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In Vitro Enhancement of Spore Germination and Early Hyphal Growth of a Vesicular-Arbuscular Mycorrhizal Fungus by Host Root Exudates and Plant Flavonoids

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Abstract

We have tested the hypothesis that root-exuded plant signals may contributed to early symbiont interactions in VAM and that flavonoids, essential to initial events in nodule symbiosis, may be active components. Root exudates of clover, a vesicular-arbuscular mycorrhizal (VAM) host plant, increased *in vitro* spore germination and hyphal growth of *Gigaspora margarita* whilst those of a non-host legume, lupin, had no effect. Two flavonones and a flavone, which activate *nod* gene expression in *Rhizobium*, induced rapid responses in fungal development from spores at concentrations as low as 0.15 to 1.5 μM. It is suggested that the presence of such molecules in root exudates may promote cell to cell contact between VAM symbionts during early pre-infection phases.

Keywords: root exudates, clover, lupin, VAM, *Gigaspora margarita*, flavonoids

1. Introduction

Establishment of the root symbiosis between vesicular-arbuscular mycorrhizal (VAM) fungi and their host plants involves a sequence of events which lead to complex interactions between the two symbionts (Bonfante-Fasolo, 1984; Gianinazzi-Pearson and Gianinazzi, 1988). During the pre-infection

phase when fungal hyphae develop out from propagules, directional growth towards a host root can occur (Powell, 1976; Koske, 1982). Furthermore, hyphal growth is considerably stimulated in the presence of living host roots before appressorium formation and infection have occurred (Hepper, 1984; Mosse, 1988). It has been shown that exudates and volatiles from growing roots promote hyphal growth from spores of *Gigaspora* species (Gemma and Koske, 1988; Bécard and Piché, 1989a,b), although energy sources from the spores are essential for continued mycelial extension. These observations raise the question of the role of root exudates in the infection process in VAM and of their eventual involvement in signalling phenomena prior to contact between the two symbionts.

The recent discovery that nod$^-$ (non nodulating) and myc$^-$ (inability to form VAM) characters in pea mutants are probably controlled by the same or closely linked monogenic recessive determinants (Duc et al., 1989, J.P. Guillaumin unpublished data) suggests that common plant functions may control certain early steps in the infection processes in the two symbiosis. In the root symbiosis between rhizobia and legumes, expression of bacterial *nod* genes essential to initial stages in the nodulation process is markedly enhanced by root exudates (Innes et al., 1985; Mulligan et al., 1985). Several active components in root exudates have been identified as early plant signals and these appear to be flavonoids or isoflavonoids (Firmin et al., 1986; Redmond et al., 1986; Kosslak et al., 1987; Rolfe, 1988; Zaat et al., 1988). Since such compounds occur widely in nature (Wollenweber and Dietz, 1981), we have studied the possibility that they may also be messenger molecules in early symbiont interactions in VAM. In the present paper we compare the influence of root exudates of a host plant, a non host plant and of three flavonoids (two flavanones and one flavone) on early events in the pre-infection phases of spore germination and hyphal growth of *Gigaspora margarita in vitro*.

2. Materials and Methods

Spores of *Gigaspora margarita* (Becker and Hall) (strain LPA2), isolated by wet-sieving soil from heavily infected *Allium porrum* cultures, were surface sterilized 15 mn in 5% chloramine T plus 0.04% streptomycin containing a few drops of Tween 80. After rinsing in sterile water, they were transferred to Petri dishes containing 10 ml test media (nine spores per dish, 10 replicates per treatment) and incubated at 25°C in the dark. The aim of the present investigation being to detect early modifications in responses of *G. margarita*, percentage of germinating spores, extent of hyphal growth and number of

vesicle clusters formed (Pons and Gianinazzi-Pearson, 1985) were assessed after 5 to 9 days incubation. However, in order to see whether effects were long term, parameters were also measured after 23 days incubation in one experiment (with naringenin). Hyphal length was estimated using a grid intersect method in which the number of intersections hyphae made with a microscope eyepiece grid was counted. The number of intersections was converted to hyphal length (mm) after calibration of the grid using curly wool fiber of a similar diameter to the hyphae.

