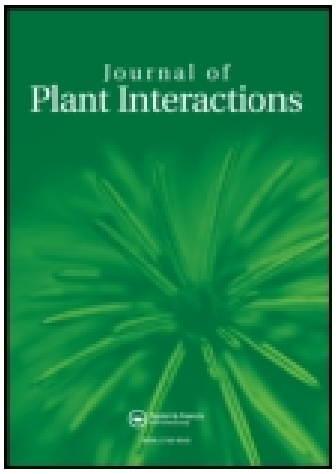

Journal of Plant Interactions
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tjpi20

Flavonoids exclusively present in mycorrhizal roots of white clover exhibit a different effect on arbuscular mycorrhizal fungi than flavonoids exclusively present in non-mycorrhizal roots of white clover

Jose M. Scervino [a], María A. Ponce [b], Rosa Erra-Bassells [b], Horst Vierheilig [c], Juan A. Ocampo [d] & Alicia Godeas [a]

[a] Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

[b] CIHIDECAR-CONICET, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

[c] Institut für Pflanzenschutz, Universität für Bodenkultur Wien, Wien, Austria

[d] Departamento de Microbiología, Estación Experimental de Zaidín, CSIC, Granada, España

Published online: 28 Sep 2010.

To cite this article: Jose M. Scervino, María A. Ponce, Rosa Erra-Bassells, Horst Vierheilig, Juan A. Ocampo & Alicia Godeas (2005) Flavonoids exclusively present in mycorrhizal roots of white clover exhibit a different effect on arbuscular mycorrhizal fungi than flavonoids exclusively present in non-mycorrhizal roots of white clover, Journal of Plant Interactions, 1:1, 15-22, DOI: 10.1080/17429140500192597

To link to this article: http://dx.doi.org/10.1080/17429140500192597

Journal of Plant Interactions, March 2005; 1(1): 15–22

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Flavonoids exclusively present in mycorrhizal roots of white clover exhibit a different effect on arbuscular mycorrhizal fungi than flavonoids exclusively present in non-mycorrhizal roots of white clover

JOSE M. SCERVINO[1], MARÍA A. PONCE[2], ROSA ERRA-BASSELLS[2], HORST VIERHEILIG[3], JUAN A. OCAMPO[4] & ALICIA GODEAS[1]

[1]*Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina,* [2]*CIHIDECAR-CONICET, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina,* [3]*Institut für Pflanzenschutz, Universität für Bodenkultur Wien, Wien, Austria, and* [4]*Departamento de Microbiología, Estación Experimental de Zaidín, CSIC, Granada, España*

(Received 9 December 2004; accepted 31 March 2005)

Abstract
The flavonoids 5,6,7,8,9-hydroxy chalcone, 3,7-hydroxy-4′methoxy flavone, 5,6,7,8-hydroxy-4′-methoxy flavone and 3,5,6,7,4′-hydroxy flavone can be detected only in non-mycorrhizal roots of white clover, but not in mycorrhizal roots, whereas the flavonoids acacetin, quercetin and rhamnetin are only present in mycorrhizal roots. We tested the effect of several concentrations of these compounds on spore germination, hyphal growth, hyphal branching, formation of clusters of auxiliary cells and of secondary spores of the arbuscular mycorrhizal fungi *Gigaspora rosea*, *Gigaspora margarita*, *Glomus mosseae* and *Glomus intraradices*. Our results indicate that depending on the flavonoid, the tested compounds are involved at different stages in the regulation of mycorrhization. This hypothesis is strengthened by their differing effect on several AM fungal growth parameters. Furthermore, our study provides more data on the AM fungus genus/species specificity of flavonoids.

Keywords: *Arbuscular mycorrhiza, glomales, flavonoid, regulation, signal molecule, symbiosis*

Introduction

Arbuscular mycorrhiza (AM) is a symbiotic plant-fungus association which can be found in nearly all land plants (Harley & Harley 1987). The establishment of the AM symbiosis is the result of a complex exchange of signals between AM fungi and the host plant (Vierheilig 2004a) initiating various metabolic changes, e.g., the alteration of the flavonoid pattern, in the host root. Interestingly, alterations of the flavonoid pattern seem to depend on the developmental stage of the AM symbiosis (Akiyama et al. 2002, Larose et al. 2002), thus, flavonoids, which are known as key signals for the establishment of the rhizobial symbiosis, have been suggested as signalling molecules in the AM symbiosis (Phillips & Tsai 1992, Vierheilig et al. 1998, Vierheilig & Piche 2002).

A number of studies on the effect of flavonoids on AM spore germination, fungal growth and root colonization are available (Siqueira et al. 1991, Kape et al. 1992b, Morandi et al. 1992, Morandi, 1996, Vierheilig et al. 1998, Juge et al. 2002), however, data on the effect on other important parameters such as hyphal branching and the formation of auxiliary cells or secondary spores are scarce (Gianinazzi-Pearson et al. 1989, Bécard et al. 1992, Phillips & Tsai 1992). In a recent study Ponce et al. (2004) showed that the flavonoid pattern in white clover is altered after colonization by the AM fungus *Glomus intraradices*. Several flavonoids such as 5,6,7,8,9-hydroxy chalcone (NM7); 3,7-hydroxy-4′methoxy flavone; 5,6,7,8-hydroxy-4′-methoxy flavone (RR4) and 3,5,6,7,4′-hydroxy flavone (RR4–2) could be exclusively detected in non-mycorrhizal white clover roots, whereas others (acacetin, quercetin and rhamnetin) were newly synthesized in mycorrhizal roots. The flavonoid 3,5,6,7,8-hydroxy-4′-methoxy flavone was detected in roots of nonmycorrhizal and mycorrhizal white clover plants.

Flavonoids isolated from roots of non-mycorrhizal white clover plants have been reported to stimulate

Correspondence: Juan A. Ocampo, Departamento de Microbiología del Suelo, Estación Experimental del Zaidín, CSIC, Prof. Albareda 1, E-18008 Granada, Spain. Fax: +34 958 129600. E-mail: jocampo@eez.csic.es

ISSN 1742-9145 print/ISSN 1742-9153 online
DOI: 10.1080/17429140500192597

AM hyphal growth and when applied to plants, to enhance root colonization by AM fungi (Nair et al. 1991, Siqueira et al. 1991), thus, the synthesis and/or suppression of flavonoids in white clover roots colonized by an AM fungus could be an indicator that these plant molecules are involved in the regulation of the AM symbiosis at different stages (Vierheilig & Piche 2002). Also there are some data on the effect of quercetin on AM fungi (Tsai & Phillips 1991, Bécard et al. 1992, Chabot et al. 1992, Kape et al. 1992a, Bel-Rhlid et al. 1993, Poulin et al. 1997), whereas no studies on the effect of the other flavonoids isolated from mycorrhizal and non-mycorrhizal white clover roots on AM fungi have been performed yet.

