Flavonoids Are Not Necessary Plant Signal Compounds in Arbuscular Mycorrhizal Symbioses

Guillaume Bécard,[1] Loverine P. Taylor,[2] David D. Douds, Jr.,[1] Philip E. Pfeffer,[1] and Landis W. Doner[1]

[1]Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, PA 19118, U.S.A.; and [2]Horticulture Dept. and Program in Plant Physiology, Washington State University, Pullman, WA 99164 U.S.A.

30 December 1993. Accepted 1 November 1994.

Some common flavonoids have been shown to greatly stimulate growth of arbuscular mycorrhizal (AM) fungi. Although exudates of Ri T-DNA transformed roots of carrot similarly stimulate growth of AM fungi, HPLC (retention time), UV spectral and ^{1}H NMR analyses of tissue detected no flavonoids but rather various derivatives of caffeic acid. A pollen germination rescue bioassay also detected no flavonoids in root extracts. The addition of polyvinypolypyrrolidone to the culture medium to sequester putative exuded flavonoids did not prevent successful mycorrhizal establishment. Maize plants deficient in chalcone synthase activity, necessary for the biosynthesis of flavonoids, were equally colonized with AM fungi as maize with chalcone synthase. These results suggest that root metabolites in addition to flavonoids may stimulate AM fungal growth and that flavonoids are not necessary for the establishment of mycorrhizal symbiosis.

Additional keywords: chalcone synthase, *Gigaspora margarita*, polyvinylpolypyrrolidone, quercetin.

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs that must establish symbioses, presumably including formation of arbuscules, to obtain a supply of carbon and energy from the host plant. A number of colonization steps, partially under the control of the plant, must be achieved as the fungus progresses from the germination of the spore to the formation of arbuscules (Gianinazzi-Pearson and Gianinazzi 1989; Koide and Schreiner 1992). One very early interaction between the partners involves root exudates that stimulate hyphal growth after spore germination (Azcon and Ocampo 1984; Elias and Safir 1987; Bécard and Piché 1989b). Plants that do not form mycorrhizal associations seem not to exude these stimulatory compounds (Glenn *et al.* 1989; Gianinazzi-Pearson *et al.* 1989; Bécard and Piché 1990).

Plant flavonoids are an important class of signaling molecules during legume nodulation. They can act as inducers of nodulation genes, chemoattractants, and growth stimulants for *Rhizobium* (Phillips 1992). The facts that some Nod$^-$ pea mutants are Myc$^-$ (Duc *et al.* 1989) and that some mycorrhizins immunologically cross-react with nodulins (Wyss *et al.* 1990) suggest that both plant-microbe associations share common mechanisms of molecular interactions.

This assumption has motivated several empirical investigations on the effect of various flavonoids on growth of AM fungi (Gianinazzi-Pearson *et al.* 1989; Bécard *et al.* 1992; Chabot *et al.* 1992; Kape *et al.* 1992). For example, the most recent studies have shown that some flavonol aglycones can considerably stimulate hyphal growth of *Gigaspora margarita* (Bécard *et al.* 1992; Chabot *et al.* 1992). A similar stimulation of growth of this fungus had been obtained in the presence of root exudates (Bécard and Piché 1989b) produced by a clone of Ri T-DNA transformed roots of carrot (*Daucus carrota* L.) used as host plant tissue to grow and study arbuscular mycorrhizal fungi *in vitro* (Bécard and Piché 1992). Two flavonols known to stimulate the growth of *G. margarita*, quercetin and kaempferol, were reported to be found in extracts and exudates of Ri T-DNA transformed roots and seedlings of carrot (Bel-Rhlid *et al.* 1993; Poulin *et al.* 1993). Nair *et al.* (1991) found the isoflavonoids formononetin and biochanin A in roots of clover (*Trifolium repens*) and showed these compounds stimulated the growth of a *Glomus* sp. (Siqueira *et al.* 1991). Harrison and Dixon (1993) found formononetin malonyl glucoside and medicarpin malonyl glucoside in roots of *Medicago truncatula*.

In this study, we address the question of the role and occurrence of flavonoids as regulatory plant metabolites in AM associations in nature. Our data show that the absence of flavonoids does not affect mycorrhizal establishment. They suggest that other plant metabolites, yet to be investigated, stimulate AM fungus growth. The study involves HPLC, NMR, and mass spectral analyses of root extracts and a pollen germination rescue bioassay to detect flavonoids; the use of polyvinylpolypyrrolidone (PVPP) to sequester the putative exuded root flavonoids (Doner *et al.* 1993); and the use of maize mutants deprived of chalcone synthase activity, the enzyme that catalyzes the first step in flavonoid production.

Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others not mentioned.

Current address of G. Becard: Centre de Biologie et Physiologie Vegetales, Univ Paul Sabatier; 118, route de Narbonne; 31062 Toulouse Cedex, France.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1995.

RESULTS

Chemical analyses of root phenolics.

