

Extensive In Vitro Hyphal Growth of Vesicular-Arbuscular Mycorrhizal Fungi in the Presence of CO₂ and Flavonols

G. BÉCARD,* D. D. DOUDS, AND P. E. PFEFFER

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118

Various flavonoids were tested for their ability to stimulate in vitro growth of germinated spores of vesicular-arbuscular mycorrhizal fungi. Experiments were performed in the presence of 2% CO₂, previously demonstrated to be required for growth of *Gigaspora margarita* (G. Bécard and Y. Piché, Appl. Environ. Microbiol. 55:2320–2325, 1989). Only the flavonols stimulated fungal growth. The flavones, flavanones, and isoflavones tested were generally inhibitory. Quercetin (10 μM) prolonged hyphal growth from germinated spores of *G. margarita* from 10 to 42 days. An average of more than 500 μm of hyphal growth and 13 auxiliary cells per spore were obtained. Quercetin also stimulated the growth of *Glomus etunicatum*. The glycosides of quercetin, rutin, and quercitrin were not stimulatory. The axenic growth of *G. margarita* achieved here under rigorously defined conditions is the most ever reported for a vesicular-arbuscular mycorrhizal fungus.

Plant factors involved in the biotrophic growth of vesicular-arbuscular (VA) mycorrhizal fungi remain to be discovered. Even before symbiosis takes place, the germinating spores need certain factors to promote hyphal growth (3). At this stage, the fungus is still trophically dependent on spore reserves, and it has been proposed that some root exudates regulate the ability of the fungus to use its endogenous reserves (3). Good candidates for such regulating compounds are plant phenolics since they have been found to be important transcriptional signals in soil bacteria. For example, specific plant phenolic molecules induce genes in *Rhizobium* spp., *Agrobacterium* spp., and *Pseudomonas syringae* involved in the formation of nodules, transformation of plants, and production of a toxin, respectively (10, 12).

Recently, several laboratories have tested the hypothesis that flavonoids play an important role in VA mycorrhizal symbioses. The flavanones hesperetin and naringenin and the flavone apigenin stimulated hyphal growth of *Gigaspora margarita* (8). The two isoflavones formononetin and biochanin A and, to a lesser extent, the flavone chrysin have been shown to stimulate formation of VA mycorrhizae between a *Glomus* sp. and white clover (11, 13). Also, the flavonol glycoside quercetin-3-*O*-galactoside, 4',7-dihydroxy flavone, and 4',7-dihydroxy flavanone, identified in seed and root exudates of alfalfa, enhanced spore germination of two *Glomus* species (14).

The objective of this study was to evaluate further the stimulatory effect of flavonoids on growth of VA mycorrhizal fungi by using pregerminated spores and controlling the carbon dioxide concentration of the cultures. Carbon dioxide was found to be a critical factor for growth of *G. margarita* in vitro (4) and for the germination and growth of other species of VA mycorrhizal fungi (unpublished results). As a result, the conditions of the bioassays for comparison of the various flavonoids were optimized by using a high CO₂ environment and have led to a discrepancy with results from some of the previous studies. Extensive axenic fungal growth was obtained with the most stimulatory flavonoids,

and a common structural feature of the active flavonoids is proposed.

MATERIALS AND METHODS

Spores. Azygospores of *G. margarita* Becker & Hall (DAOM 194757) were produced in greenhouse pot culture with *Allium porum* L. and *Paspalum notatum* Flugge as the plant hosts. Plants were fertilized weekly with Hoagland's solution without phosphorus to stimulate sporulation (5). Chlamydo-spores of *Glomus etunicatum* Becker & Gerdemann and *Glomus intraradix* Schenck & Smith were purchased from Native Plant Industries (Salt Lake City, Utah).

Spores were collected by wet sieving, several centrifugations in 40% (wt/vol) sucrose, and finally density gradient centrifugation in Renografin-60 (6). Purified spores then were surface sterilized as described by Bécard and Fortin (2).

