Research Note

The Pre-Symbiotic Growth of Arbuscular Mycorrhizal Fungi Is Induced by a Branching Factor Partially Purified from Plant Root Exudates

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Accepted 7 March 2000.

Arbuscular mycorrhizal (AM) symbiosis is an association between obligate biotrophic fungi and more than 80% of land plants. During the pre-symbiotic phase, the host plant releases critical metabolites necessary to trigger fungal growth and root colonization. We describe the isolation of a semipurified fraction from exudates of carrot hairy roots, highly active on germinating spores of *Gigaspora gigantea*, *G. rosea*, and *G. margarita*. This fraction, isolated on the basis of its activity on hyphal branching, contains a root factor (one or several molecules) that stimulates, directly or indirectly, *G. gigantea* nuclear division. We demonstrate the presence of this active factor in root exudates of all mycotrophic plant species tested (eight species) but not in those of nonhost plant species (four species). We negatively tested the hypothesis that it was a flavonoid or a compound synthesized via the flavonoid pathway. We propose that this root factor, yet to be chemically characterized, is a key plant signal for the development of AM fungi.

Arbuscular mycorrhizal (AM) symbiosis is a very ancient plant-microbe association between some Zygomycete (order Glomales) fungi and more than 80% of land plants (Smith and Read 1997). AM fungi contribute to plant health as they improve plant nutrition and, in most instances, plant resistance against pathogen attacks (St.-Arnaud et al. 1995; Gianinazzi-Pearson et al. 1996). In turn, the host plant supplies carbon to the fungal partner, an obligate biotroph incapable of accomplishing its life cycle in the absence of a host plant. In spite of this obligate biotrophism, spores of AM fungi are generally capable of spontaneous germination as long as some physical and physiological conditions are fulfilled. Germ tube growth occurs for 1 to 3 weeks (depending on the species of fungus) but ceases long before consumption of spore reserves if a host root is not present in the environment (Bécard and Piché 1989b). Several authors have described growth stimulation of germinating AM fungi in the presence of host root exudates

(Mosse and Hepper 1975; Graham 1982; Elias and Safir 1987; Bécard and Piché 1989a, 1989b; Giovannetti et al. 1993; Tawaraya et al. 1996), while exudates from nonmycotrophic species had no effect (Gianinazzi-Pearson et al. 1989; Bécard and Piché 1990; Giovannetti et al. 1994; Giovannetti and Sbrana 1998) or appeared to be inhibitory (Koide and Schreiner 1992; Vierheilig and Piché 1995). Therefore, it is conceivable that host plants release critical metabolites needed to trigger a further step in fungal growth and development. This step would be a prerequisite for the fungus to exploit its reserves entirely and to succeed in host root colonization. Signals derived from growing roots of *Ocimum basilicum*, eliciting branching of *Glomus mosseae*, were hypothesized by Giovannetti et al. (1996). The active product(s) were not isolated and their chemical structure is yet to be determined.

We have isolated a semipurified fraction from exudates of hairy roots of *Daucus carota* L. that is highly active on germinating spores of *Gigaspora gigantea* (from an agricultural field in Kutztown, PA). This fraction, isolated on the basis of its activity on hyphal branching (see below), contains a root factor (one or several molecules) that we will call the “branching factor.” Hairy roots of *D. carota* were propagated on a minimal (M) medium (Bécard and Fortin 1988) in petri dishes at 28°C for 3 weeks. They were aseptically transferred to sterile, distilled water (approximately 500 mg of equivalent root dry weight per 100 ml of water) and left to soak for 2 days at 25°C in the dark. They were oxygenated with sterile air with an aquarium pump. Crude root exudates were filtered (0.2 µm) before purification and the filtrate caused hyphal branching when tested directly on germinating spores. The filtrate was fractionated and the fractions to be tested were concentrated to 2 µl per 1 mg of equivalent root dry weight. This concentration served as a reference to compare the activities of the various fractions within one plant species, and the fractions of different species with each other. The first step in the purification of the root exudates involved a phase separation (ethyl acetate/aqueous) of root exudates where all the activity remained in the ethyl acetate. This fraction was dried under vacuum and resuspended in methanol, and an inactive insoluble fraction was removed by centrifugation. The super-

natant was then dried under vacuum, resuspended in ethyl acetate/hexane (1/4), and centrifuged. The activity remained in the supernatant. Essentially, the purification procedure eliminated macromolecules, water-soluble molecules, and the most hydrophilic compounds soluble in the ethyl acetate.