The chromatograms are shown at 365 nm (Fig. 1), the optimum wavelength to observe most flavonols. A standard mixture of taxifolin, rutin, quercitrin, fisetin, quercetin, luteolin, kaemperol, and chrysin were easily resolved at 7.1, 10.4, 13.4, 15.3, 18.9, 20.3, 23.5, and 31.4 min, respectively (Fig. 1A), using the methanol/acetic acid solvent program. Ten major peaks were resolved from the extract of the transformed carrot roots (Fig. 1B). The first nine peaks of the chromatogram exhibit very similar UV spectra, such as that in Figure 2A, suggesting they share common chemical structures. These UV spectra do not correspond to those of flavonols such as kaempferol, quercetin, and myricetin (Mabry *et al.* 1970), all three biologically active on mycorrhizal fungi. Rather, they match the UV spectrum of phenylpropanoids such as caffeic acid (Fig. 2C). Peak 10 exhibits a different UV spectrum in addition to being more hydrophobic (Figs. 1B and 2B). Acid hydrolysis of root extracts yielded compounds with neither the retention times nor the UV spectra of common flavonols (Mabry *et al.* 1970). The hydrolyzed compounds eluted earlier and with different peak heights (i.e., different extinction coefficients) than unhydrolyzed samples (Fig. 1C). This was confirmed when both H_2SO_4 and TFA hydrolysis of the compound isolated by preparative chromatography as peak 5 (in Fig. 1B) yielded a compound with less peak area. It eluted in 5 min, one half the retention time (RT) of peak 5 (data not shown). Both its RT and UV spectrum matched those of caffeic acid (data not shown). The RT and UV spectrum of hydrolyzed luteolin-7-*O*-glucoside yielded the expected later eluting luteolin, showing the acid hydrolysis method used was effective for releasing the aglycone. HPLC profiles of extracts of transformed carrot root tissue

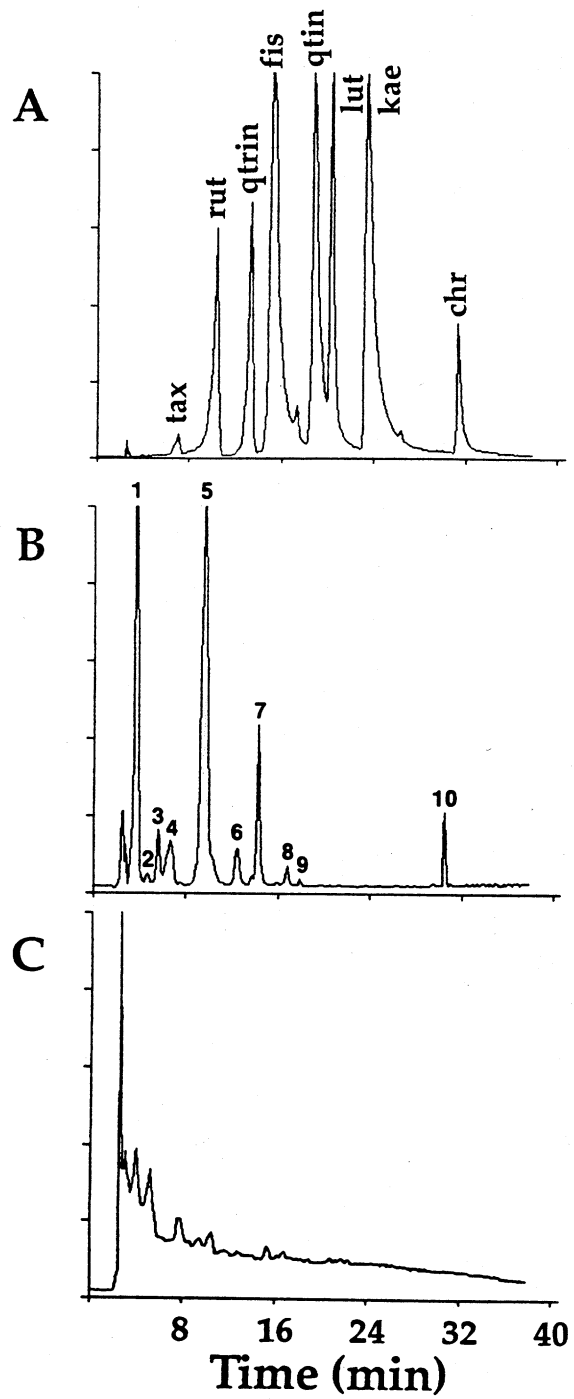

Fig. 1. High-performance liquid chromatograms of: **A,** standard mixture of taxifolin (tax), rutin (rut), quercitrin (qtrin), fisetin (fis), quercetin (qtin), luteolin (lut), kaempferol (kae), and chrysin (chr), respectively, at 219, 100, 149, 233, 197, 116, 116, and 131 μM; **B,** extract of Ri T-DNA transformed roots of carrot; and **C,** acid hydrolyzed extract of the Ri T-DNA transformed roots of carrot.