Spore germination. Spores were germinated on M medium (2) gelled with 0.4% (wt/vol) gellan gum (Gel-Gro; ICN Biochemicals, Cleveland, Ohio). Petri dishes were incubated in an inverted position, unsealed, in a CO₂ incubator (Forma Scientific, Inc., Marietta, Ohio), at 32°C. The incubator was calibrated to 2% CO₂ by using a Fyrite gas analyzer (Bacharach, Inc., Pittsburgh, Pa.; model 7% CO₂/O₂) since this concentration was found to be optimum for growth of *G. margarita* (unpublished results). Germination of *G. margarita* spores (80 to 100%) and of *Glomus* spores (over 70%) occurred after 48 and 72 h of incubation, respectively.

Conditions for fungal growth. One germinated spore of *G. margarita* or three spores of either *Glomus* sp. were axenically transferred within a core of medium to square petri dishes (9 by 9 cm). These dishes contained the same medium used for germination except that it was supplemented with various flavonoids at 10 μM. An additional experiment was conducted to determine the optimal concentration of quercetin to stimulate fungal growth. Totals of 8 to 10 and 4 experimental dishes per treatment were used for *G. margarita* and the *Glomus* sp., respectively. Petri dish lids were secured with a small piece of tape, and dishes were incubated vertically under the conditions of the germination assay. Quercetin, myricetin, kaempferol, hesperetin, biochanin A, chrysin, and rutin were obtained from Sigma

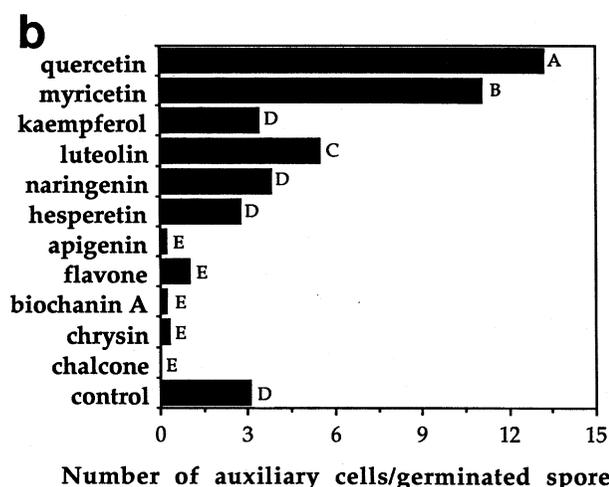
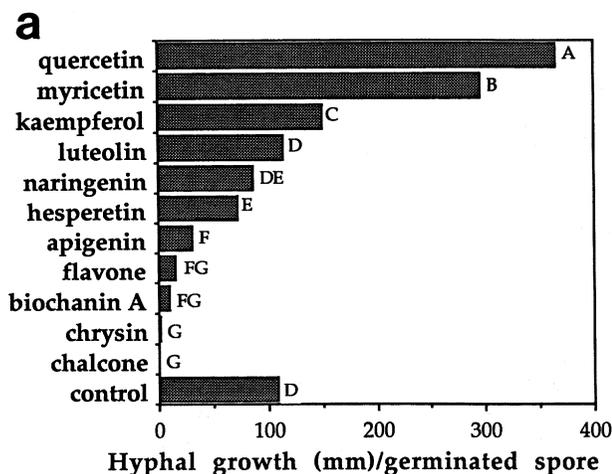


FIG. 1. Effect of various flavonoids (10 μ M) on in vitro growth of germinated spores of *G. margarita* after 6 days of culture in the presence of 2% CO₂. (A) Hyphal growth; (B) formation of auxiliary cells. Same letters indicate no significant differences ($P \leq 0.05$, Duncan's multiple range test).