In the present work we studied the effect of all flavonoids detected in mycorrhizal and non-mycorrhizal roots of white clover on spore germination and several other AM fungal growth parameter (e.g., hyphal growth, hyphal branching). In order to determine an AM fungal species or genus effect, the above mentioned parameters were determined in *Gigaspora rosea*, *Gigaspora margarita*, *Glomus mosseae* and *G. intraradices*.

Materials and methods

The flavonoids 5,6,7,8,9-hydroxy chalcone (NM7); 3,7-hydroxy-4′methoxy flavone; 5,6,7,8-hydroxy-4′-methoxy flavone (RR4) and 3,5,6,7,4′-hydroxy flavone (RR4–2) were isolated from non-mycorrhizal clover roots, the flavonoids 3,5,7,3′,4′-pentahydroxy flavone (quercetin); 5,7-dihydroxy-4′-methoxy flavone (acacetin) and 3,5,3′,4′-tetrahydroxy-7-methoxy flavone (rhamnetin) were isolated from AM mycorrhizal white clover roots and the flavonoid 3,5,6,7,8-hydroxy-4′-methoxy flavone was isolated from AM mycorrhizal and non- mycorrhizal clover roots as described by Ponce et al. (2004). The experiments were carried out in 1.5 l pots filled with a soil collected from the province of Buenos Aires. The soil (Silty-clay-loam of argiudol type, pH 5.4), with 2.28% C, contained (in mg kg^{-1}) 331 N, 9.5 P ($NaHCO_3$-extractable) and 3.2 Ca (Mingorance, 2002). After steam-sterilization the soil was mixed with sterilized perlite at a proportion of 1:1 (V:V). Seeds of white clover (*Trifolium repens* L.) were surface-sterilized with ethanol:water 1:1 during 90 sec, bleach:water 1:1 during 90 sec and thoroughly rinsed with sterilized water and sown in moistened sand. After germination, uniform seedlings were planted and grown in the greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 μE m^{-2} s^{-1}, 400–700 nm, with a 16/8 h day/night cycle at 25/19°C and 50% relative humidity. Plants were watered from below and fed with a Long Asthon nutrient solution at 20 ml per pot weekly (Hewitt, 1952), lacking phosphate for AM-inoculated clover plants. *G. intraradices* G3 Schenk and Smith from "Buenos Aires Fungal Collection" (BAFC), Argentina was the AM fungus used in these experiments. The AM fungal inoculum was a root-and-soil inoculum consisting of 5 g of rhizosphere soil containing spores and colonized root fragments of *Medicago sativa* L., which were predetermined to have achieved high levels of root colonization. Non AM inoculated pots were given a filtrate (Whatman no.1 paper) of the inoculum containing the common soil microbiota, but free of AM fungal propagules.

Plants were harvested after 10 weeks and samples of fresh roots from the five replicate pots were taken from the entire root system at random. These root samples were cleared in KOH and stained with trypan blue (Phillips & Hayman, 1970), and the percentage of root colonization was measured by the gridline intersect method (Giovannetti & Mosse 1980).

The roots were crushed after drying at 70°C for 72 h and extracted exhaustively with ethanol for 4 days (1.5 l of ethanol per gram of dried material). The extracts obtained from the dried plant material were filtered and evaporated to dryness at reduced pressure and then suspended in water. Thereafter, each sample was redistributed between water-organic solvents of increasing polarity. We obtained four extracts, from hexane (defatting), from ethyl acetate, from dichloromethane and water itself. The resultant extracts were evaporated at reduced pressure to dryness and were analysed by TLC chromatography. TLC was carried out on precoated silica gel $60F_{254}$ aluminium sheets (Merck). The solvent system used was EtOAc-CH_2Cl_2-HCO_2H (8:12:1). Chromatograms were visualized after drying (i) by UV light and (ii) by spraying with a solution containing 6 g vainillin (Aldrich) and 3 ml H_2SO_4 in 197 ml of MeOH (Bel-Rhlid et al. 1993). After visualization the compounds were recovered by treating each portion of the adsorbent with EtOAc, EtOAc-EtOH and EtOH. After evaporation of the organic solvent mixture, the samples were analysed by chromatography. The purity of the flavonoids was checked by HPLC. 1H-NMR (500 MHz) spectra were recorded in DMSO-d_6 with TMS as internal standard. EIMS were recorded at 70 eV (ionizing potential) using a direct inlet system. The flavonoids were identified by their peaks at m/s in its EIMS and HREIMS, respectively consistent with the molecular formula which was further supported by CNMR spectroscopy.

Flavonoids were tested on *G. rosea* (BEG 9), *G. margarita* (J7) from BAFC, *G. mosseae* (BEG 12) and *G. intraradices* (DAOM 197198) spores in 9 cm diameter Petri dishes. Spores of *G. margarita* Becker & Hall were isolated from Ciudad Universitaria soil (Fracchia 2002), in the province of Buenos Aires (Argentina) and identified (Bentivenga & Morton 1995).

Flavonoids were dissolved in absolute ethanol to obtain 4 mM stock solutions. Thereafter, flavonoids were filtered through a disk of filter paper and sterilized twice by filtration through a 0.20 μm Millipore membrane and transferred to 10 ml of 10 mM 2-(N-morpholin) ethane sulphonic acid (MES) buffer (pH 7) plus 0.04 g of Gel-Gro (ICN Biochemicals, Aurora, Ohio, USA). The effect of 0.05%, 0.1%, 0.5%, 1.0% ethanol-water on the percentage of germination and hyphal length of *Gigaspora* and *Glomus* spores was tested. The concentration of 0.05% ethanol was selected because it was the only substance that did not have an effect on the percentage of germination and hyphal length of spores. The flavonoids dissolved in absolute ethanol were added to 10 ml of Gel-Gro at a final concentration of 0.5, 2 and 8 μM in 0.05% ethanol.

