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Flavonoids Released Naturally from Alfalfa Promote Development of Symbiotic *Glomus* Spores In Vitro

SIU M. TSAI† AND DONALD A. PHILLIPS*

Department of Agronomy & Range Science, University of California, Davis, California 95616

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Because flavonoids from legumes induce transcription of nodulation genes in symbiotic rhizobial bacteria, it is reasonable to test whether these compounds alter the development of vesicular-arbuscular mycorrhizal (VAM) fungi that infect those plants. Quercetin-3-*O*-galactoside, the dominant flavonoid released naturally from alfalfa (*Medicago sativa* L.) seeds, promoted spore germination of *Glomus etunicatum* and *Glomus macrocarpum* in vitro. Quercetin produced the maximum increases in spore germination, hyphal elongation, and hyphal branching in *G. etunicatum* at 1 to 2.5 μM concentrations. Two flavonoids exuded from alfalfa roots, 4′,7-dihydroxyflavone and 4′,7-dihydroxyflavanone, also enhanced spore germination of this fungal species. Formononetin, an isoflavone that is released from stressed alfalfa roots, inhibited germination of both *Glomus* species. These in vitro results suggest that plant flavonoids may facilitate or regulate the development of VAM symbioses and offer new hope for developing pure, plant-free cultures of VAM fungi.

Plant phenolics, including flavonoids, are important transcriptional signals to soil bacteria (18). Legumes release various flavonoids that induce nodulation (*nod*) genes in *Rhizobium* and *Bradyrhizobium* bacteria (rhizobia) (12), and examples of one class of flavonoids, chalcones, induce *vir* genes in *Agrobacterium tumefaciens* (21).

Plant factors that affect the development of symbioses with mycorrhizal fungi are less well understood. Root exudates and extracts from pine (*Pinus sylvestris*) enhance spore germination of several *Suillus* species that form ectomycorrhizal associations with that tree, and a diterpene resin, abietic acid, probably causes the effect (3). Promotive effects of root exudates on in vitro spore germination and/or hyphal growth of vesicular-arbuscular mycorrhizal (VAM) fungi have been documented by numerous workers (1, 2, 5), and several commercially available flavonoids promote spore germination of the VAM fungus *Gigaspora margarita* (4). Whether the promotive flavonoids were actually present in the root exudates studied is unknown, and whether the fungal response is specific, in the sense that only certain flavonoids released by its host can trigger developmental events, has not been explored. Addressing these questions requires a detailed understanding of flavonoids that are released from a host plant.

An array of natural flavonoids that induce *nod* genes in *Rhizobium meliloti* has been identified from alfalfa (*Medicago sativa* L.) (9, 13, 17). Studies that separated flavonoids released by alfalfa seeds from those exuded by roots during the first 72 h of germination show that roots exude 5-deoxy flavonoids (flavone numbering system; Fig. 1B) (13), while flavonoids from seeds are substituted at the C-5 position (9). Nearly half (46%) of the flavonoid fraction released from alfalfa seeds contains the aglycone quercetin, which does not induce *nod* genes in *R. meliloti* but which does increase the growth rate of that organism (6). The single most prevalent flavonoid in that fraction is quercetin-3-*O*-galactoside (6). Whether quercetin compounds from alfalfa seeds influence other microbial symbionts of alfalfa has not been reported.

This study was initiated to test whether any or all of the flavonoids released from alfalfa seedlings affect spores of two VAM fungi that infect that plant. To simplify experimental variables, studies were done in vitro under sterile conditions. Because initial tests showed that an isolate of *Glomus etunicatum* formed markedly more effective symbioses with 'Moapa 69' alfalfa than an isolate of *Glomus macrocarpum*, the most extensive studies were done with *G. etunicatum*.

MATERIALS AND METHODS

Fungal isolates. Spores of *G. etunicatum* (Native Plants Inc., Salt Lake City, Utah) and *G. macrocarpum*, kindly supplied by G. Bethlenfalvay (U.S. Department of Agriculture, Albany, Calif.) from 6-month-old pot cultures of sorghum (*Sorghum bicolor* (L.) Moench), were stored for 14 to 28 days at −12°C to synchronize spore germination (20). After cold storage, spores were isolated by wet sieving followed by centrifugation on a 20:40:60% (wt/vol) sucrose step gradient for 5 min. Spores were removed quickly from the upper interface of the 40% sucrose, washed several times with tap water, transferred into sterile Eppendorf tubes, and surface sterilized with 0.1% sodium hypochlorite for 10 min. Spores were then transferred to a sterile streptomycin sulfate (Sigma, St. Louis, Mo.) solution (200 $\mu g \cdot ml^{-1}$) for 10 min and washed five times with sterile distilled water. Groups of 8 to 15 spores were transferred with a micropipette into culture media for experiments.