Chemical Co., St. Louis, Mo.; luteolin was from ICN Biochemicals; naringenin, flavone, and chalcone were from Aldrich Chemical Co., Milwaukee, Wis.; apigenin was from Carl Roth KG, Karlsruhe, Germany; and quercitrin was from Pfaltz & Bauer, Stamford, Conn. Stock solutions (4 mM) of flavonoids were normally prepared in absolute ethanol, except for glycosides where the more-polar solvent methanol was used. Flavonoid stocks were added axenically (400 \times dilution) to the culture medium after it had been autoclaved and allowed to cool to 50°C. Media for control dishes were supplied with an equivalent volume of the solvent used for the flavonoid stock solution. Incubators were calibrated at 0.0, 0.6, and 2.0% CO₂ by using a Fyrite gas analyzer to study the effect of three concentrations of CO₂, with or without quercetin (10 μ M), upon the growth of *G. margarita*.

Assessment of fungal growth. Hyphal length (in millimeters) of *G. margarita* was measured 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 (thin-walled vesicles produced on coiled hyphae [7]) was also determined. For more accuracy, the hyphal length

TABLE 1. Effect of various CO₂ concentrations, in the presence and absence of quercetin, on growth of *G. margarita* after 5 days of culture

Treatment		Hyphal growth (mm) ^a	No. of auxiliary cells ^a
CO ₂ (%)	Quercetin (μ M)		
0.0	0	2.2D	0.0C
	10	43.6C	2.8B
0.6	0	34.4C	2.5B
	10	166.2B	7.7A
2.0	0	38.4C	2.8B
	10	206.2A	8.7A

^a Means followed by the same letter did not differ significantly ($P \leq 0.05$, Duncan's multiple range test).

(in millimeters) of the *Glomus* sp. was measured after being magnified on the screen of a video system coupled with the dissecting microscope. Data were analyzed by using analysis of variance after SQRT ($x + 1$) transformation. Significant treatment effects were further characterized by using Duncan's multiple-range test ($\alpha = 0.05$). The experiments were performed twice with similar results.

RESULTS

The growth of *G. margarita* varied with the flavonoid present. In the presence of 2% CO₂, flavonoids either stimulated (quercetin, myricetin, kaempferol), slightly inhibited (hesperetin), greatly inhibited (chrysin, biochanin A, flavone, apigenin), or had no effect (naringenin, luteolin) upon hyphal growth (Fig. 1A). Chalcone totally stopped fungal growth. A three- to fourfold stimulation of growth was obtained with myricetin and quercetin within 6 days. Almost 400 mm of hyphal length per germinated spore, including more hyphal branching, was obtained with quercetin in this period. The pattern for the production of auxiliary cells was the same as that for hyphal growth, except that luteolin

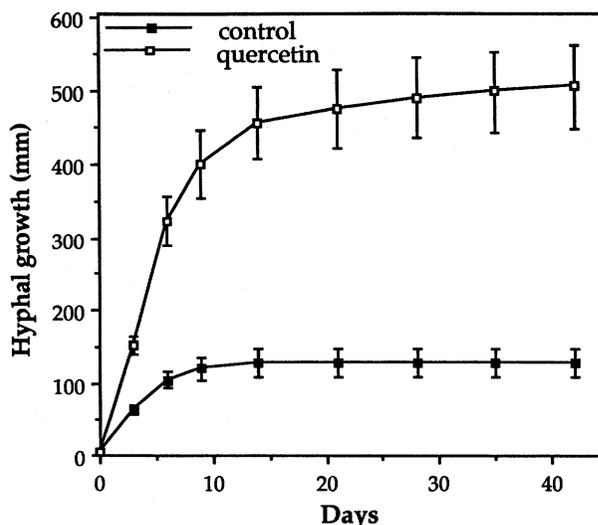


FIG. 2. Time course of hyphal growth of germinated spores of *G. margarita* in the absence or presence of 10 μ M quercetin and in the presence of 2% CO₂. Vertical bars correspond to the standard errors of the means.

