



Arbuscular mycorrhizal colonization of tomato by *Gigaspora* and *Glomus* species in the presence of root flavonoids

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Summary

The effect of flavonoids isolated from arbuscular mycorrhizal (AM) colonized and noncolonized clover roots on the number of entry points and percentage of root colonization of tomato (*Lycopersicon esculentum* L.) by *Gigaspora rosea*, *G. margarita*, *Glomus mosseae* and *G. intraradices* symbionts was determined. With fungi of both genera, a correlation between the number of entry points and the percentage of root colonization was found in the presence of some of the tested flavonoids. The flavonoids acacetin and rhamnetin, present in AM clover roots, inhibited the formation of AM penetration structures and the AM colonization of tomato roots, whereas the flavonoid 5,6,7,8,9-hydroxy chalcone, which could not be detected in AM clover root, inhibited both parameters. The flavonoid quercetin, which was present in AM clover roots, stimulated the penetration and root colonization of tomato by *Gigaspora*. However, the flavonoids 5,6,7,8-hydroxy-4'-methoxy flavone and 3,5,6,7,4'-hydroxy flavone, which was not found in AM clover root, increased the number of entry points and the AM colonization of tomato roots by *Gigaspora*. These results indicated that flavonoids could be implicated in the process of regulation of AM

Abbreviations: AM:arbuscular mycorrhiza; BAFC:Buenos Aires Fungal Collection

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colonization in plant root, but its role is highly complex and depend not only on flavonoids, but also on AM fungal genus or even species.

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Introduction

Flavonoids constitute a family of compounds that can be found in most plants and play an important role as signal molecules between plant and soil microorganisms such as legumes and *Rhizobium* symbiosis (Phillips and Tsai, 1992; Vierheilig and Piche, 2002).

In the arbuscular mycorrhizal (AM) symbiosis the plant regulate the fungal development before and after the contact and penetration of the fungus inside the root. Flavonoids can influence the preinfective state of the AM fungus (Akiyama et al., 2002; Guenouné et al., 2002), and some of these compounds can increase the level of AM colonization of roots (Siqueira et al., 1991; Kape et al., 1992; Morandi et al., 1992; Vierheilig et al., 1998). In a previous study it has been shown that colonization by AM fungi changes the flavonoid pattern in clover roots. 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 could be detected in non-AM colonized roots, whereas these compounds were absent in roots colonized by AM fungi. 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 newly synthesized in AM colonized clover root (Ponce et al., 2004). The flavonoid 3,5,6,7,8-hydroxy-4'-methoxy flavone was not affected by AM colonization (Ponce et al., 2004). The flavonoids 5,6,7,8,9-hydroxy chalcone, 3,7-hydroxy-4'-methoxy flavone, 5,6,7,8-hydroxy-4'-methoxy flavone, 3,5,6,7,4'-hydroxy flavone and 3,5,6,7,8-hydroxy-4'-methoxy flavone have been detected recently in plants and its role in plant physiology and in plant-microbial interactions are unknown (Ponce et al., 2004). There are some data available on acacetin and rhamnetin, which have been reported to exhibit an antimicrobial activity (Serra-Bonvehi et al., 1994; Fawe et al., 1998). In several in vitro experiments the flavonoid quercetin showed a stimulatory effect on AM fungi (Tsai and Phillips, 1991; Bécard et al., 1992; Chabot et al., 1992; Kape et al., 1992; Bel-Rhliid et al., 1993; Poulin et al., 1997), whereas some chalcone molecules exhibited a negative effect (Bécard et al., 1992; Larose et al., 2002).

A number of studies showed that depending on the compound applied to AM fungal inoculated plants, root colonization is enhanced, decreased or not affected (Nair et al., 1991; Siqueira et al., 1991; Xie et al., 1995; Vierheilig et al., 1998). Vierheilig and Piché (2002) tested the effect of biochanin A, a flavonoid isolated from clover roots grown under phosphate stress, on root colonization of clover and tomato. Interestingly, they found that the compound not only stimulated root colonization in clover, the plant where it was isolated from, but also in tomato plants, suggesting that the observed stimulatory effect did not depend on the host plant but on the AM fungi. Thus, the application of flavonoids isolated from other plants to tomato plants seems a valid system to test their effect on colonization parameters.