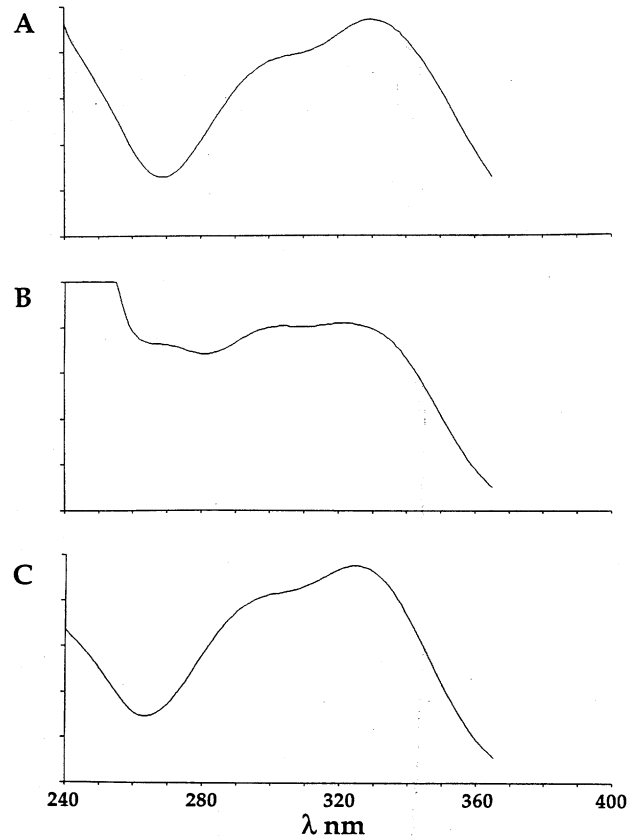

Fig. 2. UV spectrum of: **A,** peak 5 of the chromatogram of Figure 1A. Peaks 1 to 9 exhibited an almost identical UV spectrum. **B,** Peak 10 of the chromatogram of Figure 1A. **C.** caffeic acid.

grown in low P media and roots colonized by *G. margarita* were identical to those shown in Figure 1B and characteristic of phenylpropanoids. Profiles of root exudates were the same as for extracts, but peak heights indicated lower concentrations. The predominance of phenylpropanoids and the absence of flavonoids in extracts and exudates was verified by using an additional solvent program based on an acetonitrile/pH 3.0 water gradient (Graham 1991).

Further structural analyses of the root phenolics that shared the same UV spectra were made by NMR and mass spectrometry. These analyses were performed on peak 5 as it represented the major peak in the root extracts. The 400 MHz ^1H spectrum of preparative HPLC purified peak 5 showed two doublets centered at 7.47 δ and 7.45 δ and two doublets at 6.26 δ and 6.12 δ, each with a coupling constant of 15.9 Hz characteristic of *trans* olefinic protons adjacent to an aromatic ring. The ratio of the two sets of *trans* olefinic protons was 1:1. In addition two sets of three aromatic protons were observed at 7.02 δ (s), 6.87 δ (d) J = 8.1 Hz, 6.76 δ (d) J = 8.1 Hz, and 6.96 δ (s), 6.75 δ (d) J = 8.1 Hz, 6.65 δ (d) J = 8.1 Hz. The connectivity of these two separate sets of shifts were established with a 2D homonuclear COSY spectrum. The chemical shifts for both sets of resonances were in good agreement with those reported for the two caffeoyl ring protons of 1,6 di-*O*-caffeoylquinic acid (Merfort 1992). Additional shifts in the upfield region of the spectrum could not be identified. No evidence of OCH_3 substitution was observed at 3.6 δ.

The molecular mass of peak 5 was determined by positive ion FAB mass spectrometry to be 518 [m + 1 = 519] (15%) with additional seen ions at 497 (17%), 207 (63%), and 185 (100%).

Table 1. Failure of carrot root extracts and hydrolyzed extracts to restore germination of CMF pollen of petunia

Treatment	Germination[a] (%)
CMF pollen control	0%
CMF pollen + extract[b]	0%
CMF pollen + extract + 0.5 μM kaempferol	53%
CMF pollen + hydrolyzed extract[b]	0%
CMF pollen + Hyd. Extr. + 0.5 μM kaempferol	38%
CMF pollen + 0.5 μM kaempferol	85%

[a] Means of three replicates.
[b] See Methods for procedure.

Table 2. Growth of *Gigaspora margarita* after 5 days in the presence of quercetin (10 μM) and/or Polyvinylpolypyrrolidone (PVPP, 1 g/L)

	Hyphal length (mm)	Number of clusters of auxiliary cells
Control	37 (6.6)[a]	2.8 (0.4)
+ PVPP	39 (9.7)	3.3 (1.0)
Quercetin	108 (23.0)	8.7 (2.2)
Quercetin + PVPP	32 (2.5)	2.5 (1.4)

[a] Numbers in brackets are standard deviations of the means.

Pollen rescue bioassay.