The effect of root exudates of clover (*Trifolium pratense* L.), a VAM host plant, and lupin (*Lupinus albus* L.), a non mycorrhizal plant species, on spore germination and hyphal growth were tested. Clover and lupin seeds were surface sterilized 9 and 20 mn, respectively, in 7% calcium hypochlorite solution, rinsed in sterile water and germinated 3 days in the dark at 25°C on 0.75% water Difco bacto-agar (8 seeds per Petri dish). Dishes were then transferred to a growth chamber and seedlings grown 7 days in the light (250 μE $m^{-2}s^{-1}$, 16 h day, 25°C), after which they were carefully removed from the agar. Control dishes without seedlings, containing 0.75% water agar only, were treated in the same way. Surface sterilized spores of *G. margarita* were placed where seedlings had developed.

Three commercially available flavonoids, known to induce *nod* gene expression in *Rhizobium* (Firmin et al., 1986; Zaat et al., 1987), were tested for their influence on the *in vitro* behaviour of *G. margarita* : naringenin (4',5,7-trihydroxyflavanone), hesperitin (3',5, 7-trihydroxy-4'-methoxyflavanone) and apigenin (4',5, 7-trihydroxyflavone) obtained from Sigma Chemical Co. Flavonoid solutions were prepared in 50% ethanol and prediluted prior to their use such that the final ethanol concentration never exceeded 0.05% (this concentration had no effect on fungal development in prior tests). They were sterilized using a 0.2 μm Millipore filter and added to autoclaved liquid 0.75% water agar to give concentrations ranging from 15 nM to 15 μM. Flavonoids were omitted in control dishes. The possibility of a chemotaxic effect of the flavonoids was examined by introducing different concentrations into wells cut in the solidified water agar medium and placing surface sterilized spores at a distance of 2 mm from one side of each well.

All tests were carried out at least twice and results were statistically analyzed by ANOVA and Duncan's test. Results for replicate tests were

very similar and therefore only data of one are presented for each test.

3. Results

Influence of root exudates of host and non-host plants

Results are presented in Table 1. Root exudates of the non-host plant, lupin, did not affect spore germination or hyphal growth of *G. margarita* as compared to that on water agar. On the contrary, a rapid increase in spore germination was already observed in the presence of root exudates of seedlings of the VAM host plant clover after 5 days incubation, and a significant effect on hyphal growth was evident.

No vesicle clusters were formed in any treatment at 9 days but observations from a duplicate test showed that after 18 days significantly more had developed in the presence of clover root exudates (15.0 ± 1.0) then with either water agar (4.7 ± 0.5) or lupin root exudates (6.7 ± 1.7). Vesicle number was found to be correlated with hyphal length in later experiments (see below). Other aspects of hyphal morphology were similar in the different treatments.

Effect of apigenin, naringenin and hesperitin

Hyphae developing from spores germinating close to wells containing any of the three flavonoids grew in all directions and showed no preferential growth towards the source of the compounds.

Table 1. Influence of root exudates of clover and lupin on spore germination and hypha development of *G. margarita*

Root exudates	Days	Germinated spores (%)	Hyphal length (mm) per germinated spore	Number of vesicle clusters per germinated spore
0	5	22.0 a	0.8 a	0 a
	9	27.0 a	1.7 a	0 a
lupin	5	16.1 a	1.1 a	0 a
	9	28.6 a	2.0 ab	0 a
clover	5	60.1 b	2.9 bc	0 a
	9	62.0 b	6.5 d	0 a

Values in each column followed by the same letter do not differ significantly ($P = 0.05$; Duncan's test).

Naringenin (Fig. 1) did not have a pronounced early effect on *G. margarita* development. Although the presence of 1.5 μM of this flavanone consistently

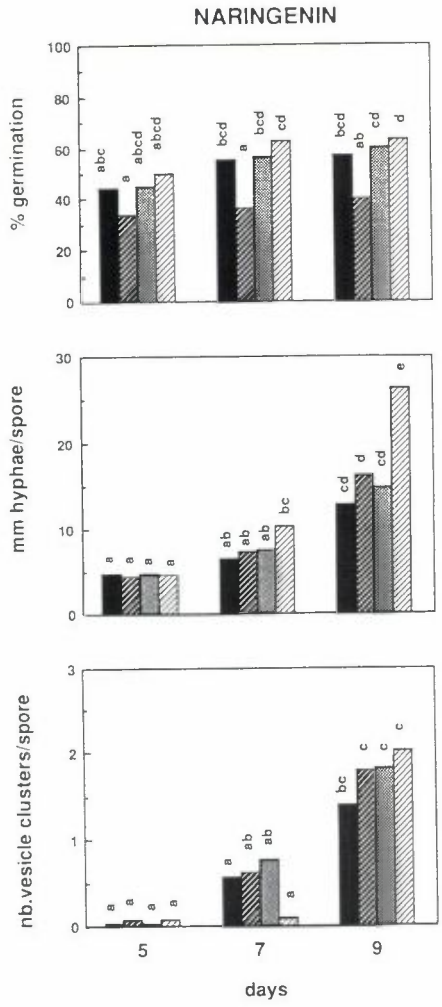

Figure 1. Effect on spore germination, hypha and vesicle cluster development per spore, of naringenin concentrations of 0 (■), 15 nM (▨), 150 nM (▩) and 1.5 μM (▨). Columns with the same letter(s) do not differ significantly (P = 0.05; Duncan's test).