Flavonoids. Stock solutions were prepared gravimetrically to 10 $\mu g \cdot ml^{-1}$ in methanol from the following compounds: luteolin (3′,4′,5,7-tetrahydroxyflavone), luteolin-7-*O*-glucoside, quercetin (3,3′,4′,5,7-pentahydroxyflavone), quercetin-3-*O*-galactoside, 4′,7-dihydroxyflavone, liquiritigenin (4′,7-dihydroxyflavanone), formononetin (7-hydroxy-4′-methoxyisoflavone) (all obtained from Spectrum Chemical, Gardena, Calif.), and 4,4′-dihydroxy-2′-methoxychalcone (generously supplied by R. E. Carlson, Ecochem Research, Chaska, Minn.). Standard conventions for carbon numbers of flavonoids were used (Fig. 1).

* Corresponding author.

† Permanent address: Centro de Energia Nuclear na Agricultura/USP—Caixa Postal 96, Piracicaba, 13400 Sao Paulo, Brazil.

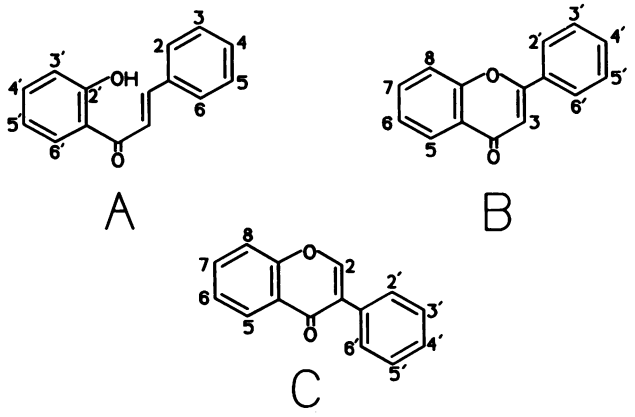

FIG. 1. Carbon numbering conventions for flavonoids in this study. (A) Chalcones; (B) flavones, flavanones, and flavonols (3-hydroxyflavones); (C) isoflavones.

Culture medium. The culture medium contained the following ingredients in 1.0 liter: KCl, 4.0 mg; KNO_3, 6.4 mg; $MgSO_4 \cdot 7H_2O$, 4.0 mg; $Ca(H_2PO_4)_2 \cdot H_2O$, 0.8 mg; FeNaEDTA, 0.19 mg; thiamine, 0.4 mg; biotin, 0.04 mg; sucrose, 1.0 g; and Bacto-Agar (Difco Laboratories, Detroit, Mich.), 7.5 g. The medium was adjusted to pH 6.4 and sterilized by autoclaving. Six milliliters of medium was dispensed into each disposable plastic petri dish (60 by 15 mm). Methanol stocks of flavonoids were added to 56°C agar medium to supply the indicated concentrations and stirred under a laminar-flow hood for 30 min to maximize methanol evaporation. No medium was supplemented with more than 0.4% methanol, and control media always were supplied with the maximum amount of methanol used for dispensing flavonoids in a particular experiment. Each dish was sealed with Parafilm (American National Can, Greenwich, Conn.), which was changed weekly to restore ambient atmospheric conditions. Plates were incubated in the dark at 25 ± 1°C.

Quercetin effects on fungal development. Quercetin (0, 0.05, 0.1, 0.25, 0.5, 1.0, 1.25, 2.5, 5, or 10 μM) was tested in the defined culture medium with *G. etunicatum* spores to identify an optimum concentration and to determine whether spores developed normally with this flavonol. Each treatment consisted of four replicate plates, and each plate contained 5 drops of spores with an average of 10 spores per spot. The experiment was scored for germination (percent) and hyphal branching (percent) with a dissecting microscope at ×80 magnification 3, 7, 15, and 21 days after plating. A scale with 0.1-mm divisions was used to measure hyphal length (in millimeters). Ungerminated spores were not included in calculations of either the mean hyphal length or the percentage of branched hyphae. Data were tested for significant treatment effects by analysis of variance (Statistix, NH Analytical Software, Roseville, Minn.). The experiment was performed twice with similar results.

Flavonoid effects on germination. Nine treatments, consisting of eight flavonoids at a concentration of 2.5 μM and a flavonoid-free control, were tested in the defined culture medium for effects on germination of *G. etunicatum* and *G. macrocarpum*. Every treatment contained three replicate plates of each fungal species, and each plate had 5 droplets with an average of 10 spores per drop. After 21 days of incubation, data on germination (percent) were collected with a dissecting microscope, and treatments were tested for statistically significant effects with the analysis used in the quercetin experiments. The study was conducted twice with similar results.