The flavonoid concentrations 0.5, 2 and 8 μM of were selected for showing significant effects on different steps of the AM fungal development with other flavonoids (Morandi 1996, Vierheilig et al. 1998).

Petri dishes with 0.05% ethanol or without ethanol were used as control. Spores of *G. rosea*, *G. margarita*, *G. intraradices* and sporocarps of *G. mosseae* were isolated by wet sieving (Gerdemann 1955) soil from a clover pot culture (*T. repens*) and were stored in water at 4°C until use. Spores of *G. mosseae* were obtained by dissecting the sporocarps and surface-sterilization (Mosse 1962). Spores were selected with the aid of a stereomicroscope and aseptically transferred to Petri dish with 10 ml of Gel-Gro. In the experiments ten replicates and 10 controls of each treatment were prepared. Ten surface sterilized spores of *G. rosea*, *G. margarita*, *G. mosseae* or *G. intraradices* were placed onto the surface of the medium. The Petri plates were sealed with parafilm to reduce dehydration and contamination risks and incubated in the dark at 25°C for 2 weeks. The spore germination, hyphal length, hyphal branches, the number of cluster of auxiliary cells of *Gigaspora* spores and the number of secondary spores of *Glomus mosseae* were determined. Hyphal length of germinated spores was assessed using the gridline intersect method (Marsh 1971). Experimental data were statistically analysed by an ANOVA and Tukey's test ($p = 0.05$). Each experiment was repeated at least twice.

Results

Microscopic observations of stained roots showed no presence of AM fungi in the uninoculated clover roots. In inoculated clover plants the percentage of root colonization reached 80+9%. The flavonoid 3,5,6,7,8-hydroxy-4′-methoxy flavone present in mycorrhizal and non-mycorrhizal clover roots exhibited no effect on any of the AM fungal parameters tested (data not shown).

Flavonoids from non-mycorrhizal clover roots

The flavonoid 3,7-hydroxy-4′methoxy flavone exhibited no effect on any of the presymbiotic steps of the AM fungi studied (data not shown).

As shown in Figure 1, the flavonoids RR4 and RR4–2 increased spore germination of *G. rosea* and *G. margarita* in the presence of all the doses tested, whereas they did not have any effect on the percentage of germination of *G. mosseae* and *G. intraradices*. The flavonoid NM7 did not affect the percentage of spore germination of any of the tested mycorrhizal fungi.

The flavonoids RR4 and RR4–2 increased the hyphal length of *G. rosea* in the presence of all tested doses, but the highest increase of the hyphal length of this endophyte was observed in the presence of 0.5 μM. The application of 0.5 μM of RR4 and RR4–2 increased the hyphal length of *G. margarita* spores whereas the application of 2 and 8 μM decreased the hyphal length. The hyphal length of *G. mosseae* and *G. intraradices* were not affected by the flavonoids RR4 and RR4–2. All doses of the flavonoid NM7 decreased the hyphal length of *G. rosea* but only the application of 8 μM of NM7 reduced the hyphal length of *G. margarita*. The flavonoid NM7 decreased the hyphal length of *G. mosseae* and *G. intraradices* spores (Figure 2).

As Figure 3 shows the flavonoids RR4 and RR4–2 increased the number of hyphal branches of *G. rosea* when applied at 0.5, 2, or 8 μM. Moreover, these flavonoids only increased the number of hyphal branches of *G. margarita* spores when 0.5 μM was applied. The number of hyphal branches of *G. mosseae* and *G. intraradices* spores was not

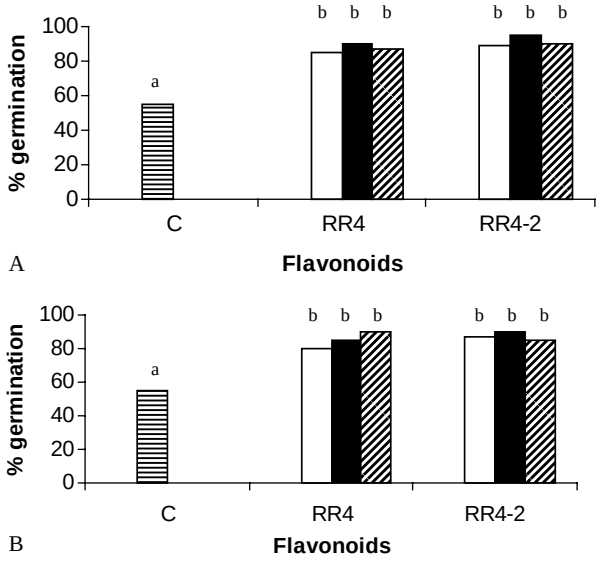

Figure 1. Effect of 5,6,7,8-hydroxy-4′-methoxy flavone (RR4) and 3,5,6,7,4′-hydroxy flavone (RR4–2) on the percentage of germination of (A) *Gigaspora rosea* and (B) *G. margarita*. Column values followed by the same letter are not significantly different as determined by Tukey's test ($p = 0.05$). ≡, Control without flavonoids; □, 0.5 μM of flavonoids; ■, 2 μM of flavonoids; and ▨, 8 μM of flavonoids.