improved spore germination as compared to that on water agar (+11.2 to +13.4%), differences were not significant up to 9 days (Fig. 1). This concentration of naringenin began to enhance hyphal growth from spores after 7 days incubation. At 9 days, hyphal length was twice that obtained on water

agar and significantly greater than that in the presence of lower amounts of the flavanone. The number of vesicle clusters developing on hyphae increased with time but there was no significant effect of the different naringenin concentrations on their production. In another experiment, spore germination and hyphal growth were compared on water agar and 1.5 μM naringenin after 23 days incubation in order to see whether these flavonoid effects persisted. As shown in Fig. 2, percentage spore germination was no longer different,

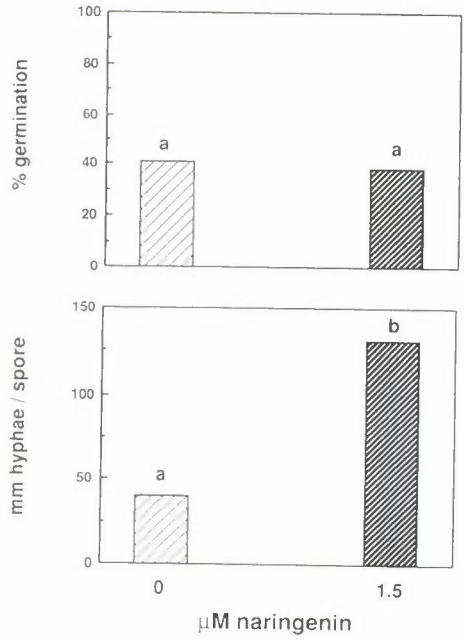

Figure 2. Percentage spore germination and hyphal growth per spore after 23 days in the absence (0) and presence (1.5 μM) of naringenin. Columns with the same letter do not differ significantly (P = 0.001; Duncan's test).

whilst hyphal growth continued to be significantly increased in the presence of the flavanone.

Apigenin (Fig. 3) had a more rapid effect on spore germination and hyphal growth of *G. margarita* than naringenin with both parameters showing significant increases after 5 days incubation in the presence of 150 nM of the flavone. This concentration remained the most efficient in promoting fungal development and stimulated highest vesicle cluster formation. Although 15 nM of apigenin also enhanced hyphal elongation and vesicle cluster development as compared to that occurring on water agar, this concentration was less active than that of 150 nM. Higher concentrations of the flavone were

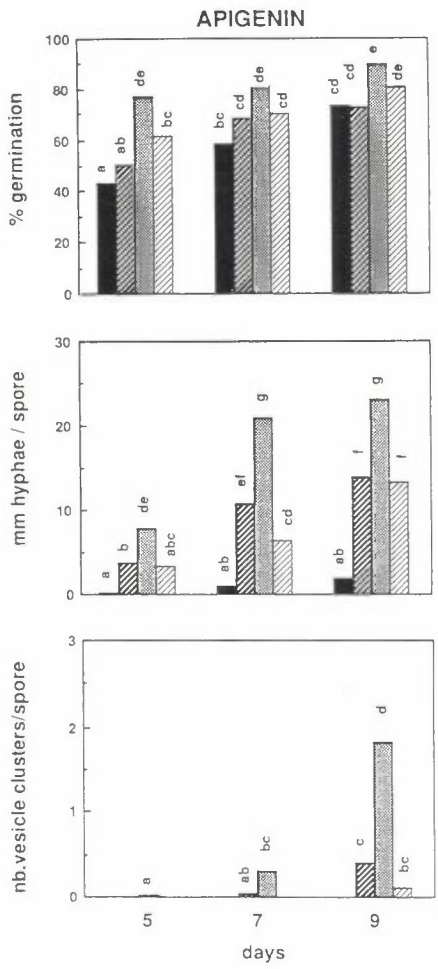

Figure 3. Effect on spore germination, hypha and vesicle cluster development per spore, of apigenin concentrations of 0 (■), 15 nM (▨), 150 nM (▩) and 1.5 μM (▧). Columns with the same letter(s) do not differ significantly (P = 0.05; Duncan's test).

antagonistic to its promoting effect on fungal development and at 1.5 μM the positive influence on hyphal growth and vesicle cluster formation significantly decreased.