Experiments with PVPP and pollen germination bioassays were conducted in the event flavonoids were present in amounts undetectable by HPLC. Hydrolyzed and nonhydrolyzed extracts of Ri T-DNA transformed carrot roots failed to restore germination of pollen of petunia mutants deficient in flavonoids and hence, unable to germinate (Table 1). Addition of the flavonol kaempferol restored germination in the presence of the extracts, but not to the same levels as kaempferol alone. This may be due to inhibitory effects of phenolics present in the extracts.

Effect of PVPP on growth of *G. margarita*.

Flavonoids such as quercetin stimulate the growth of AM fungi (Bécard *et al* 1992; Chabot *et al* 1992). It has also been shown that polyvinylpolypyrrolidone effectively binds flavonoids such as quercetin. At a mass ratio of 35:1 [PVPP:quercetin], 86% of quercetin was bound in 60 min (Doner *et al* 1993). Quercetin and/or PVPP were added to petri plates of media (10 μM quercetin and 1g/L PVPP, mass ratio 320 :1 [PVPP: quercetin]) with a pregerminated spore of the AM fungus *G. margarita*. Quercetin alone stimulated the growth of the fungus threefold, but in the presence of added PVPP, growth was equivalent to controls (Table 2). The insoluble PVPP particles—originally white—turned yellowish, the color of the quercetin solution.

Polyvinylpolypyrrolidone was also added to the media at the time of establishment of dual cultures of *G. margarita* and Ri T-DNA transformed roots of carrot. Under these culture conditions compounds are exuded which stimulate AM fungus hyphal growth (Bécard and Piché 1989b). Should these compounds be flavonoids and only flavonoids, the development of the symbiosis, as characterized by eventual sporulation by the fungus, should be affected by the addition of

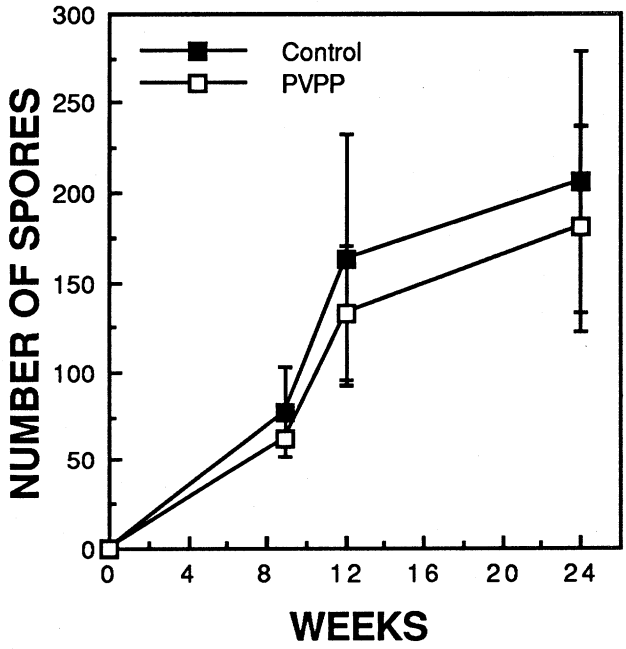

Fig. 3. Number of spores of *Gigaspora margarita* per dish of root-host culture (transformed roots of carrot) produced during 24 wk in the presence (PVPP) or the absence (Control) of 1 g/L of polyvinylpolypyrrolidone. Vertical lines indicate standard errors of the means.

PVPP. The presence of PVPP in the dual cultures did not significantly affect the production of spores (Fig. 3). During the course of the experiment, the PVPP particles turned slightly brownish.

Mycorrhizal colonization of CHS⁺ and CHS⁻ maize plants. The colonization of the roots of maize by *G. margarita* and *G. etunicatum* was low, indicating that the measurement was made in an early stage (7 wk) of root-fungal interaction (Table 3). The heavier inoculation (400 spores/pot) of the plants with *G. mosseae* led to a greater value of colonization. In any case, mycorrhizal colonizations were not significantly different between CHS⁻ and CHS⁺ maize plants. No differences in morphology of the fungal infection were noted for CHS⁻ *vs* CHS⁺ mycorrhizas.

CONCLUSION

Transformed roots of carrot have served as a model root-host for several years to study *in vitro* interactions between host roots and AM fungi, including the mechanisms of stimulation of growth of the AM fungus *G. margarita*. In particular, these roots exude compounds that stimulate the growth of this fungus (Bécard and Piché 1989a, 1989b). Since growth of the same fungus was stimulated and prolonged in a similar manner by some very common flavonoids (Bécard *et al.* 1992; Chabot *et al.* 1992), it was hypothesized that these were the stimulatory compounds exuded by the roots. Flavonol aglycones such as quercetin, myricetin, and kaempferol were the expected candidates since they were the most stimulatory and almost ubiquitous in the plant kingdom. Despite trying several methods of extraction, including that of Bel-Rhlid *et al* (1993), the flavonoid analyses of the carrot roots, based on HPLC retention time (Fig. 1), UV spectral and ^{1}H NMR analyses (Fig. 2), were negative. Extracts of transformed roots of *Medicago sativa* did yield several flavonoids in an independent study using the same analytical protocols (data not shown). Acid hydrolysis, performed to liberate aglycones from their sugar moiety, also did not reveal the presence of glycosides in the root extracts. An *in vitro* bioassay based on the ability of flavonol aglycones to restore the germination of *Petunia* pollen deficient in CHS activity (and as a result, all flavonoids) (Mo *et al.* 1992) confirmed the absence of flavonols in our carrot root extracts (Table 1). This assay is more than five times more sensitive than HPLC (UV detection) analysis. Therefore, we are confident no flavonoids were present and, since mycorrhizas developed normally *in vitro* (Fig. 3) and in corn plants (Table 3) in the absence of flavonoids, we conclude that flavonoids are not necessary plant signals for AM symbiosis.

