 1938). Plants were allowed to grow for three weeks, removed from the pots, and roots were rinsed in water to remove the vermiculite. The roots of the intact plants were placed in a beaker containing 80 ml water and allowed to sit for 3 h at room temperature to collect the exudate. The exudate was concentrated on a C18 cartridge and fractionated exactly like the *in vitro* carrot root exudate described above.

Root cytosolic components

Cultured carrot roots were washed three times with cold, deionized distilled water to remove soluble compounds (remaining exudate) from the apoplast or cell wall freespace. Roots (8 g fresh weight) were then ground with a mortar and pestle in 0.1 M Tris–HCl, Trizma Base at pH 7.5 with 5 mM dithiothreitol at 4 °C. The homogenization ratio of grinding medium (ml) to fresh weight of roots (g) was 10:1. The homogenate was strained through cheesecloth and centrifuged at 80000 ***g*** for 45 min at 4 °C to pellet the organelles and microsomes. The resulting supernatant contained the soluble cytosolic compounds. For plant tissue, the term cytosol is loosely used as it also contains soluble compounds from the vacuole. The cytosolic fraction was diluted to 250 ml with distilled water, passed through a C18 cartridge, and fractionated the same way as the exudate. The dried fractions were dissolved in 1 ml of the appropriate alcohol concentration so the activity in the exudate and cytosol were assayed at the same level relative to the fresh weight of roots.

Bioassays for BFs and inhibitors

When *Gigaspora gigantea* was used as the test organism, the bioassay was performed as described earlier (Nagahashi & Douds 1999) except that 8 μl of sample was injected per well. The assay was usually terminated 3 d after the injections by pouring a Trypan blue stain (0.47 g trypan blue, 250 ml lactic acid, 417 ml glycerol, 250 ml H_2O) over the gel. For *G. intraradices*, nine to 12 spores were inserted into round Petri plates (100 × 15 cm) containing M medium which was solidified with gellan. The spores usually germinated in 4–6 d. When some of the spores germinated, the new hyphal growth from the subtending hypha curled and stayed near the spore and no major hyphae could be readily identified. These spores were not treated as the analysis was impossible to do. Germinated spores with a dominant germ tube hypha growing away from the spore were chosen for treatment. Various ASFs were injected into wells placed near this major hypha and growth was terminated after 10 d as described above. When the hyphal branching patterns of both species were compared, the same sample and same volume (8 μl per well) were used. Quantification was done by counting the number of branches at ×20 with a stereomicroscope (Zeiss STEMI SR) after the hyphae were stained. Overall growth measurements were made by using the grid-line intersect method (Newman 1966).

A Nikon DXM1200 F microscope was used to count the number of branches in the area between the tip and the origin of the main germ tube coming out of the subtending hypha of the spore. Hyphal branches arising from this major hypha were defined as secondary branches (2°), those arising from the 2° were defined as 3°, and so forth. Spore germination and hyphal growth after various treaments were performed in a carbon dioxide (2.5 %) incubator to maximize growth (Bécard & Piché 1989).