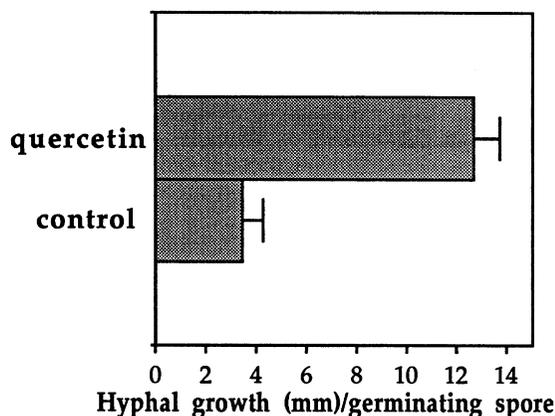


FIG. 3. Stimulatory effect of quercetin (10 μM) on hyphal growth of germinating spores of *G. etunicatum* after 12 days of culture in the presence of 2% CO_2 . Horizontal bars correspond to the standard deviations.

significantly stimulated the production of auxiliary cells while kaempferol did not (Fig. 1B).

In the absence of both CO_2 and quercetin, almost no fungal growth was obtained (Table 1). CO_2 (0.6 or 2.0%) or quercetin (10 μM) stimulated fungal growth, but the stimulation in the presence of both was greater than an additive model would have predicted. Similarly, growth in the absence of quercetin was not significantly greater at 2% CO_2 than that at 0.6%, but it was significantly enhanced (20%) in the presence of quercetin. These results indicate that the stimulatory effects of these two factors on the growth of *G. margarita* were not additive but synergistic.

The fungal growth period was increased from 10 to 42 days in the presence of quercetin and 2% CO_2 over that of 2% CO_2 alone (Fig. 2). Indeed, none of the spores treated with CO_2 alone showed hyphal growth after day 9, and most (75%) regerminated on day 14. In contrast, all germinated spores growing in the presence of quercetin continued hyphal growth

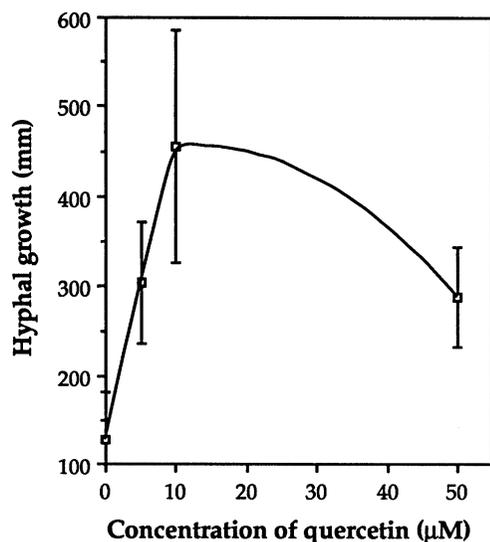


FIG. 4. Effect of concentration of quercetin on hyphal growth from germinating spores of *G. margarita* in a 2-week-old culture in the presence of 2% CO_2 . Vertical bars correspond to the standard deviations.

TABLE 2. Effect of quercetin and myricetin, alone or in combination, in the presence of 2% CO_2 , on growth of *G. margarita* after 5 days of culture

Treatment	Hyphal growth (mm) ^a	No. of auxiliary cells ^a
Control	44C	1.3C
Quercetin		
10 μM	265A	8.8AB
20 μM	253A	8.4AB
Myricetin		
10 μM	196B	6.6B
20 μM	188B	6.9AB
Quercetin (10 μM) + myricetin (10 μM)	235AB	9.4A

^a Means followed by the same letter did not differ significantly ($P \leq 0.05$, Duncan's multiple range test).

from day 14 through day 42, with individual additional hyphal elongation of 4 to 14% over that present on day 14 (average, 10.8%). Quercetin also stimulated (3.7 fold) the growth of *G. etunicatum* (Fig. 3) and *G. intraradix* (data not shown). The optimum concentration for stimulation of growth with quercetin appeared to be 10 μM (Fig. 4). Quercetin was not completely soluble in the culture media at concentrations greater than 50 μM , making the data unreliable. The presence of both quercetin and myricetin at 10 μM in the culture medium did not stimulate *G. margarita* fungal growth above that of either compound alone at a concentration of 20 μM (Table 2). The two quercetin glycosides, quercitrin and rutin, were not stimulatory to *G. margarita* fungal growth (Table 3).