The flavanone hesperitin was also much more active than naringenin in increasing spore germination, hyphal growth and vesicle cluster formation of *G. margarita* (Experiment 1, Table 2). Early promoting effects were observed after 5 days incubation in the presence of 150 nM and 1.5 μM, when hyphal

growth was particularly improved. Significant increases in spore germination in the presence of hesperitin disappeared at 9 days, but these two concentrations of the flavanone continued to significantly enhance hyphal growth as compared to 15 nM or water agar. Vesicle clusters were more abundant in the presence of 150 nM and 1.5 μM hesperitin. In a second series of experiments, raising the concentration of hesperitin to 15 μM increased spore germination but significantly reduced the promoting effect of the flavanone on hyphal growth and vesicle cluster formation (Experiment 2, Table 2).

Exposure to the flavonoids not only accelerated spore germination and hyphal growth but also reduced variations in fungal behaviour between the different spore populations isolated from pot cultures for each replicate experiment. This is illustrated by the results presented in Table 2. Whilst considerably less hyphal growth occurred on water agar in experiment 1 than experiment 2, values obtained in the presence of 1.5 μM hesperitin were very similar. The promoter effect of the three flavonoids was not additive and when the spores were exposed 9 days to a mixture of each compound at its

Table 2. Effect of varying concentrations of hesperitin on spore germination and mycelium development of *G. margarita*

		Germinated spores (%)		Hyphal length (mm) per germinated spore		Number of vesicle clusters per germinated spore	
Experiment: Days		1	2	1	2	1	2
5	0	43.4a	22.7a	0.8a	3.5a	0.0a	0.0a
	15.0 nM	51.1ab		4.9abc		0.0a	
	150.0 nM	57.7abc		11.33cd		0.0a	
	1.5 μM	74.5cde	43.4cd	9.8bcd	11.0abc	0.39ab	0.1a
	15.0 μM		53.7cde		9.4ab		0.0a
7	0	58.8abc	39.9bcd	0.9a	9.9ab	0.0a	0.2a
	15.0 nM	64.5bcd		16.1cd		0.4ab	
	150.0 nM	79.8dc		24.7f		0.6b	
	1.5 μM	78.9dc	51.1cd	27.7fg	27.2f	1.5c	1.5b
	15.0 μM		66.9f		19.2de		0.4a
9	0	79.5abc	47.5cd	4.8ab	17.7cde	0.0e	1.1b
	15.0 nM	78.6dc		23.5ef		1.0c	
	150.0 nM	79.9dc		33.1gh		2.6d	
	1.5 μM	91.3c	56.3cd	38.4h	40.5g	2.6d	3.5c
	15.0 μM		75.7f		24.1ef		1.5b

Values in each column followed by the same letter do not differ significantly (P = 0.05; Duncan's test).

optimal concentration, no significant effect on spore germination (24.5%) or hyphal growth (16.1 mm) was observed as compared to that occurring on water agar alone (27.3% and 11.0 mm respectively).

None of the flavonoids significantly affected the number of germ tubes produced by each spore of *G. margarita*, nor did they induce changes in the growth pattern or morphology of hyphae. In all the tests, a significant correlation ($P = 0.01$) was found between hyphal length and number of vesicle clusters formed.

4. Discussion

The positive effect of clover root exudates on *in vitro* spore germination and hyphal growth of *G. margarita* confirms previous reports that host roots produce factors which enhance VAM fungal growth prior to infection (Graham, 1982; Hepper, 1984; Gemma and Koske, 1988; Mosse, 1988; Bécard and Piché, 1989a,b). The fact that the root exudates from lupin, which does not form mycorrhiza with any fungi, neither inhibited nor promoted development of *G. margarita* suggests that VAM host plants produce promoter molecules which influence fungal behaviour during early pre-infection phases prior to symbiont contact. These observations also support the hypothesis that roots of non mycorrhizal species do not produce fungal growth inhibitors but rather lack a diffusable growth stimulus for VAM fungi so that, unlike with host plants, hyphal proliferation is not elicited in their rhizosphere (Glenn et al., 1988). Since initial steps of fungal colonization can occur sparsely in such plants (Tommerup, 1984), inhibition of VAM fungal development in their roots must occur after initial contact events (Ocampo et al., 1980). Stem grafting experiments between host and non-host legumes indicated that this may be, at least partly, under control of a shoot produced factor (Gianinazzi-Pearson and Gianinazzi, 1989; Gianinazzi and Gianinazzi-Pearson, 1990).