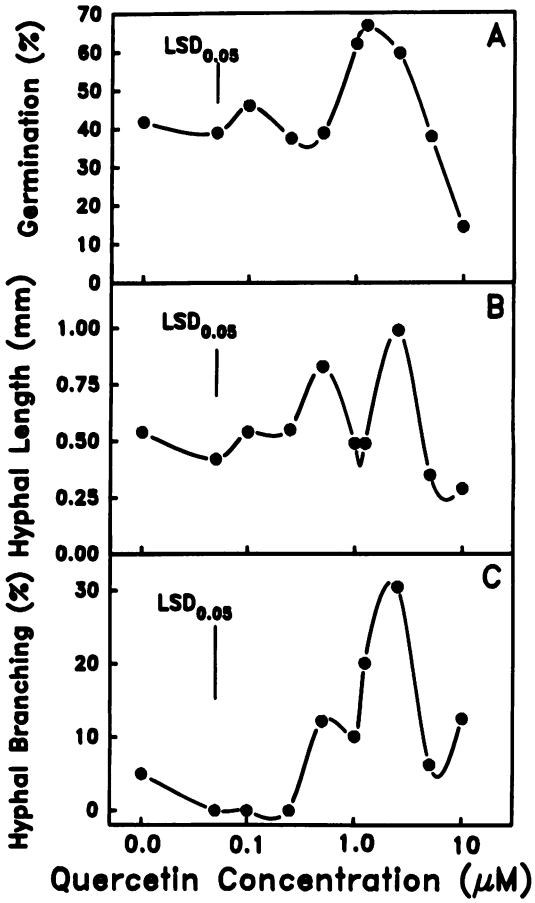

FIG. 2. Effects of quercetin concentration on development of *G. etunicatum* spores after 21 days in vitro. $LSD_{0.05}$, least significant difference ($P \leq 0.05$).

RESULTS

Quercetin effects on fungal development. When different concentrations of quercetin aglycone were tested, maximum spore germination was produced in 1.0 to 2.5 μM after 21 days (Fig. 2). Hyphal length and the percentage of hyphae with branches were maximized by the 2.5 μM quercetin treatment, and both were significantly greater than in the untreated control ($P \leq 0.05$). Some individual hyphae reached a length of 7 mm, but population means never exceeded 1 mm.

Within 7 days, nearly 60% of the *G. etunicatum* spores exposed to 2.5 μM quercetin had germinated, while untreated controls were still significantly below that level after 21 days (Fig. 3A). Hyphal length in spores developing with and without quercetin increased to a maximum within 15 days, but the quercetin treatment promoted mean hyphal elongation by nearly 100% ($P \leq 0.05$) (Fig. 3B). Hyphal branching in the presence of 2.5 μM quercetin was much greater than in the untreated control after 21 days because in the control treatment, new spores germinated after day 7 but no additional branching occurred (Fig. 3C). This combination of events produced a decrease in the branching param-

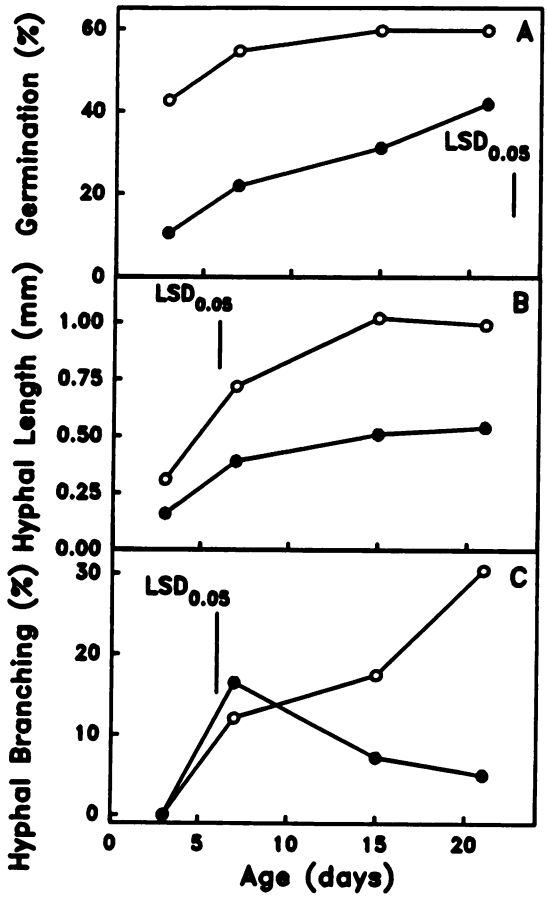

FIG. 3. Time course of *G. etunicatum* development in vitro with (○) or without (●) 2.5 μM quercetin. $LSD_{0.05}$, least significant difference ($P \leq 0.05$).

eter measured as a percentage of total hyphae. Similar promotive effects of quercetin also were observed in preliminary tests with *G. etunicatum* on 0.75% (wt/vol) water agar medium, pH 6.5.