DISCUSSION

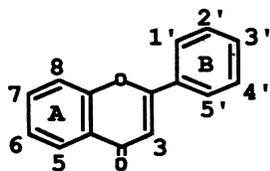
We have obtained the most extensive growth yet reported for a VA mycorrhizal fungus in pure culture by using 2% CO_2 and 10 μM quercetin. Over 500 mm of hyphal growth per germinating spore of *G. margarita* was produced in culture. Over 100 mm of growth was produced in the presence of carbon dioxide alone. Not only was the hyphal growth rate increased (5 mm/h on some days), but the duration of growth was increased from 10 days to 6 weeks. The synergistic stimulatory effect of CO_2 and quercetin is similar to that obtained with CO_2 and root exudates in a previous report (4). In the latter, it was proposed that CO_2 is an essential carbon source for hyphal growth of VA mycorrhizal fungi when catabolism of lipids is involved. Lipids are almost the exclusive source of energy during spore germination if the negligible relative amount of trehalose is not considered (1), and we expect no growth in the absence of

TABLE 3. Effect of quercetin and two quercetin glycosides (10 μM), in the presence of 2% CO_2 , on growth of *G. margarita* after 4 days of culture

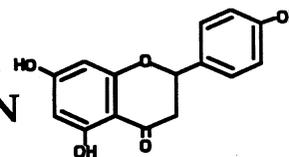
Treatment	Hyphal growth (mm) ^a	No. of auxiliary cells
Control	37 B	1.0 B
Quercetin	136 A	4.1 A
Quercitrin	35 B	0.6 B
Rutin	30 B	0.4 B

^a Means followed by the same letter did not differ significantly ($P \leq 0.05$, Duncan's multiple range test).

FLAVONE



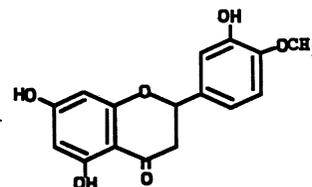
NARINGENIN



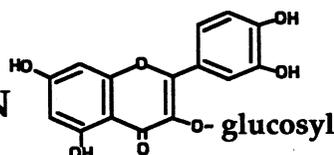
QUERCETIN



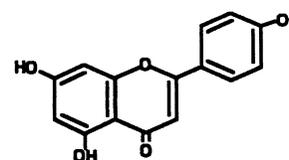
HESPERETIN



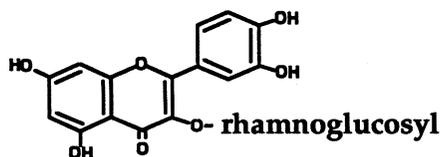
QUERCITRIN



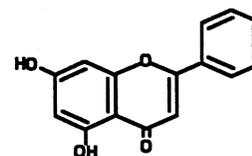
APIGENIN



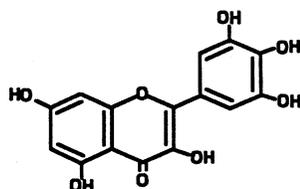
RUTIN



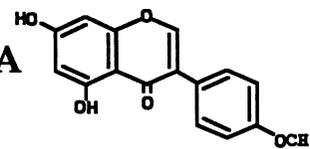
CHRYSIN



MYRICETIN



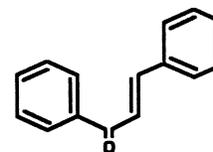
BIOCHANIN A



KAEMPFEROL



CHALCONE



LUTEOLIN

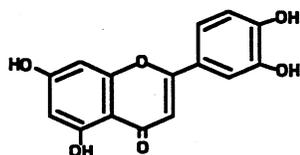


FIG. 5. Chemical structures of the flavonoids used in the study and a generic structure showing the numbering system used to identify rings and carbon atoms.