To our knowledge, apart from quercetin, nothing is known on the effect of the flavonoids discussed above on AM fungi. The new synthesis and suppression of some flavonoids in clover roots colonized by the AM fungus *G. intraradices* suggests a specific role of these plant molecules in the regulation process of the AM symbiosis (Vierheilig and Piche, 2002). In the present study we examined the effect of the molecules described above on the number of entry points and the percentage of root length colonization of tomato by *Gigaspora* and *Glomus*.

Materials and methods

The flavonoids 5,6,7,8,9-hydroxy chalcone (NM7), 3,7-hydroxy-4'-methoxy flavone (RR7), 5,6,7,8-hydroxy-4'-methoxy flavone (RR4) and 3,5,6,7,4'-hydroxy flavone (RR4-2) were isolated from non-inoculated clover root, 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 inoculated clover root and the flavonoid 3,5,6,7,8-hydroxy-4'-methoxy flavone (NM8) was isolated from AM colonized and noncolonized clover roots as described by Ponce et al. (2004). For that 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 (mg kg^{-1}) 331 N, 9.5 P (NaHCO_3 -extractable) and 3.2 Ca (Mingorance, 2002). This was steam-ster-

ilized and mixed with sterilized perlite at a proportion of 1:1 (V:V). Seeds of white clover (*Trifolium repens*) were surface-sterilized with ethanol:water 1:1 during 90s, bleach:water 1:1 during 90s and thoroughly rinsed with sterilized water and sown in moistened sand. After germination, uniform seedlings were planted and grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, $400 \text{ E m}^{-2} \text{ s}^{-1}$, 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 and Hayman, 1970), and the percentage of root colonization was measured by the line intersect method (Giovanetti and 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 each 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. Each sample was then redistributed between water–organic solvents of increasing polarity. We obtained four extracts: from hexane (defatting), ethyl acetate, 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 60F₂₅₄ aluminium sheets (Merck). The solvent system used was EtOAc–CH₂Cl₂–HCO₂H (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 H₂SO₄ in 197 ml of MeOH (Bel-Rhliid 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-d₆ 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.

The effect of the flavonoids of clover plants roots on the number of AM entry points and percentage of root length colonization of tomato (*Lycopersicon esculentum*) by *Gi rosea* (BEG 9), *Gi margarita* (J7) from BAFC, *G. mosseae* (BEG 12) and *G. intraradices* (DAOM 197,198) spores were tested in 5 cm diameter Petri dishes using a monosporic culture technique (Fracchia et al., 2001). Spores of *Gi margarita* (J7) Becker & Hall were isolated from Ciudad Universitaria soil (Fracchia, 2002), in the province of Buenos Aires (Argentina) and identified (Bentivenga and Morton, 1995). Spores of *Gi. rosea*, *Gi. margarita*, *G. intraradices* and sporocarps of *G. mosseae* were isolated by wet sieving (Gerdemann, 1955) soil from a leek pot culture (*T. repens*) and were stored on water at 4 °C until used. The spores of *G. mosseae* were obtained by dissecting the sporocarps. All spores were surface-sterilized (Mosse, 1962). One surface sterilized spore was transferred with a sterilized Pasteur capillary pipette to a 5 cm diam Petri dish with 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, OH, USA). Petri dishes were incubated at 25 °C for 8 days and spore germination and hyphal development were observed under a binocular microscope. Petri dishes contaminated with other microorganisms were discarded. Petri dishes with hyphal length about 5 mm for *Glomus* or 2 cm for *Gigaspora* strains were selected.

Tomato seedlings were grown in 5 cm diam Petri dishes with 10 ml of autoclaved (120 °C, 20 min) vermiculite–perlite mixture (1/1, v/v). The vermiculite and perlite were previously sieved through 500 µm mesh. Seeds were surface sterilized with 10% sodium hypochlorite for 2 min and one seed was sown on each vermiculite:perlite Petri dish. Plants were grown in a chamber with Sylvania incandescent and cool-white lamp 400–700 nm, with a 16/8 h day/dark cycle at 25/19 °C and 50% relative humidity.

All flavonoids tested were dissolved in absolute ethanol to obtain 4 mM stock solutions. The flavonoids were filtered through a disk of filter paper and sterilized twice by filtration through a

0.20 μm Millipore membrane. The effect of 0.05%, 0.1%, 0.5%, 1% 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 that did not have effect in 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. These concentrations of flavonoids were selected for being the concentrations more suitable that have a significant effect on the different steps of the AM fungal development (Morandi, 1996; Vierheilig et al., 1998). Petri plates with 0.05% ethanol or without ethanol were used as controls.