Activity of root exudates was tested according to a bioassay (Fig. 1) developed on *G. gigantea* by Nagahashi and Douds (1999). Three to five surface-sterilized spores of *G. gigantea* were placed inside a gel (20 ml of solidified 0.4% Phytigel, M medium) in 80-mm-square, plastic petri dishes (Bécard and Fortin 1988). By incubating (2% CO₂, 32°C) the dishes vertically, the negative geotropism of *G. gigantea* germ tubes was exploited to direct hyphal growth upward. The germinated spores were allowed to grow 4 to 6 days before the addition of the exudates. Sterile Pasteur pipettes were used to make two small wells (2 mm in diameter) by suction in the gelled medium, one on each side of the tip of the growing germ tubes. Five microliters (exudates produced by 2.5 mg of equivalent root dry weight) of the different root exudate fractions, dissolved in water/methanol (1:1), was injected into each well. Injections of water/methanol (1:1) were used for controls. Only one germinating spore per dish was stimulated. The two to four neighboring germinating spores were subjected only to diffusing fractions. The intensity and the delay of their response (18 h after injection) provided additional information that we used to make a semiquantitative interpretation of fraction activity. The fungal response was observed 18 h after stimulation and characterized by the formation of new hyphal branches along the germ tube. Control hyphae appeared straight with few lateral branches (Fig. 1A) whereas the treated hyphae showed different patterns of hyphal branching, depending on the proximity of branching factor injection. Near the stimulation area (less than 20 mm), hyphal branching was very intense, with the formation of many clusters of short and curled hyphae (Fig. 1B). Beyond 30 mm from the injection zone, the branching pattern was less twisted and curled (Fig. 1C), showing fewer but longer branches. The first branches appeared between 5 and 8 h after the addition of the branching factor. For the experiments of growth quantification and nuclear staining (see below), five injections were made in the gelled medium (four at the corner and one in the middle) just prior to spore inoculation.

Four days after germination in the presence of the branching factor, the total hyphal length of the stimulated fungi was threefold greater than the total hyphal length of controls (Fig. 2). After 10 days of growth, two methods of quantification, including image processing, showed that the treatment caused an approximately 4.5-fold increase in total hyphal length (Fig. 2 and Table 1). This hyphal growth stimulation was characterized by the formation of new primary branches (Table 1), each exhibiting intense branching activity. For example, 5 days after stimulation the mean number ($n = 5$) of hyphal apices per germinating spore was 205 ± 17 ($\pm$ SEM) in the presence of the branching factor and 7 ± 1 ($\pm$ SEM) for the control. In contrast, the elongation of the germ tubes and the number of auxiliary cells were not significantly different between the control and the treatment (Table 1).

To correlate the proliferation of hyphal branches with cell growth stimulation, the nuclear status of *G. gigantea* was examined 5 days after spore germination in the presence or absence of branching factor. Growing hyphae were fixed in the

culture dishes with 70% ethanol for 1 h then rinsed several times with deionized water. The dishes were dried in an oven at 60°C for 3 h to obtain a very thin-layer of gel. Nuclei were stained with 4' 6-diamidino-2-phenylindol (DAPI; Sigma, St. Louis, MO) dissolved in liquid M medium to a final concentration of 5 μ M. After 12 h of staining in the dark at 4°C, samples were rinsed and the number of nuclei were counted under UV light with an inverted fluorescence microscope. For each condition ($\pm$ exudates) the nuclear density was calculated in hyphae of five germinating spores after random sampling of 50 hyphal segments (10 segments of 150 to 200 μ m per germinating spore). Prior to counting the nuclei, microscopic images were acquired with an inverted Leica DMIRBE microscope equipped with epifluorescence illumination (100 W mercury lamp, dichroic mirror 400 nm, excitation 340 to 370 nm, barrier 430 nm) and a color cool view device camera (Photonic Science, Robertsbridge, U.K.). The number of nuclei per millimeter of hyphae remained constant, between 101 ± 6 ($\pm$ SEM) and 113 ± 5 ($\pm$ SEM) in control and treated hyphae, respectively, but the total number of nuclei was threefold higher for the treated fungi. The staining intensity of the nuclei was also consistently stronger in the branching structures and at the hyphal tips. To exclude the possibility that the more numerous nuclei we observed were of spore origin, i.e., existed before the branching factor was added, we removed the spores before adding the branching factor and observed the severed germ tubes after 15 h. They exhibited the same branching response as hyphae still attached to their spores. The number of nuclei per millimeter of hyphal length was the same in control and treated germ tubes: 128 ± 2 ($\pm$ SEM) and 130 ± 6 ($\pm$ SEM), respectively. Therefore, the treated hyphae contained a proportionally larger number of nuclei. Hyphal branching in filamentous fungi is physiologically equivalent to cell proliferation for unicellular organisms like yeast. Every new hyphal tip is like a new life unit where most of the cell machinery is located. When a fungus is under stress, branching is also a common response. Several of our data support the cell proliferation hypothesis rather than the hypothesis of a stress response. New apices, which appeared 5 h after the addition of the root factor, emerged laterally while the tip of the main hypha was still actively growing. The overall hyphal length increased considerably and, more importantly, the number of nuclei increased proportionally. Nuclear division gives a good indication of active growth for a filamentous fungus, particularly when it is coenocytic. It will be important to determine whether the branching factor affects the fungal cell cycle directly, or if the rise in the number of nuclei is simply a consequence of hyphal branching.