Since the two sets of characteristic *trans* olefinic protons and two independent sets of three aromatic protons per aromatic ring (established by a 2D ^{1}H COSY experiment), each corresponding to shifts for caffeic acid, were observed in the proton NMR spectrum, we conclude that peak 5 is a dicaffeoyl substituted derivative. The absence of an OCH_3 resonance at 3.64 δ confirmed that this compound is a caffeic rather than a ferulic acid derivative. This conclusion was verified by the results of the positive-ion FAB mass spectrum which was consistent with a structure with not more than two caffeoyl groups per molecule (m + 1 ion = 519).

The results of our analyses of carrot root extracts are different than those of Poulin *et al.* (1993) and Bel-Rhlid *et al.* (1993), who reported to have found quercetin and kaempferol in these roots. Although we did not detect flavonoids in our roots, possibly because they do not accumulate, the hypothesis that they were exuded and active in a very low concentration in the medium was tested with the use of PVPP. The experimental conditions were optimum for potential PVPP binding of flavonoids since the dual culture medium was 100% aqueous at pH 6.0 after autoclaving (Doner *et al.* 1993). Furthermore, the concentration of PVPP was 9 mM (f. wt. of the PVPP monomer unit = 111.1), i.e., in large excess, assuming that the putative flavonoids were at low concentration. We are confident that PVPP should have intercepted very effectively any flavonoids present in the culture medium especially those with a hydroxyl group at the 4′- and 7-position for which the binding is the best (Doner *et al.* 1993), a structural feature common to quercetin, myricetin, and kaempferol. Quercetin (10 μM) was efficiently intercepted by PVPP, leading to the suppression of its stimulatory effect on growth of *G. margarita* (Table 2). The presence of PVPP did not affect the production of spores in the root-host/*G. margarita* dual culture system (Fig. 3). The production of spores is the ultimate sign, from the fungal viewpoint, of a successful mycorrhizal establishment. Sporulation is highly correlated with degree of root colonization in this association (Diop *et al.* 1992).

These results strongly suggest that the stimulatory compounds exuded by the transformed roots of carrot (Bécard and Piché 1989b) are not flavonoids. Other root metabolites, yet to be investigated, must stimulate growth of AM fungi. These results also raise the more general question regarding the role of flavonoids in the mycorrhizal association. Bel-Rhlid *et al.* (1993) proposed that flavonoids are fundamental regulating factors during the early events of AM symbiosis establishment. The stimulatory effect of some flavonoids on the growth of AM fungi shown *in vitro* and a reported positive analysis of their presence in the root are two independent findings, however, and not sufficient to prove *in vivo* significance of flavonoids in AM symbioses. The *in vivo* experiment showing that, at an early stage of plant-microbe interaction in which flavonoids are expected to play a role, maize plants deprived of the ability to produce flavonoids were equally susceptible to mycorrhizal infection as "wild-type" plants reinforces our view that these metabolites are not essential for the establishment of this plant-microbe association. However,

Table 3. Root colonization (% root length) of wild type maize (CHS⁺) and of mutants deprived of chalcone synthase activity (CHS⁻), inoculated for 6 wk with three different species of arbuscular mycorrhizal fungi

	Plant type	
Fungal species	**CHS⁺**	**CHS⁻**
G. margarita	7.8 (4.7)[a]	7.5 (4.2)
G. mosseae	46.5[b] (5.5)	39.6[b] (6.3)
G. etunicatum	4.8 (5.9)	6.3 (3.9)

[a] Numbers in brackets are standard deviations of the means.
[b] Values between the two treatments are not significantly different (Student's *t* test, $P > 0.10$).

the *in vitro* stimulatory effect of flavonol aglycones on growth of AM fungi remains. It is conceivable that this stimulation of growth occurs occasionally in the rhizosphere when the proper flavonoids are present or released by a plant in proximity. Seeds of alfalfa, for example, release flavonoids that can promote spore germination of *Glomus* (Tsai and Phillips 1991).

Interestingly, the same flavonols that actively promoted the growth of AM fungi, i.e. kaempferol, quercetin, and myricetin (Bécard *et al.* 1992; Chabot *et al.* 1992) are essential for germination of pollen and growth of the germ tube in petunia, maize, and tobacco (Mo *et al.* 1992; Ylstra *et al.* 1992). It is possible that these compounds have some regulatory effect upon cell elongation in general. We conclude that they are not a part of a specific plant-fungus relationship in mycorrhizae.