The flavonoids and the content of a vermiculite-perlite dish with 2-week-old tomato seedling were transferred at the same time onto the Gel-Gro medium with the germinated spore. The hyphal development was observed every 2 days through the bottom of the Petri dish under the binocular microscope. All plants inoculated with single germinated spores developed mycorrhizas. With this system non-destructive observation of hyphal development and hyphal contact of AM fungi with the plant root was possible.

Plants were harvested and the root system was cleared and stained (Phillips and Hayman, 1970). Material from each replicate was cut into 1 cm segments that were mixed and repeatedly subdivided to yield random samples of 50 root segments. These were mounted on slides and examined under a compound microscope at $\times 160$ magnifications, and the number of entry points (appresoria) per 30 cm of root was estimated (Ocampo et al., 1980). The percentage of root colonization was measured by the line intersect method (Giovannetti and Mosse, 1980). In the experiment, 20 replicates per treatment and controls were used. Ten replicates per treatment and control plants were harvested when hyphal contact of the AM fungi with the plant root was observed (usually about 2 weeks after seedling transplanting), and the number of entry points was assessed. The rest of the plants were harvested 6 weeks after transplanting and the percentage of root colonization was measured.

Experimental data were statistically analysed by a one-way analysis of variance and standard deviation ($P = 0.05$) to detect significant differences between treatment means. Percentage data were subjected to arcsine transformation before analysis. Each experiment was repeated at least twice.

Results

Microscopic observations of stained roots showed no presence of AM fungi either in uninoculated clover roots and only AM structures in AM inoculated clover plants which reached $80 \pm 9\%$ of root length colonization.

Under our experimental conditions the dry weights of tomato plants grown in Petri dishes were similar in all treatments (data not shown).

The flavonoid NM 8 isolated from mycorrhizal and non-mycorrhizal colonized roots did not affect the number of entry points and the percentage of root length colonized by the symbionts *Gi. rosea*, *Gi. margarita*, *G. mosseae* and *G. intraradices* (Fig. 1).

Flavonoids from AM inoculated roots

Figure 1 shows that the number of entry points and the percentage of tomato root colonized by *Gi. rosea* increased when 2 or 8 μM of the flavonoid quercetin were applied. On the other hand, all doses of the flavonoid acacetin and rhamnetin inhibited the number of entry points and AM root colonization of tomato by *Gi. rosea*. *Gi. margarita* produced more number of entry points and higher percentage of root length colonization when 2 μM of quercetin was applied, while 8 and 2 μM of acacetin and rhamnetin decreased the number of entry points and the mycorrhizal colonization caused by this symbiont (Fig. 1). Quercetin had no effects on the number of entry points and on the percentage mycorrhizal colonization of root by the *Glomus* species. Acacetin and rhamnetin decreased the number of entry points and the AM root length colonization by *G. mosseae* or *G. intraradices* when 2 or 8 μM of these flavonoids were applied (Fig. 1).

Flavonoids from non-AM inoculated roots

Figure 2 shows that the number of entry points and the percentage of tomato root length colonized by *Gi. rosea* were not affected by the flavonoid RR7. However, the flavonoids RR4 and RR4-2 increased the number of entry points and AM root length colonization by *Gi. rosea* when 0.5 and 2 μM doses were applied. The flavonoid NM7 decreased the number of entry point and the percentage of AM root length colonization of this symbiont when 0.5, 2 and 8 μM were applied. The application of 0.5 μM of the flavonoids RR4 and RR4-2 increased the number of entry points and the AM root length colonization by *Gi. margarita*, whereas the flavonoid RR7 did not affect these fungi (Fig. 2). On the other hand, the application of NM7 decreased the