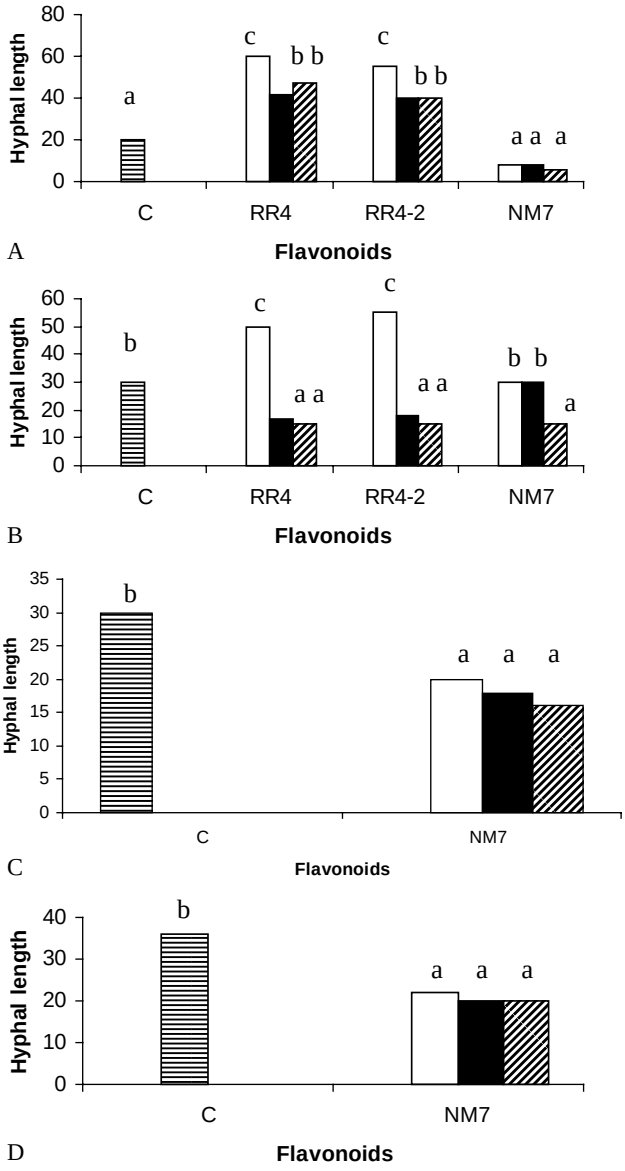

Figure 2. Hyphal length (mm) of (A) *Gigaspora rosea* and (B) *G. margarita* spores in presence of 5,6,7,8-hydroxy-4′-methoxy flavone (RR4), 3,5,6,7,4′-hydroxy flavone (RR4–2) and 5,6,7,8,9-hydroxy chalcone (NM7), and (C) *Glomus mosseae* and (D) *G. intraradices* spores in presence of NM7. Column values followed by the same letter are not significantly different as determined by Tukey's test ($p = 0.05$). ≡, Control without flavonoids; □, 0.5 μM of flavonoids; ■, 2 μM of flavonoids; and ▨, 8 μM of flavonoids.

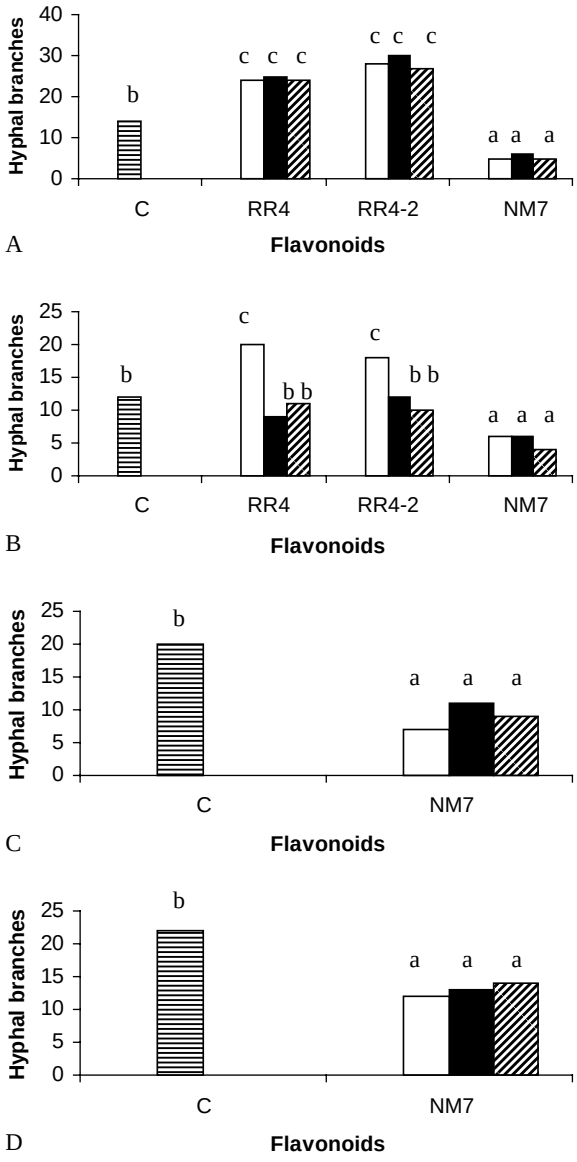

Figure 3. Number of hyphal branches of (A) *Gigaspora rosea* and (B) *G. margarita* spores in presence of 5,6,7,8-hydroxy-4′-methoxy flavone (RR4), 3,5,6,7,4′-hydroxy flavone (RR4–2) and 5,6,7,8,9-hydroxy chalcone (NM7), and (C) *Glomus mosseae* and (D) *G. intraradices* spores in presence of NM7. Column values followed by the same letter are not significantly different as determined by Tukey's test ($p = 0.05$). ≡, Control without flavonoids; □, 0.5 μM of flavonoids; ■, 2 μM of flavonoids; and ▨, 8 μM of flavonoids.

affected by the flavonoids RR-4 and RR4–2. The flavonoid NM7 decreased the number of hyphal branches from all AM fungi tested.

All doses of the flavonoids RR4 and RR4–2 increased the number of clusters of auxiliary cells of *G. rosea* but only 0.5 μM of these flavonoids increased the number of cluster of *G. margarita*. The flavonoid NM7 decreased the number of clusters of auxiliary cells of *G. rosea* at all doses tested and of *G. margarita* at the 8 and 2 μM doses. The number of secondary spores of *G. mosseae* was not affected by the flavonoids RR4 and RR4–2 but the application of 2 and 8 μM of NM7 decreased the number of secondary spores of this endophyte (Figure 4).

Flavonoids from mycorrhizal roots

Figure 5 show that quercetin increased the spore germination of *G. rosea* and *G. margarita* in the presence of the 2 and 8 μM doses, whereas this flavonoid did not affect the spore germination of *G. mosseae* and *G. intraradices* at any of the doses tested. Acacetin and rhamnetin showed no effect on the percentage of spore germination.