MATERIALS AND METHODS

Root organ culture.

A clone of Ri T-DNA transformed root of carrot was propagated at 28° C on minimal (M) medium (Bécard and Fortin 1988) in petri dishes. The gelling agent used was gellan gum (0.2%, w/v, Gel-Gro, ICN Biochemicals, Cleveland, OH).

Azygospores of *G. margarita* Becker & Hall (DAOM 194757) were produced in greenhouse pot culture as already described (Bécard *et al.* 1992). They were collected, purified, and sterilized as described by Bécard and Piché (1992). Spores were germinated on M medium gelled with 0.4% gellan gum in an incubator at 2% CO_2 (Bécard *et al.* 1992).

Quercetin/PVPP bioassay.

The effect of quercetin (10 µM, Sigma Chemical Co., St. Louis, MO), the flavonoid previously found to exhibit the greatest stimulation of hyphal growth of germinating spores of *G. margarita* (Bécard *et al.* 1992; Chabot *et al.* 1992), was bioassayed on growth of *G. margarita* in the presence and absence of polyvinypolypyrrolidone (PVPP 1 g/L). PVPP is water insoluble but extremely hydrophilic and it binds very efficiently to phenolic compounds, including a wide variety of flavonoids (Doner *et al.* 1993). Purified PVPP (Doner *et al.* 1993) was added to M medium before autoclaving. Quercetin was filter sterilized and added to the M medium after autoclaving to a concentration of 10 µM (Bécard *et al.* 1992). Germinated spores were axenically transferred within a core of medium to experimental square petri dishes (9 × 9 cm). Petri dishes were incubated vertically in a 2% CO_2 incubator at 32° C (Bécard *et al.* 1992). Nine experimental dishes were used per treatment with one spore per dish. Hyphal length (in millimeter) was measured after 5 days of incubation by using a 2-mm grid while observing the bottom half of the petri dish through a dissecting microscope. The number of clusters of auxiliary cells (vesicles produced on coiled hyphae) was also determined.

Effect of PVPP on mycorrhizal development.

Dual cultures of transformed roots of carrot and *G. margarita* were designed as described in Diop *et al.* 1992. Three germinated spores per square dish (9 × 9 cm) were used to inoculate one root explant in the presence and the absence of PVPP (1 g/L). Seven dishes were used per treatment. The dual cultures were maintained for 24 wk after which the production of daughter spores was assessed through a dissecting microscope.

Pollen rescue bioassay.

Pollen from conditional male fertile (CMF) *Petunia* do not germinate when self pollinated. Germination of pollen and pollen tube production is restored when pollen are placed on a wild-type pistil or in the presence of flavonoids (Mo *et al.* 1992). A bioassay was conducted to determine if extracts of Ri T-DNA transformed carrot roots contained flavonoids and could restore CMF pollen germination. Powdered roots were extracted in methanol (2 mg/600 µl). A 200-µl aliquot was dried and redissolved in 10 µl DMSO. A second 200-µl aliquot was acid hydrolyzed (200 µl 4*N* HCl, 95° C, 1 hr), neutralized, extracted with ethyl acetate, evaporated to dryness, and dissolved in 10 µl of DMSO. One microliter of either of these solutions was added to CMF *Petunia* pollen. In addition, certain assay volumes were made to 0.5 µM kaempferol to provide the necessary controls. Three replicate extracts/assays were conducted.

***In vivo* colonization of maize roots.**

Chalcone synthase efficient (CHS^+) plants of maize (*Zea mays* L.) and chalcone synthase-deficient mutants (CHS^-) were compared for their susceptibility to mycorrhizal infection. The CHS^- mutants (white kernels) are characterized by stable recessive mutations at the two chalcone synthase genes, *c2c2/whpwhp* (Coe *et al.* 1981). The CHS^+ strain (purple kernels) differs from the mutants by a single dominant allele at chalcone synthase, *C2c2/whpwhp*. This leaves the mutant unable to produce flavonoids, thereby inhibiting growth of pollen germ tubes (Mo *et al.* 1992). HPLC analysis of acid hydrolyzed extracts of CHS^+ and CHS^- root tissue yielded a flavonol in the wild type but no flavonoids in roots of the mutant (data not shown). One surface sterilized (5 min H_2O, 20 min 5% sodium hypochlorite, H_2O rinsings) seed of maize was introduced into a conic plastic pot (21 by 4 cm; Super Cell C-10, Stuewe & Sons, Corvallis, OR) containing a potting mixture inoculated with either *G. margarita*, *Glomus mosseae* (Nicol. & Gerd) Gerdemann & Trappe [INVAM 156], or *Glomus etunicatum* Becker & Gerdemann #8961 (from Native Plant Industries, Salt Lake City, UT) with 45, 400, and 180 spores, respectively, to test whether the lack of flavonoid production may inhibit colonization of roots by AM fungi in the soil. The potting mixture was a 1:1 (v/v) mixture of sand and calcined clay ("Turface," Applied Industrial Materials Corp., Deerfield, IL) except for the treatment with *G. margarita* where it was a 0.75:1:1:0.75 (v/v/v/v) mixture of field soil/sand/vermiculite and calcined clay. Four to six plants were grown per fungal inoculation × maize genotype combination. Plants were grown in a greenhouse under natural photoperiods beginning 23 April 1993 and fertilized once a week with half strength Hoagland nutrient solution (Hoagland and Arnon 1938) containing P/10 or no P for plants inoculated with the *Glomus* species or with *G. margarita*, respectively. After 7 wk of culture, root colonization (percentage root length) was estimated for the three fungi by using the gridline-intersect method (Newman 1966).