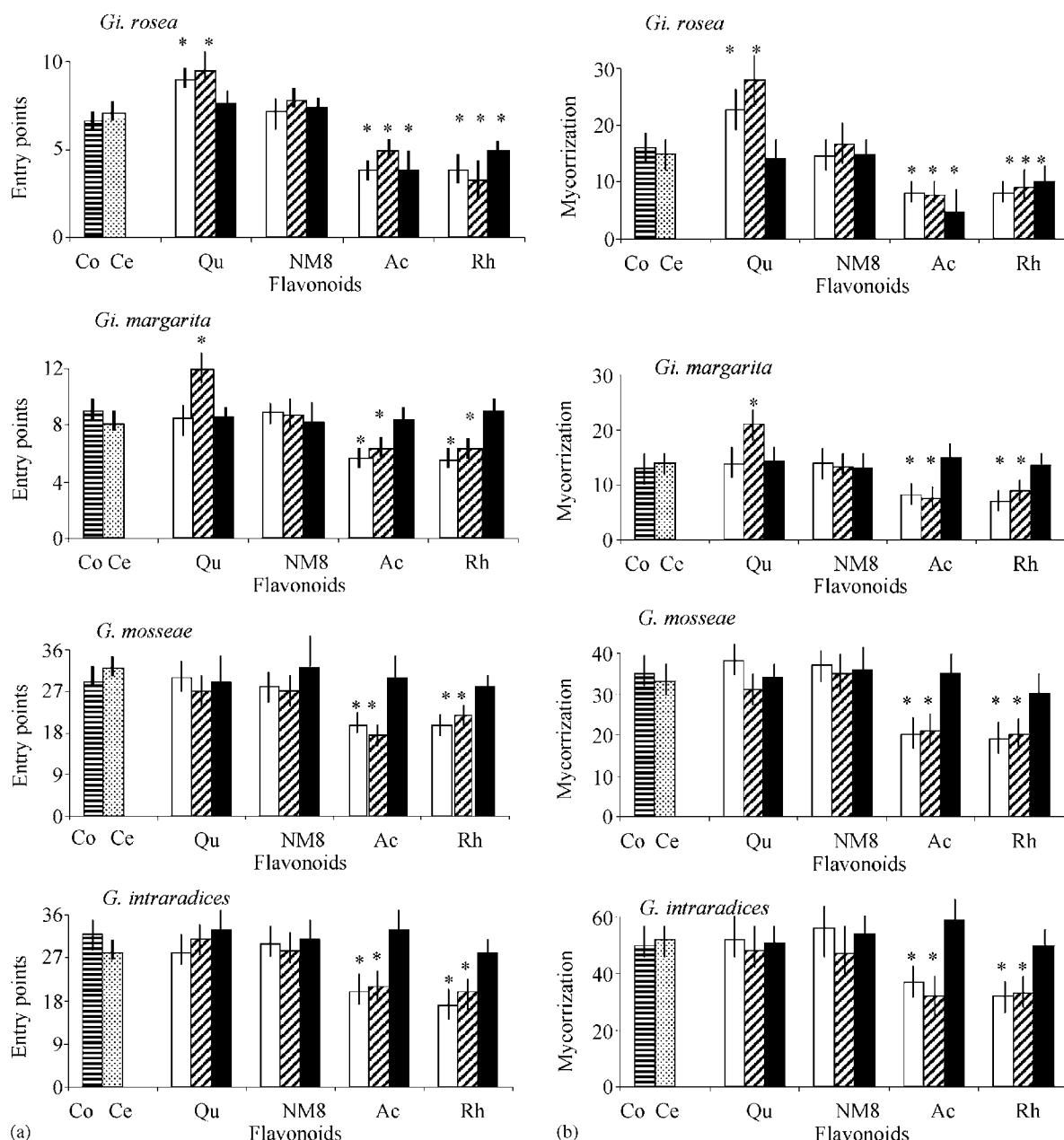


Figure 1. Effect of 3,5,6,7,8-hydroxy-4'-methoxy flavone (NM8), acacetin, quercetin and rhamnetin on the number of entry points per 30cm of root (Fig. 1a) and on the percentage of root length colonization (Fig. 1b) of tomato by *Gigaspora rosea*, *Gi. margarita*, *Glomus mosseae* and *G. intraradices* spores. Vertical bars are the standard deviation ($P = 0.05$). *Significantly different from control. Co = Control without flavonoids and without ethanol; Ce = Control plus 0.05% ethanol. (□) 8 μ M of flavonoids, (▨) 2 μ M of flavonoids and (■) 0.5 μ M of flavonoids.

number of entry points and the AM root length colonization of *Gi. margarita* when 0.5, 2 and 8 μ M were applied. None of the flavonoids tested affected the number of entry points or the AM root length colonization produced by *G. mosseae* or *G. intraradices* symbionts, exception was the flavonoid NM7 that decreased the number of entry points and AM root length colonization caused by both *Glomus* species (Fig. 2).