As can be observed in Figure 6, the hyphal length of *G. rosea* and *G. margarita* was increased by 2 μM of quercetin, but this flavonoid did not affect the hyphal length of *G. mosseae* and *G. intraradices*. Acacetin and rhamnetin inhibited the hyphal length of *G. rosea* at all doses tested, but only 2 and 8 μM of

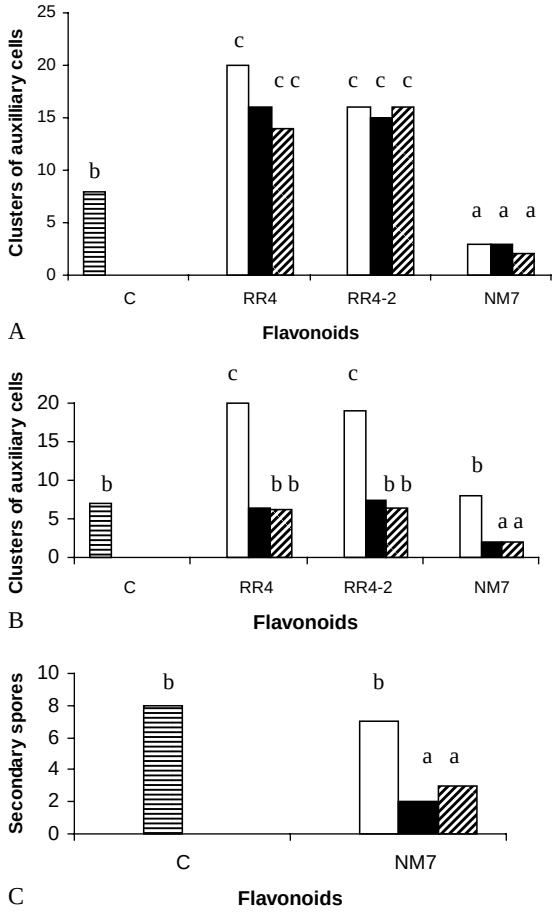

Figure 4. Number of clusters of auxiliary cells of (A) *Gigaspora rosea* and (B) *G. margarita* spores in presence of 5,6,7,8-hydroxy-4′-methoxy flavone (RR4), 3,5,6,7,4′-hydroxy flavone (RR4–2) and 5,6,7,8,9-hydroxy chalcone (NM7), and secondary spores of (C) *Glomus mosseae* spores in the presence of NM7. Column values followed by the same letter are not significantly different as determined by Tukey's test ($p = 0.05$). ≡, Control without flavonoids; □, 0.5 μM of flavonoids; ■, 2 μM of flavonoids; and ▨, 8 μM of flavonoids.

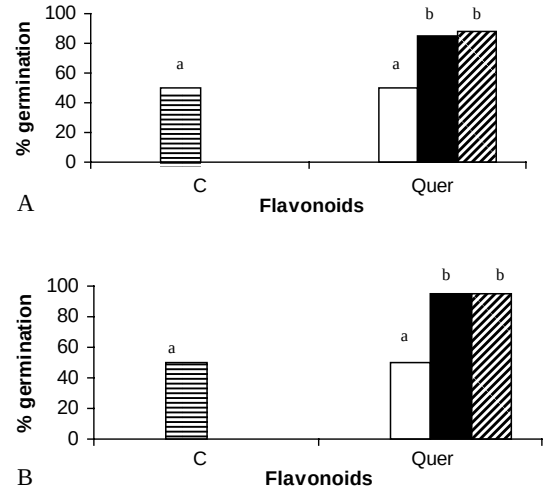

Figure 5. Effect of quercetin (Quer) on the percentage of germination of (A) *Gigaspora rosea* and (B) *G. margarita*. Column values followed by the same letter are not significantly different as determined by Tukey's test ($p = 0.05$). ≡, Control without flavonoids; □, 0.5 μM of flavonoids; ■, 2 μM of flavonoids; and ▨, 8 μM of flavonoids.

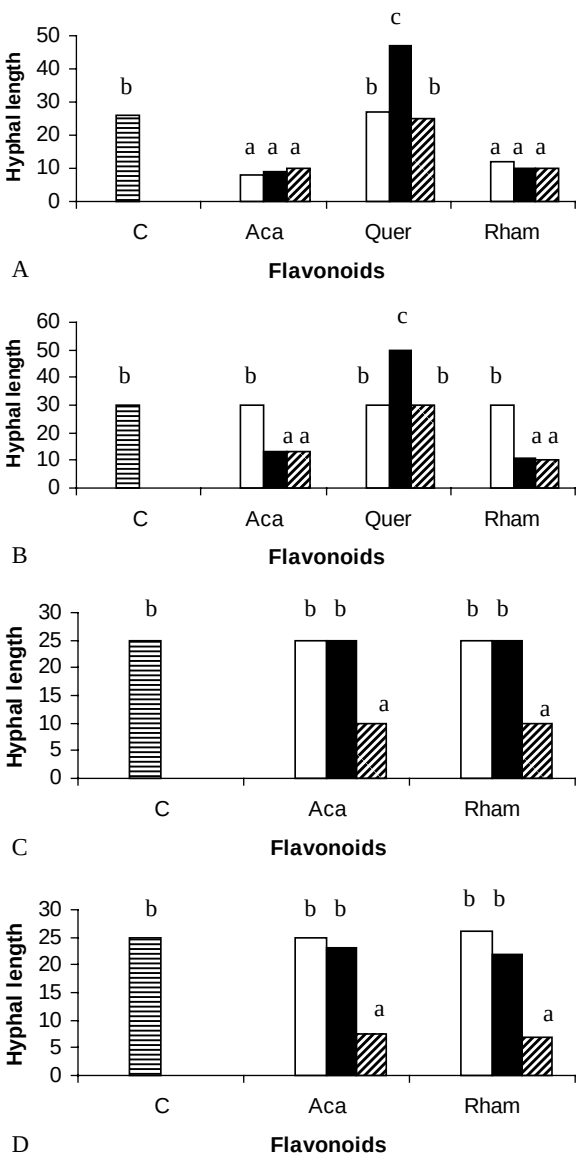

Figure 6. Hyphal length (mm) of (A) *Gigaspora rosea* and (B) *G. margarita* spores in the presence of acacetin (Aca), quercetin (Que) and rhamnetin (Rham), and (C) *Glomus mosseae* and (D) *G. intraradices* spores in presence of acacetin (Aca) and rhamnetin (Rham). Column values followed by the same letter are not significantly different as determined by Tukey's test ($p = 0.05$). ≡, Control without flavonoids; □, 0.5 μM of flavonoids; ■, 2 μM of flavonoids; and ▨, 8 μM of flavonoids.

these flavonoids decreased the hyphal length of *G. margarita*. Acacetin and rhamnetin decreased the hyphal length of *G. mosseae* and *G. intraradices* spores at 8 μM.