Two more species of AM fungi, *Gigaspora margarita* (BEG34, purchased from Biorhize, Dijon, France) and *Gigaspora rosea* (DAOM 194757, maintained in pot culture with leek) were treated with the branching factor purified from the *D. carota* hairy roots as previously described for *G. gigantea*. The two *Gigaspora* species responded by strong hyphal branching in the same way as *G. gigantea*. The growth stimulation of *G. margarita* was particularly intense, although this fungus showed a slightly delayed response (24 h). Moreover, *G. margarita* seemed to exhibit enhanced longevity.

We also tested whether the branching factor present in exudates of carrot hairy roots was present in root exudates of a wide variety of plant species. Twelve species that belong to

Fig. 1. In vitro experimental system showing stimulation of growth and branching in a *Gigaspora gigantea* germ tube after microinjection (5 μ l per well) of root exudates, and microscopy images showing different growth and branching patterns of *G. gigantea* in the presence of branching factor. **A**, Nontreated hypha. **B**, Treated hypha: strong branching with an important number of arborescent structures (arrows). **C**, Fungal branching observed in a distant area of the branching factor injection. Scale bars = 200 μ m.

six different families were selected (transformed roots or seedlings; Table 2). As previously described with *D. carota*, clones of Ri T-DNA-transformed roots of *Medicago sativa*, *Medicago truncatula*, *Solanum esculentum*, and *Beta vulgaris* were propagated on an M medium (Bécard and Fortin 1988) in petri dishes at 28°C. Axenic roots of various plant species were also obtained from seedlings (excised, nontransformed roots). Seeds were surface sterilized by immersion in chloramine T (2%) for 2 to 20 min, depending on the plant species, and were washed with sterile water and sterile solution containing antibiotics (100 mg · l⁻¹ gentamicin and 200 mg · l⁻¹ streptomycin). Transformed and nontransformed roots were used after 3 weeks of growth to produce root exudates. Exudates from undifferentiated carrot cells were also tested. Carrot cell cultures (*D. carota* subsp. *sativus*) were maintained in Murashige and Skoog medium with 0.4 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D) as described by Widholm (1977). The standard exudation and purification protocols developed for the carrot hairy root exudates were applied to all exudates except for those of carrot cells, which were purified directly from the culture medium after 10 days of growth. Less than 24 h after the stimulation, we observed strong branching of *G. gigantea* with the purified exudates of all host plant species (transformed roots or not) including those of the Myc⁻¹ and Myc⁻² pea mutants. However, exudates from non-

host roots (*Brassicaceae* and *Chenopodiaceae*) were inactive. Dilution experiments with carrot hairy root exudates showed that the nonhost root exudates contain, if any, less than 1/5,000 the amount of branching factor present in host-root exudates. Our purification procedure allowed high concentrations of exudate fractions to be tested without concentrating a number of potentially inhibitory molecules. This result reinforces previous studies suggesting that nonmycotrophic plants lack stimulatory compounds normally found in host plants (Glenn et al. 1988; Bécard and Piché 1990; Giovannetti et al. 1993, 1994; Giovannetti and Sbrana 1998). In contrast to the nonmycotrophic plants tested, the nonmycorrhizal pea mutants affected in the early (Myc⁻¹) and late (Myc⁻²) steps of mycorrhizal formation (Duc et al. 1989; Gianinazzi-Pearson 1996) seemed to produce the branching factor as well as did the wild type. This result was expected, as the fungus forms numerous appressoria, i.e., does not seem to be affected during its pre-symbiotic growth even with the early mutant (Myc⁻¹) (Duc et al. 1989; Gianinazzi-Pearson 1996). In contrast to exudates from carrot root cultures and to previous reports (Carr et al. 1985; Paula and Siqueira 1990), exudates prepared from carrot cell cultures, crude or purified, had no effect on *G. gigantea*.