CO₂ unless some, released during respiration, is reutilized in metabolism. We suspect that the growth obtained in the incubator without CO₂ but in the presence of quercetin (Table 1) was the result of this phenomenon or of the

utilization of trehalose (1). We observed about 10-fold more hyphal growth than did Gianinazzi-Pearson et al. (8) and Tsai and Phillips (14) with *G. margarita* and *G. etunicatum*, respectively. No CO₂ was provided in these studies other

than what accumulated in the sealed cultures because of respiration of the germinating spores. We believe that it is not reliable to study the effect of flavonoids on germination and on hyphal growth in an experiment in which CO₂ concentrations are not controlled. Germination in a sealed chamber causes an increase in CO₂ which, in turn, stimulates more germination and hyphal growth. Here we have removed these complications by exposing only pregerminated spores to the flavonoids in unsealed dishes in a controlled-atmosphere chamber. Our results of hyphal growth inhibition by hesperetin, apigenin, and biochanin A and the neutral effect of naringenin are in contrast with those of Gianinazzi-Pearson et al. (8) and Nair et al. (11) who reported a stimulatory effect of these compounds on germination and hyphal growth of *G. margarita* and *Glomus* sp., respectively. Since the percentage of spore germination and hyphal growth were measured in the same experiment, one explanation might be that these compounds activated the germination only and that the resulting increase of CO₂ in the culture was entirely responsible for further stimulation of hyphal growth. It is also possible, in the case of Nair et al. (11), that the *Glomus* isolate used reacted differently than did *G. margarita*.

Interestingly, the stimulation of hyphal elongation and production of auxiliary cells were not always positively correlated. Luteolin, for example, stimulated the production of auxiliary cells but not hyphal growth. The inverse occurred with kaempferol. It is possible that the differentiation leading to the formation of an auxiliary cell requires a different mechanism of stimulation or induction than does hyphal elongation and that some flavonoids are involved in only one of these mechanisms.

An initial hypothesis on the relationship between the chemical structure of the flavonoids used in the different experiments and their respective abilities to stimulate growth of *G. margarita* can be formulated (Fig. 5). The two flavonols, quercetin and kaempferol, stimulated hyphal growth, while luteolin and apigenin, deprived of a hydroxyl group on position 3, did not. The glycosides quercitrin and rutin were also not stimulatory, perhaps because of the glycosylation on position 3. Therefore, we hypothesize that the hydroxyl group in position 3 is essential to confer stimulatory activity to the molecule, and one might predict, for this reason, that flavonols in general should be more stimulatory than flavones. Among the flavonols, quercetin, with hydroxyl groups on positions 3' and 4', gave the greatest stimulation, while the addition of a hydroxyl group on 2' (myricetin) or the lack of one on 4' (kaempferol) led to a lower level of stimulation. As expected, biochanin A, which is an isoflavonoid, as are most of the phytoalexins, demonstrated a strong inhibitory effect on the growth of *G. margarita*. Tsai and Phillips (14) also found another isoflavonoid (formononetin) to inhibit germination of spores of *G. etunicatum*.

The fact that no additional stimulation was obtained when both quercetin and myricetin were provided together in the culture medium suggests that these two very closely related molecules acted similarly and may have shared the same fungal receptors or stimulatory pathways. Further investigations are needed to accurately determine the structure-function relationship of these biologically powerful molecules.