Figure 7 shows that quercetin at 2 μM increased the number of hyphal branches of *G. rosea* and *G. margarita*, while the same flavonoid decreased the number of hyphal branches of *G. margarita* at 8 μM. However, quercetin did not affect the number of hyphal branches of *G. mosseae* and *G. intraradices*. All doses of acacetin and rhamnetin inhibited the number of hyphal branches of *G. rosea* but, only the application of 2 and 8 μM decreased hyphal branching of *G. margarita*. At 8 μM acacetin and rhamnetin decreased the number of hyphal branches of *G. mosseae* and *G. intraradices.*

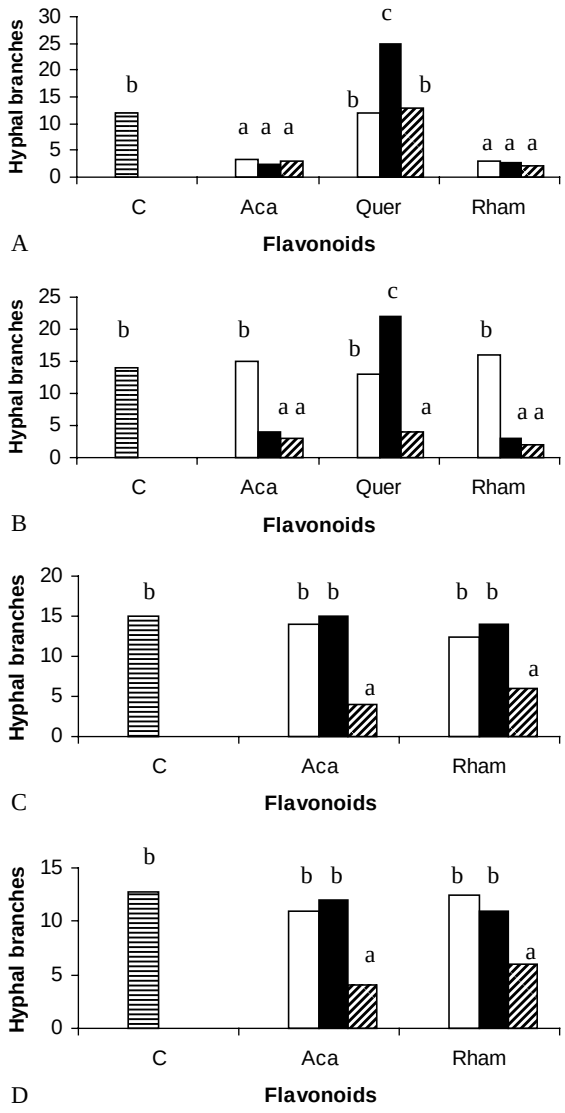

Figure 7. Number of hyphal branches of (A) *Gigaspora rosea* and (B) *G. margarita* spores in presence of acacetin (Aca), quercetin (Que) and rhamnetin (Rham), and (C) *Glomus mosseae* and (D) *G. intraradices* spores in presence of acacetin (Aca) and rhamnetin (Rham). Column values followed by the same letter are not significantly different as determined by Tukey's test ($p = 0.05$). ≡, Control without flavonoids, □, 0.5 μM of flavonoids; ■, 2 μM of flavonoids; and ▨, 8 μM of flavonoids.

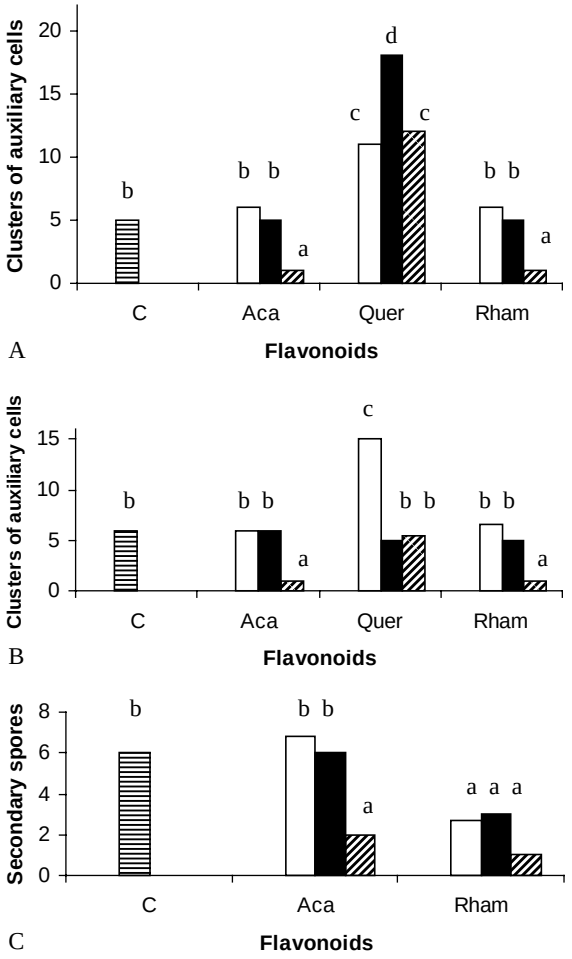

Figure 8. Number of clusters of auxiliary cells of (A) *Gigaspora rosea* and (B) *G. margarita* spores in presence of acacetin (Aca), quercetin (Que) and rhamnetin (Rham), and secondary spores of (C) *Glomus mosseae* spores in presence of acacetin (Aca) and rhamnetin (Rham). Column values followed by the same letter are not significantly different as determined by Tukey's test ($p = 0.05$). ≡, Control without flavonoids; □, 0.5 μM of flavonoids; ■, 2 μM of flavonoids; and ▨, 8 μM of flavonoids.

Quercetin increased the number of cluster of auxiliary cells of *G. rosea,* when applied at the concentration 0.5, 2 and 8 μM and those of G. *margarita* at 0.5 μM. On the other hand, the application of 8 μM of acacetin and rhamnetin decreased the number of clusters of auxiliary cells of *G. rosea* and *G. margarita*. The number of secondary spores of *G. mosseae* was decreased with 8 μM acacetin and by all doses of rhamnetin, whereas it was unaffected by all quercetin concentrations (Figure 8).

Discussion

Recently, Ponce et al. (2004) reported that, depending on the mycorrhizal status in roots of white clover plants, different flavonoids can be detected, suggesting the disappearance and/or the new synthesis of flavonoids during mycorrhization as an indicator for their involvement in the mycorrhizal regulation.