HPLC analyses of root phenolics.

Root phenolics were extracted as described by Graham (1991). Typically, 100 mg of fresh root tissue was ground

thoroughly in 80% ethanol and immediately centrifuged at 18,000 *g* for 3 min prior to injection. Occasionally, extractions were carried out from freeze-dried root tissue, with 100% methanol or ethanol and for longer periods (several days). No noticeable difference was found between the various methods. Acid hydrolyses of the root extracts were performed with trifluoroacetic acid (TFA, 2*N*) for 3 hr at 100° C. The TFA was evaporated under N_2 and the extract resuspended in methanol, sonicated for a few seconds, and centrifuged (18,000 *g*) prior to injection. Analytical HPLC separations were carried out on a Spectra-Physics (San Jose, CA) system equipped with a scanning Spectra-Focus detector, a Rainin (Woburn, MA) C18 reverse-phase packing analytical column (4.6 × 250 mm) and a 20-L loop injector. The phenolics were separated at low pH (constant acetic acid concentration, 4%) by linear gradient elution for 30 min of 30–70% methanol and 8 min of isocratic elution of 70% methanol, at 1.0 ml/min. Preparative HPLC separations were carried out by applying the above solvent program and using a Rainin Dynamax-60A 8 μm (10 × 250 mm) column at a flow of 4.0 ml/min. Collected peaks were evaporated overnight under N_2 in preparation for NMR and Mass Spectral analyses. Root extracts and exudates were also chromatographed on the C18 analytical column using a linear gradient of 0–55% acetonitrile in pH 3.0 water (adjusted with H_3PO_4) for 25 min, followed by a step increase to 100% acetonitrile, which was held for 2 min before a step return to pH 3 water (Graham 1991). Standard flavonoids were purchased either from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) except for luteolin (ICN Biomedicals, Cleveland, OH). Caffeic acid was purchased from Sigma.

NMR and mass spectral analyses.

^{1}H NMR was performed on a JEOL GX-400 NMR spectrometer. Each sample was dissolved in CD_3OD in a 5-mm tube. All shifts were referenced to CD_3OD at 3.3 δ. Spectra were obtained with a 60-degree pulse, a repetition rate of 1 sec and a spectral width of 5,000 Hz. The 2D Homonuclear COSY spectrum was obtained with a 256 × 512 matrix zero filled to 512. Each spectrum required 164 scans and a repetition rate of 1.16 sec with 1,024 data points. The spectra were transformed with a sine bell apodization (3.54 Hz broadening factor) and symmetrized.

Fast atom bombardment (FAB) analysis was carried out on a VGZAB-2SE high field mass spectrometer (VG Analytical, Manchester, UK) operating at 8 KeV. A cesium ion gun was used to generate ions for the acquired mass spectra which were recorded using a PDP 11-250J data system. Mass calibration was performed using cesium iodide.

LITERATURE CITED

Azcon, R., and Ocampo, J. A. 1984. Effect of root exudation on VA mycorrhizal infection at early stage of plant growth. Plant Soil 82:133-138.

Bécard, G., and Fortin, J. A. 1988. Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol. 108:211-218.

Bécard, G., and Piché, Y. 1989a. New aspects on the acquisition of biotrophic status by a VAM fungus, *Gigaspora margarita*. New Phytol. 112:77-83.

Bécard, G., and Piché, Y. 1989b. Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl. Environ. Microbiol. 55:2320-2325.

Bécard, G., and Piché, Y. 1990. Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and non-host Ri T-DNA transformed roots. Can. J. Bot. 68:1260-1264.

Bécard, G., and Piché, Y. 1992. Establishment of VA mycorrhizae in root organ culture: Review and proposed methodology. Pages 89-108 in: Methods in Microbiology. Vol. 24. J. R. Norris, D. J. Read, and A. K. Varma eds. Academic Press, UK.

Bécard, G., Douds, D. D., and Pfeffer, P. E. 1992. Extensive hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. Appl. Environ. Microbiol. 58:821-825.

Bel-Rhlid, R., Chabot, S., Piché, Y., and Chênevert, R. 1993. Isolation and identification of flavonoids from Ri T-DNA transformed roots (*Daucus carota*) and their significance in vesicular-arbuscular mycorrhiza. Phytochemistry 33:1369-1371.