Additional work is also necessary to verify the occurrence of these molecules in VA mycorrhizae and their function as plant signals to control the activity of the mycosymbiont. This is very likely since quercetin, for example, is the most widely distributed flavone in the plant kingdom (9), where mycorrhizal associations are ubiquitous. Recently, quercetin-3-O-galactoside was found to be the dominant flavonoid

released naturally from alfalfa seeds and to promote spore germination of *G. etunicatum* and *Glomus macrocarpum* (14). The discovery that formononetin and biochanin A, previously identified from clover roots (11), stimulated colonization and growth of clover is also very promising (13). We are presently investigating the role that flavonoids play in a functional symbiotic system developed by Bécard and Fortin (2), involving transformed roots and *G. margarita*. The type of growth stimulation caused by some of the flavonoids reported here is very similar to that obtained with the exudates of transformed roots (3, 4).

The recent evidence that flavonoids play an important role in the biology of VA mycorrhizal fungi is giving new impetus to research directed toward growing these microorganisms in pure culture. It is possible that flavonoids are one of the clues to the growth requirements of these fungi which has eluded researchers for decades.

ACKNOWLEDGMENTS

We thank Selena McLaurin for her technical assistance and Stanley F. Osman, William F. Fett, David K. Brauer, and Landis W. Doner for helpful discussions.

REFERENCES

- Bécard, G., L. W. Doner, D. B. Rolin, D. D. Douds, and P. E. Pfeffer. 1991. Identification and quantification of trehalose in vesicular-arbuscular fungi by *in vivo* ¹³C NMR and HPLC analyses. *New Phytol.* **118**:547-552.
- Bécard, G., and J. A. Fortin. 1988. Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytol.* **108**:211-218.
- Bécard, G., and Y. Piché. 1989. New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. *New Phytol.* **112**:77-83.
- Bécard, G., and Y. Piché. 1989. Fungal growth stimulation by CO₂ and root exudates in vesicular-arbuscular mycorrhizal symbiosis. *Appl. Environ. Microbiol.* **55**:2320-2325.
- Douds, D. D., and N. C. Schenk. 1990. Increased sporulation of vesicular-arbuscular mycorrhizal fungi by manipulation of nutrient regimes. *Appl. Environ. Microbiol.* **56**:413-418.
- Furlan, V., H. Bartschi, and J. A. Fortin. 1980. Media for density gradient extraction of endomycorrhizal spores. *Trans. Br. Mycol. Soc.* **75**:336-338.
- Gerdemann, J. W., and J. M. Trappe. 1974. The Endogonaceae in the Pacific Northwest. *Mycol. Mem.* **5**:1-76.
- Gianinazzi-Pearson, V., B. Branzanti, and S. Gianinazzi. 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.
- Gripenberg, J. 1962. Flavones, p. 406-440. *In* T. A. Geissman (ed.), *The chemistry of flavonoid compounds*. Pergamon Press, Inc., Elmsford, N.Y.
- Mo, Y. Y., R. F. Bonsall, and D. C. Gross. 1990. Characterization of plant signals that induce the SYR B gene required for syringomycin production by *Pseudomonas syringae* PV. *Syringae*. *Abstr. Annu. Meet. Am. Phytopathol. Soc. Can. Phytopathol. Soc. Phytopathology* **80**:1038.
- Nair, M. G., G. R. Safir, and J. O. Siqueira. 1991. Isolation and identification of vesicular-arbuscular mycorrhiza-stimulatory compounds from clover (*Trifolium repens*) roots. *Appl. Environ. Microbiol.* **57**:434-439.
- Peters, N. K., and D. P. S. Verma. 1990. Phenolic compounds as regulators of gene expression in plant-microbe interactions. *Mol. Plant-Microbe Interact.* **3**:4-8.
- Siqueira, J. O., G. R. Safir, and M. G. Nair. 1991. Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. *New Phytol.* **118**:87-93.
- Tsai, S. M., and D. A. Phillips. 1991. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores *in vitro*. *Appl. Environ. Microbiol.* **57**:1485-1488.