Flavonoid effects on spore germination. Some, but not all, flavonoids released by alfalfa seeds and roots increased spore germination significantly in *G. etunicatum* and *G. macrocarpum* after 21 days (Table 1). Among seed flavonoids, quercetin aglycone and quercetin-3-*O*-galactoside produced the maximum promotion of germination in both species. Two flavonoids in root exudates, 4',7-dihydroxyflavone and liquiritigenin, also increased spore germination in *G. etunicatum*, but they had no statistically significant effect on germination of *G. macrocarpum*. One flavonoid exuded by stressed alfalfa roots, formononetin, inhibited spore germination significantly in both fungal species.

TABLE 1. Effects of alfalfa flavonoids on spore germination in vitro of two *Glomus* species[a]

Flavonoid (2.5 μM)	% Increase in spore germination in:	
	G. etunicatum	*G. macrocarpum*
None	18	32
Released by seeds		
Luteolin-7-*O*-glucoside	29*[b]	35
Luteolin (3',4',5,7-tetrahydroxyflavone)	27	38
Quercetin-3-*O*-galactoside	44**	57**
Quercetin (3,3',4',5,7-pentahydroxyflavone)[c]	54**	63**
Exuded by roots		
4,4'-Dihydroxy-2'-methoxychalcone	14	4*
4',7-Dihydroxyflavone	49**	42
Liquiritigenin (4',7-dihydroxyflavanone)	37**	32
Formononetin (7-hydroxy-4'-methoxyisoflavone)[d]	4*	7*

[a] Development was measured after 21 days on a defined agar medium.
[b] * or **, flavonoid effect significant at $P \leq 0.05$ or 0.01, respectively.
[c] Synthesized by seed tissue but stored in conjugated, not aglycone, form.
[d] Exuded by stressed roots (14).

DISCUSSION

Results from this study show clearly that some flavonoids released naturally from alfalfa seeds and roots promote spore germination of *G. etunicatum* under defined in vitro conditions (Table 1). By identifying 4',7-dihydroxyflavone as a natural promotive molecule in alfalfa root exudate, these results add new definition to earlier observations (1, 2, 4, 5, 16) that plant roots, root exudates, or arbitrary flavonoids enhance spore germination in VAM fungi. The fact that quercetin-3-*O*-galactoside released from seeds also promotes germination of *G. etunicatum* suggests that ecochemical factors in the seed zone deserve more emphasis in VAM and rhizosphere studies.

An interesting feature of quercetin-3-*O*-galactoside and 4',7-dihydroxyflavone is that they are the most prevalent compounds in the flavonoid fraction of seed and root exudates, respectively (6, 13). Although these compounds have other effects on alfalfa rhizosphere microbes (6, 13), enhancement of VAM fungal spore germination clearly could be advantageous to the plant. Clover roots also exude 4',7-dihydroxyflavone (19), and that flavone probably contributed to the enhancement of VAM fungal spore germination observed with clover root exudates (2). A direct quantitative relationship between these in vitro studies and those employing crude exudates cannot be assumed, however, because mixtures of alfalfa flavonoids show both inhibitory (7) and synergistic (8) effects on *nod* gene transcription in *R. meliloti*. Thus, formononetin, which inhibited spore germination in this study (Table 1), may play a similar role in the rhizosphere.

Low micromolar concentrations of quercetin aglycone promoted three developmental processes measured in this study: spore germination, hyphal elongation, and hyphal branching (Fig. 2). However, because quercetin was added only at the beginning of the experiments, rather than after germination, one cannot conclude that quercetin independently enhanced hyphal elongation and branching. Thus, the very rapid quercetin-induced increase in spore germination after 3 days (Fig. 3A) probably contributed to the quercetin promotion of hyphal growth and branching (Fig. 3B and C).

Data from this report should expedite the technically difficult task of defining conditions for the pure in vitro culture of VAM fungi (10, 11). Beneficial effects of carbon dioxide on hyphal growth of VAM fungi (1) also will contribute to that goal and probably are equally relevant ecological factors in the rhizosphere. Flavonoids tested in this study were identified as products from alfalfa seedlings

developing in the absence of VAM fungi. Previous experiments have shown that VAM fungi alter flavonoid metabolism in plants (15), and compounds produced as a result of infection may cause differentiation of vesicles, arbuscules, and/or spores. As various factors are identified, some combination of them may allow development of the pure in vitro culture methodologies which are crucial for advancing our fundamental understanding of VAM fungi.

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