Chabot, S., Bel-Rhlid, R., Chênevert, R., and Piché, Y. 1992. Hyphal growth promotion *in vitro* of the VA mycorrhizal fungus, *Gigaspora margarita* Becker & Hall, by the activity of structurally specific flavonoid compounds under CO_2-enriched conditions. New Phytol. 122:461-467.

Coe, E. H., McCormick, S. M., and Modena, S. A. 1981. White pollen in maize. J. Hered. 72:318-320.

Diop, T. A., Bécard, G., and Piché, Y. 1992. Long-term *in vitro* culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed roots of carrot. Symbiosis 12:249-259.

Doner, L. W., Bécard, G., and Irwin, P. 1993. Binding of flavonoids by polyvinylpolypyrrolidone. J. Agric. Food Chem. 41:753-757.

Duc, G., Trouvelot, A., Gianinazzi-Pearson, V., and Gianinazzi, S. 1989. First report on non-mycorrhizal plant mutants (Myc$^-$) obtained in pea (*Pisum sativum* L.) and fafabean (*Vicia faba* L.). Plant Sci. 60:215-222.

Elias, K. S., and Safir, G. R. 1987. Hyphal elongation of *Glomus fasciculatum* in response to root exudates. Appl. Environ. Microbiol. 53:1928-1933.

Gianinazzi-Pearson, V., and Gianinazzi, S. 1989. Cellular and genetical aspect of interactions between host and fungal symbionts in mycorrhizae. Genome 31:336-341.

Gianinazzi-Pearson, V., Branzanti, B., and Gianinazzi, S. 1989. *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243-255.

Glenn, M. G., Chew, F. S., and Williams, P. H. 1988. Influence of glucosinolate content of *Brassica* (Cruciferae) roots on growth of vesicular-arbuscular mycorrhizal fungi. New Phytol. 110: 217-225.

Graham, T. L. 1991. A rapid, high resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites. Plant Physiol. 95:584-593.

Harrison, M. J., and Dixon, R. A. 1993. Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular-arbuscular mycorrhizal associations in roots of *Medicago truncatula*. Mol. Plant-Microbe Interact. 6:643-654.

Hoagland, D. R., and Arnon, D. I. 1938. The water-culture method for growing plants without soil. Agriculture Experimental Station circular 347. University of California College of Agriculture, Berkeley.

Kape, R., Wex, K., Parniske, M., Görge, E., Wetzel, A., and Werner, D. 1992. Legume root metabolites and VA-mycorrhiza development. J. Plant Physiol. 141:54-60.

Koide, R. T., and Schreiner, R. P. 1992. Regulation of the vesicular-arbuscular mycorrhizal symbiosis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:557-81.

Mabry, T. J., Markham, K. R., and Thomas, M. B. 1970. The systematic identification of flavonoids. Springer Verlag, Berlin.

Merfort, I. 1992. Caffeoylquinic acids from flowers of *Arnica montana* and *Arnica chamissonis*. Phytochem.istry 31:2111-2113.

Mo, Y., Nagel, C., and Taylor, L. P. 1992. Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc. Natl. Acad. Sci. USA 89:7213-7217.

Nair, M. G., Safir, G. R., and Siqueira, J. O. 1991. Isolation and identification of vesicular-arbuscular mycorrhiza-stimulatory compounds from clover (*Trifolium repens*) roots. Appl. Environ. Microbiol. 57:434-439.

Newman, E. I. 1966. A method of estimating total length of root in a sample. J. Appl. Ecol. 3:139-145.

Phillips, D. A. 1992. Flavonoids: Plant signals to soil microbes. Pages 201-231 in: Recent Advances in Phytochemistry. Vol. 26. H. A. Staf-

ford and R. K. Ibrahim, eds. Plenum Press, New-York.
Poulin, M. J., Bel-Rhlid, R., Piché, Y., and Chênevert, R. 1993. Flavonoids released by carrot (*Daucus carota*) seedlings stimulate hyphal development of vesicular-arbuscular mycorrhizal fungi in the presence of optimal CO_2 enrichment. J. Chem. Ecol. 19:2317-2327.
Siqueira, J. O., Safir, G. R., and Nair, M. G. 1991. Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavanoid compounds. New Phytol. 118:87-93.
Tsai, S. M., and Phillips, D. A. 1991. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl. Environ. Microbiol. 57:1485-1488.
Wyss, P., Mellor, R. B., and Wiemken, A. 1990. Vesicular-arbuscular mycorrhizas of wild-type soybean and nonnodulating mutants with *Glomus mosseae* contain symbiosis-specific polypeptides (mycorrhizins), immunologically cross-reactive with nodulins. Planta 182: 22-26.
Ylstra, B., Touraev, A., Benito Moreno, R. M., Steger, E., van Tunen, A. J., Vicente, O., Mol, J. N. M., and Heberle-Bors, E. 1992. Flavonols stimulate development, germination, and tube growth of tobacco pollen. Plant Physiol. 100:902-907.