The fact that hyphal branching was stimulated by exudates from plant roots from all the mycotrophic plant species tested (eight species in six families), with no exceptions, suggests that this branching factor is produced by a much larger spectrum of plant species and perhaps by all plants capable of living in symbiosis with AM fungi. The absence of growth and branching stimulation with the exudates from cell culture also suggests that the branching factor is specifically synthesized by root organs whether they are transformed or not. In the root vicinity of *Ocimum basilicum* and other host plants, Giovannetti et al. (1993, 1996) also described a strong branching activity of *Glomus mosseae*. Interestingly, Giovannetti et al.

Fig. 2. Total hyphal length per germinating spores of *Gigaspora gigantea* in the (●) presence and (□) absence of the branching factor. Vertical lines correspond to the standard errors of the means ($n = 7$).

Table 1. Effect of the branching factor, purified from exudates of *Daucus carota* hairy roots, on hyphal growth of germinating spores of *Gigaspora gigantea* after 10 days of culture

Growth variables	Control	Treatment
Total hyphal length (mm) ^a	318 (± 12) ^b	1,361 (± 190) ^c
Sampled hyphal length (mm) ^d	3.1 (± 0.2)	14.2 (± 1.5) ^c
Total germ tube length (mm)	75.3 (± 1.9)	74.3 (± 2.4)
No. of primary hyphal branches	18 (± 2.8)	39 (± 2.8) ^c
No. of auxiliary cells	7 (± 1.5)	8.9 (± 1.2)

^a Mean of total hyphal length per germinating spore ($n = 7$), obtained by manual measurement.

^b Numbers in parentheses are standard errors of the means.

^c Values between the two treatments are significantly different (Student's t test, $P < 0.01$).

^d Mean length, obtained by image processing, of 31 hyphal samples made from petri dishes containing seven germinating spores.

Table 2. Branching activity of purified exudates, obtained from root or cell cultures of various plant species, on hyphae of germinating spores of *Gigaspora gigantea*

Family	Species	Activity
Plant root exudates (seedlings)		
Ombelliferae	<i>Daucus carota</i>	+
Solanaceae	<i>Nicotiana tabacum</i>	+
Gramineae	<i>Zea mays</i>	+
Gramineae	<i>Sorghum bicolor</i>	+
Leguminosae	<i>Pisum sativum</i>	+
Leguminosae	<i>Pisum sativum</i> Myc ⁻¹ ^a	+
Leguminosae	<i>Pisum sativum</i> Myc ⁻² ^a	+
Brassicaceae	<i>Arabidopsis thaliana</i>	0
Brassicaceae	<i>Brassica napus</i>	0
Brassicaceae	<i>Brassica oleracea</i>	0
Transformed root exudates		
Ombelliferae	<i>Daucus carota</i>	+
Leguminosae	<i>Medicago sativa</i>	+
Leguminosae	<i>Medicago truncatula</i>	+
Solanaceae	<i>Solanum esculentum</i>	+
Chenopodiaceae	<i>Beta vulgaris</i>	0
Cell culture exudates		
Ombelliferae	<i>Daucus carota</i>	0

^a Seeds of pea mutants: Myc⁻¹ (appressoria formation, but no penetration of the root; Duc et al. 1989) and Myc⁻² (appressoria formation and internal growth of the fungus, but no arbuscules or vesicles; Gianinazzi-Pearson 1996) were kindly provided by Vivienne Gianinazzi-Pearson (INRA/CNRS, Dijon, France).

(1993) reported that *G. mosseae* hyphae were chemoattracted to the root. We did not observe such chemotropism with *G. gigantea* hyphae either in the presence of a growing root or in the presence of the branching factor. These differences were observed with two different fungi. They can also be explained by the two experimental systems used.