Hyphae developing from spores of *Gigaspora* species respond positively to volatile compounds produced by growing roots (Gemma and Koske, 1988; Bécard and Piché, 1989b), and it has been suggested that carbon dioxide is one of the compounds critical for enhancement of VAM fungal growth during pre-infection phases. The present observations that exudates from host roots, but not those from non-host roots, can promote early responses in hyphal growth indicates that other plant factors must also be active. Flavonoids, which are widely distributed in higher plants and activate *nod* gene expression in *Rhizobium*, appear to be potential candidates contributing to these effects. All three flavonoids that were tested in the present work enhanced hyphal growth from *G. margarita* spores and two of them (hesperitin and

apigenin) significantly increased the rate at which spores germinated. This property has been used to break dormancy in VAM fungal spores of *Gigaspora* species otherwise failing to germinate *in vitro* (Bartissol, Gianinazzi-Pearson and Gianinazzi, unpublished results). The promoter effects on hyphal growth varied, both in terms of rapidity and amplitude, between the compounds. Naringenin had the slowest effect, although maximum hyphal length after 9 days exposure was comparable to that in the presence of apigenin where fungal growth was stimulated early but slowed down subsequently. The most active promoter molecule was hesperitin which rapidly increased hyphal growth as spores germinated and continued to have a stimulating effect at 9 days. The three molecules were active at low concentrations (0.15 to 1.5 μM) comparable to those reported to activate the induction of *nod* genes in *Rhizobium* (Firmin et al., 1986; Zaat et al., 1988). The physiological basis of the promoting effects of the flavonoids on *G. margarita* development is not known; they do not appear to be chemotaxic attractants for this fungus.

The magnitude and rapidity of the promoting effects of hesperitin and apigenin on hyphal growth of *G. margarita* (maximum 8 to 10 fold increase after 9 days) can be compared to the approximately 7 fold increases in hyphal growth observed by Bécard and Piché (Fig. 2, 1989a; Fig. 3, 1989b) after spores had been incubated for a similar period (9 to 10 days) in the presence of growing roots or root exudates plus volatiles. However, overall hyphal growth (maximum 40.5 mm in 9 days and 130.6 mm in 23 days) was less under the present experimental conditions than that obtained by the previous authors (approximately 150 mm in 9 to 10 days and 350 mm after 20 days; Figs. 3 and 5, in Bécard and Piché, 1988b). Since root volatiles, and in particular CO_2, may be an essential carbon source for germinating spores (Bécard and Piché, 1989b), their effect on the responses to flavonoid promoters of VAM fungal development is presently being investigated.

The flavonoid compounds which activate VAM fungal development *in vitro* are derived from the phenylpropanoid pathways (Hahlbrock and Scheel, 1989), which are also responsible for the synthesis of isoflavonoids, including phytoalexins. Colonization of soybean roots by VAM fungi considerably increases the production of glyceollin and related isoflavonoids (Morandi and Gianinazzi-Pearson, 1986; Morandi, 1989). A general activation of this biosynthetic pathway may lead to a more important synthesis of other phenylpropanoid compounds like promoter flavanones or flavones, and so in fact favour fungal-plant interactions. This may, furthermore, be a contributing factor to the greater rate of nodulation observed in VAM-infected legumes (Barea and Azcon-Aguilar, 1983). Isoflavonoids have been reported to be potent antagonists of *nod* gene inducers and it has been proposed that the ratio

of stimulator to inhibitor molecules in the rhizosphere or root tissues may be a key factor for nodule initiation (Firmin et al., 1986; Rolfe, 1988). It is tempting to speculate that such a phenomenon may also exist in interactions between VAM symbionts, and contribute to variations in the rates of hyphal development and contact between symbionts during the initial phases of the infection process with different host and non-host plants. Investigations are actually underway to test the influence of the isoflavonoids glyceollin, coumestrol and daidzein on spore germination and hyphal growth of VAM fungi.

In conclusion, plant exuded flavonoids may act as signals which induce rapid VAM fungal development from propagules as an infectible host root grows close to them. The sensitivity of *G. margarita* to small amounts of these plant molecules could reflect the situation in the rhizosphere where such plant factors would favour cell to cell contact essential to the initiation of the infection process between the VAM symbionts. The present observations together with those reported for *Rhizobium nod* gene activation by flavonoid molecules suggest a widespread role for these biofactors in the biology of microbe-plant interactions.

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