In the study of Ponce et al. (2004), the flavonoids RR4, RR4–2 and NM7 were only detected in non-mycorrhizal roots. Interestingly, in our work RR4 and RR4–2 stimulated most of the presymbiotic stages of *Gigaspora*. Recently, Akiyama and coworkers (2002) reported that a flavonoid, stimulating root colonization by AMF, was only present in non-mycorrhizal but not in mycorrhizal melon roots. From these results they postulated a regulatory effect of this compound for root colonization at an early stage of the symbiosis, but not at a later stage. In our study the two flavonoids RR4 and RR4–2 showed a similar pattern (only detectable in non-mycorrhizal roots and a stimulatory effect on AM fungi) pointing towards a role of these two compounds during early stages of the AM establishment, but not during later stages.

The other flavonoid detectable only in non-mycorrhizal white clover roots, NM7 (5,6,7,8,9-hydroxy chalcone) inhibited the presymbiotic development of all AM fungi tested. Chalcones are a family of compounds which are considered precursors in the flavonoid synthesis (Winkel-Shirley 2002). Enzymes implicated in the chalcone synthesis, such as chalcone synthase have been linked with the defence response in plants during the development of the AM symbiosis and the inhibition of AM hyphal development by some chalcones has been reported before (Bécard et al. 1992, Garcia-Garrido & Ocampo 2002).

In contrast to RR4, RR4–2 and NM7, quercetin, acacetin and rhamnetin were only detectable in mycorrhizal, but not in non-mycorrhizal white clover roots (Ponce et al. 2004). Our observation of a stimulatory effect of quercetin (on the *Gigaspora* species) on spore germination and/or hyphal growth of AM fungi is well documented (Tsai & Phillips 1991, Bécard et al. 1992, Chabot et al. 1992, Kape et al. 1992a, Bel-Rhlid et al. 1993, Poulin et al. 1997). However, no data on the effect of acacetin and rhamnetin on AM fungi are available. These two flavonoids are widely distributed in plants (Santos et al. 1995, Santos & Salatino 2000) and exhibit antifungal and bacteriostatic activities (Serra-Bonvehi et al. 1994, Fawe et al. 1998). Interestingly, both newly synthesized compounds exhibit an inhibitory effect on nearly all fungal parameters of both AM genera (*Gigaspora* and *Glomus*) tested, pointing towards a possible implication in the autoregulation of mycorrhization (Pinior et al. 1999, Vierheilig et al. 2000a, Vierheilig et al. 2000b, Catford et al. 2003, Vierheilig 2004b). This could mean that once the AM symbiosis is well established the observed suppression of further root colonization by AM fungi is at least partially due to newly synthesized compounds exhibiting an inhibitory effect on AM fungi such as acacetin and rhamnetin in white clover. Two compounds (3,7-hydroxy-4′methoxy flavone and 3,5,6,7,8-hydroxy-4′-methoxy flavone) showed no effect on any of the AM fungal parameters determined, thus a regulatory role of these two compounds in the AM symbiosis is unlikely.

The effect of flavonoids on AM fungi is not only dependent on the flavonoid type but also on the flavonoid concentration. A concentration-dependent effect of flavonoids on AMF has been reported before (Tsai & Phillips 1991, Bécard et al. 1992, Baptista & Siqueira 1994). Our results showed no clear link between the concentration of the tested flavonoids and their effect on the AM fungal parameters tested. For example quercetin at a dose of 2 μM stimulated hyphal growth and hyphal branching of two *Gigaspora* species, however, a higher (8 μM) and lower (0.5 μM) dose showed no effect.

Based on a number of reports, Vierheilig et al. (1998) suggested an AM fungal genus or even species-specific effect of flavonoids. Our data confirm this hypothesis. An AM fungal genus-specific effect could be detected with the flavonoids RR4 and RR4–2. In the presence of the two compounds the hyphal length, the number of branches and of clusters of auxiliary cells of the two *Gigaspora* species were increased, but none of the two flavonoids showed an effect on any of the fungal parameters of the two *Glomus* species.

A species-specific effect was observed with RR4–2. The hyphal length and the number of branches of *G. rosea* were increased by 2 μM and 8 μM of RR4–2 whereas with *G. margarita* the two parameters were decreased by the two concentrations of the flavonoid.

The data obtained with RR4 even indicated that the compound might be involved in one stage of the fungal regulation, but not in another, as RR4 increased the percentage of spore germination of *G. margarita*, however, inhibited the hyphal growth (at 2 μM and 8 μM) and showed no effect on hyphal branching and the cluster formation of auxiliary cells.

Hyphal branching of AM fungi has been described as one of the first events in host recognition by AM fungi during the presymbiotic phase (Giovannetti et al. 1996). The flavonoids tested in our study had variable effects on branching of *Gigaspora* and *Glomus*. The compounds RR4, RR4–2 and quercetin increased hyphal branching of the *Gigaspora* species but had no effect on hyphal branching of the *Glomus* species. The similar effect of some flavonoids on hyphal growth, hyphal branching and the formation of cluster of auxiliary cells/secondary spores indicates a more general role of these flavonoids in the mycorrhizal regulation.

To summarize, our results indicate that flavonoids present only in mycorrhizal or non-mycorrhizal root are involved in the regulation of mycorrhization at different stages. This hypothesis is strengthened by their different effect on several AM fungal growth parameters. Furthermore, our study provides more data on the AMF genus/species specificity of flavonoids. This is the first report linking compound changes in mycorrhizal roots directly to regulatory processes of the AM symbiosis, however, further studies are needed to elucidate the exact mechanisms involved.

Acknowledgements

Financial support for this study was provided by the Comisión Interministerial de Ciencia y Tecnología, Spain and by CONICET, Argentina.

References

Akiyama K, Matsuoka H, Hayashi H. 2002. Isolation and identification of a phosphate deficiency-induced C-glycosylflavonoid that stimulates arbuscular mycorrhiza formation in melon roots. Mol Plant Microbe Interaction 15:334–340.

Baptista MJ, Siqueira JO. 1994. Efeito de flavonóides na germinação e no crescimento assimbiótico de fungos micorrí-

zicos vesículo-arbusculares. Rev Brasil Fisiolog Vegetal 6:127–134.

Bécard G, Douds DD, Pfeffer PE. 1992. Extensive in vitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. Appl Environment Microbiol 58:821–825.