Since a number of authors have shown that some flavonoids stimulate the growth of AM fungi (Gianinazzi-Pearson et al. 1989, Tsai and Phillips 1991; Bécard et al. 1992; Chabot et al. 1992; Fries et al. 1997; Vierheilig et al. 1998), these plant chemicals have often been presented as fundamental signals in plant-fungus communication during the pre-infection stages of endomycorrhizal symbiosis (Hirsch and Kapulnik 1998; Vierheilig et al. 1998). The well-established importance of flavonoids as signals in other plant-microbe interactions (Long 1989; Shirley 1996), the observation that these metabolites stimulate growth of AM fungi in vitro, and the fact that plant roots contain flavonoids are not sufficient arguments that naturally occurring flavonoids are necessary plant signals in endomycorrhizal symbiosis. Significant amounts of quercetin, myricetin, and kaempferol, flavonoids with strong stimulatory activity on some AM fungi, have been detected in nonmycorrhizal plants such as *Arabidopsis thaliana* (Burbulis et al. 1996). The only study that attempted to demonstrate this natural occurrence had to conclude that flavonoids were not necessary plant signals for AM symbiosis (Bécard et al. 1995). Maize mutants deficient in the biosynthesis of flavonoids were colonized by several AM fungi, as well as the wild type. These data suggested that other plant molecules, yet to be discovered, are responsible for the fungal growth stimulation and could be the plant signals needed to trigger a developmental stage in AM fungi that precedes successful root colonization. In the present study, we used the same chalcone synthase-deficient mutants of maize to determine whether the branching factor was synthesized via the flavonoid pathway. We found no difference in the capacity of mutant and wild-type maize to produce stimulatory exudates, strongly suggesting that the branching factor does not include flavonoids.

By taking advantage of the sensitivity and the strength of the fungal branching bioassay, a series of chemicals known to have biological activity in plant-microbe interactions were directly tested on *G. gigantea* (Table 3). For each chemical, we tested different concentrations based on the literature and we assayed the fungal response 18 to 96 h after injection into the medium near the hyphal tip. None of the chemicals caused fungal branching nor did they have any inhibitory effect, ex-

cept 50 μ M salicylic acid, which had an inhibitory effect on hyphal growth. This suggests that these chemicals are not natural hyphal branching factors or that the concentrations tested were not able to trigger a biological response. The chemical identification of the branching factor will determine whether it corresponds to already described plant metabolites or not.

Growth stimulation and hyphal branching of AM fungi in the vicinity of a host root have long been described as one of the first events in host root recognition by the fungus during the pre-symbiotic phase (Mosse and Hepper 1975; Powell 1976; Bécard and Piché 1989a; Giovannetti et al. 1993, 1994, 1996). A number of studies have attempted to characterize the exuded root compound(s) responsible for stimulation of growth and branching (Nair et al. 1991; Bel Rhlid et al. 1993) but none have tried to isolate the active molecules from crude material. For the first time, we isolated a semipurified root extract (branching factor) that can mimic the presence of a host root by triggering a typical pre-symbiotic fungal response within a few hours. At this stage of our knowledge, we propose that plant root exudates contain some lipophilic compound(s) that we call branching factor, yet to be chemically characterized, with an activity (direct or indirect) on cell proliferation of AM fungi. We hypothesize that this branching factor is a plant signal needed to trigger the developmental stage of the fungus that precedes successful root colonization.

ACKNOWLEDGMENTS

We thank Natacha Bourgarel for her technical assistance and Valeria Bianciotto and Sonja Kosuta for critical reading of the manuscript. We also thank L. Taylor and V. Gianinazzi-Pearson for providing seeds of CHS⁻ maize and Myc⁻ pea, respectively. This research was supported by the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie (MENRT), and by the company Premier Tech (Rivière-du-loup, Québec, Canada).

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Table 3. Chemicals^a with no branching activity on hyphae of germinating spores of *Gigaspora gigantea*

Compound	Concentration	References
Abietic acid	1, 10, and 100 μ M	Fries et al. 1987
Brassinolide	1, 10, and 100 nM	Yokota 1997
17 β -estradiol	10 and 100 nM	Poulin et al. 1997
Jasmonic acid	5 and 10 μ M	Regvar et al. 1996
Nod factors ^b	10 nM, 100 nM, and 1 μ M	Xie et al. 1995
Quercetin	1, 10, and 100 μ M	Bécard et al. 1992
Salicylic acid	100 nM and 50 μ M	Rhodes 1994

^a Phenolic compounds were obtained from Sigma Chemical (St. Louis, MO). Nod factors were kindly provided by D. Barker (UMR 215 CNRS/INRA, Castanet-Tolosan, France) and brassinolide was obtained from CIDtech (Mississauga, Ontario, Canada).

^b Sulfated or not.

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