Bel-Rhlid R, Chabot S, Piché Y, Chenevert 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.

Bentivenga SP, Morton JB. 1995. A monograph of the genus *Gigaspora*, incorporating developmental patterns of morphological characters. Mycologia 87:720–732.

Catford JG, Staehelin C, Lerat S, Piché Y, Vierheilig H. 2003. Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors. J Exp Botany 54:1481–1487.

Chabot S, Bel-Rhlid T, Chenevert R, Piche Y. 1992. Hyphae 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 Phytologist 122:461–467.

Fawe A, Abou-Zaid M, Menzies J, Belanger RR. 1998. Silicon-mediated accumulation of flavonoid phytoalexins in cucumber. Phytopathology 88:396–401.

Fracchia S. 2002. Hongos saprotrofos del suelo como microorganismos auxiliares de la micorrización. PhD Thesis, Universidad de Buenos Aires, Argentina.

Garcia-Garrido JM, Ocampo JA. 2002. Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. J Exp Botany 53:1377–1386.

Gerdemann JW. 1955. Relation of a large soil-borne spore to phycomycetous mycorrhizal infections. Mycologia 47:619–632.

Gianinazzi-Pearson V, Branzanti B, 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.

Giovannetti M, Mosse B. 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytologist 84:489–500.

Giovannetti M, Sbrana C, Citernesi AS, Avio L. 1996. Analysis of factor involved in fungal recognition responses to host-derived signals by arbuscular mycorrhizal fungi. New Phytologist 133:65–71.

Harley JL, Harley EL. 1987. A check-list of mycorrhiza in the British flora. New Phytologist (Suppl.) 105:1–102.

Hewitt EJ. 1952. Sand water culture methods used in the study of plant nutrition. Wallingford, UK: Commonwealth Agriculture Bureau, Technical communication. p 22.

Juge C, Samson J, Bastien C, Vierheilig H, Coughlan A, Piche Y. 2002. Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intraradices*: A critical cold-storage period. Mycorrhiza 12:37–42.

Kape R, Parniske M, Brandt S, Werner D. 1992a. Isoliquiritigenin, a strong nod gene- and glyceollin resistance-inducing flavonoid from soybean root exudate. Appl Environment Microbiol 58:1705–1710.

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

Larose G, Chenevert R, Moutoglis P, Gagne S, Piche Y, Vierheilig H. 2002. Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. J Plant Physiol 159:1329–1339.

Marsh BAB. 1971. Measurement of length in random arrangements of lines. J Applied Ecol 8:265–270.

Mingorance MD. 2002. Focused microwave-assisted digestion of vegetal materials for the determination of essential mineral nutrients. Ann Bioanalyt Chem 373:153–158.

Morandi D. 1996. Occurrence of phytoalexins and phenolic compounds in endomycorrhizal interactions, and their potential role in biological control. Plant Soil 185:241–251.

Morandi D, Branzanti B, Gianinazzi-Pearson V. 1992. Effect of some plant flavonoid on in vitro behavior of an arbuscular mycorrhizal fungus. Agronomie 12:811–816.

Mosse B. 1962. The establishment of vesicular arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520.

Nair MG, Safir GR, Siqueira JO. 1991. Isolation and identification of vesicular-arbuscular mycorrhizal stimulatory compounds from clover (*Trifolium repens*) roots. Appl Environment Microbiol 57:434–439.

Phillips JM, Hayman DS. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycologic Soc 55:158–161.

Phillips DA, Tsai SM. 1992. Flavonoids as plant signals to the rhizosphere microbes. Mycorrhiza 1:55–58.

Pinior A, Wyss U, Piche Y, Vierheilig H. 1999. Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation. Can J Botany-Rev Can Botanique 77:891–897.

Poulin MJ, Simard J, Catford JG, Labrie F, Piche Y. 1997. Response of symbiotic endomycorrhizal fungi to estrogens and antiestrogens. Mol Plant Microbe Interaction 10:481–487.

Ponce MA, Scervino JM, Erra Balsells R, Ocampo JA, Godeas A. 2004. Flavonoids from shoots and roots of *Trifolium repens* (white clover) grown with and without the arbuscular mycorrhizal fungus *Glomus intraradices*. Phytochemistry 65:1925–1930.

Santos DYAC, Salatino MLF. 2000. Foliar flavonoids of Annonaceae from Brazil: Taxonomic significance. Phytochemistry 55:567–573.

Santos DYAC, Salatino MLF, Salatino A. 1995. Flavonoids of species of Cuphea (Lythraceae) from Brazil. Biochem Systemat Ecol 23:99–103.

Serra-Bonvehi J, Ventura-Coll F, Escola-Jorda R. 1994. The composition, active components and bacteriostatic activity of propolis in dietetics. J Am Oil Chem 71:529–532.

Siqueira JO, Safir GR, Nair MG. 1991. Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. New Phytologist 118:87–93.

Tsai SM, Phillips DA. 1991. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl Environment Microbiol 57:1485–1488.

Vierheilig H. 2004a. Regulatory mechanisms during the plant-arbuscular mycorrhizal fungus interaction. Can J Botany 82:1166–1176.

Vierheilig H. 2004b. Further root colonization by arbuscular mycorrhizal fungi in already mycorrhizal plants is suppressed after a critical level of root colonization. J Plant Physiol 161:339–341.

Vierheilig H, Garcia-Garrido JM, Wyss U, Piché Y. 2000a. Systemic suppression of mycorrhizal colonization of barley roots already colonized by AM fungi. Soil Biol Biochem 32:589–595.

Vierheilig H, Maier W, Wyss U, Samson J, Strack D, Piché Y. 2000b. Cyclohexenone derivative- and phosphate-levels in split-root systems and their role in the systemic suppression of mycorrhization in precolonized barley plants. J Plant Physiol 157:593–599.

Vierheilig H, Bago B, Albrecht C, Poulin MJ, Piche Y. 1998. Flavonoids and arbuscular-mycorrhizal fungi. In: Manthey JA, Buslig BS, editors. Flavonoids in the living system. New York: Plenum Press. p 9–33.

Vierheilig H, Piche Y. 2002. Signalling in arbuscular mycorrhiza: facts and hypotheses. In: Buslig B, Manthey J, editors. Flavonoids in cell function. New York: Kluwer Academic/Plenum Press. p 23–39.

Winkel-Shirley B. 2002. Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5:218–223.