Discussion

Root metabolites are implicated in the differentiation and recognition process of plant-AM fungal interaction (Bécard and Piché, 1989; Paula and Siqueira, 1990). The fungal penetration structures and the first events of fungal development into the roots are stimulated by compounds from the host cell (Paula and Siqueira, 1990). We provide

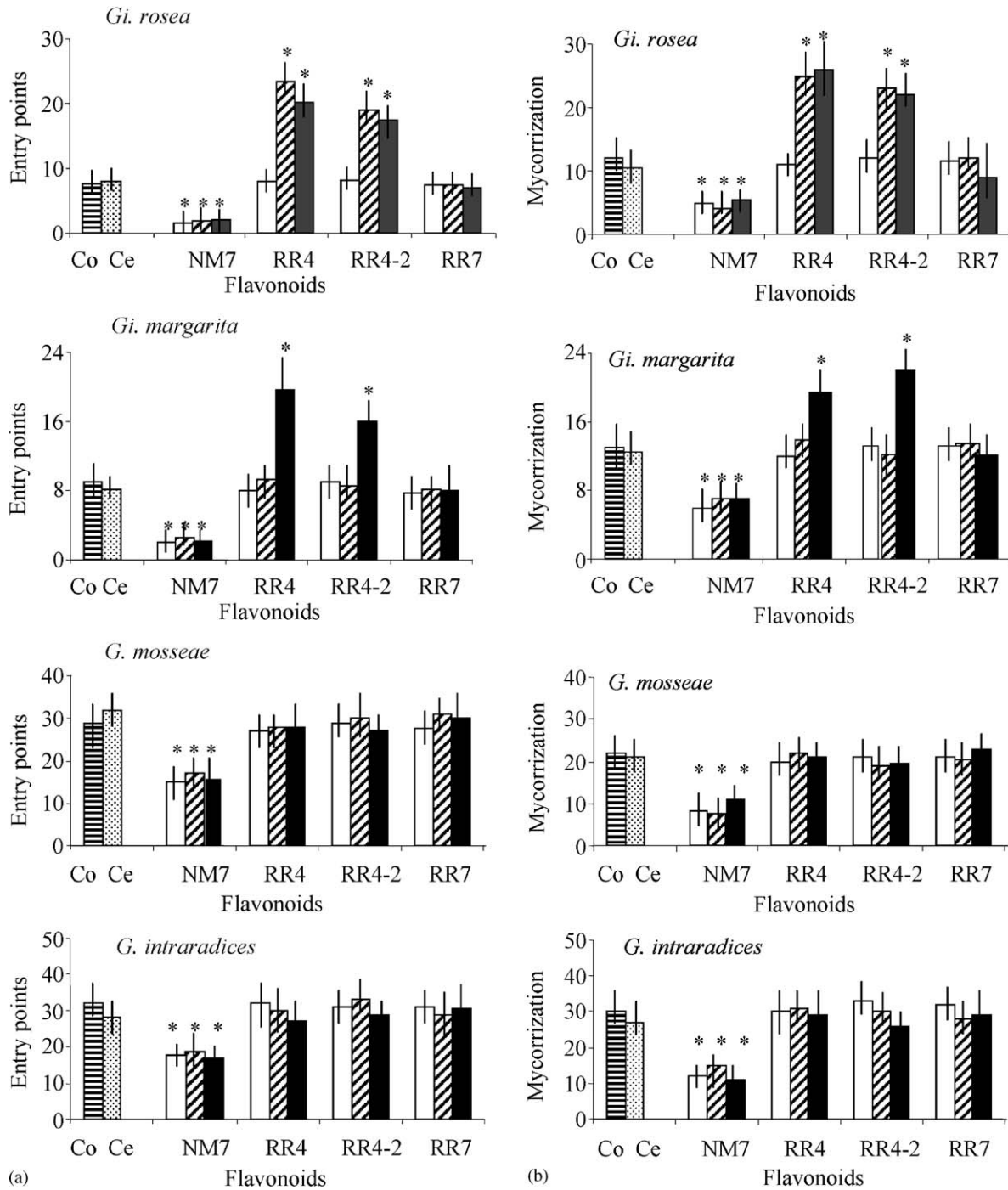


Figure 2. Effect of 5,6,7,8,9-hydroxy chalcone (NM7), 3,7-hydroxy-4'-methoxy flavone (RR7), 5,6,7,8-hydroxy-4'-methoxy flavone (RR4) and 3,5,6,7,4'-hydroxy flavone (RR4-2) on the number of entry points per 30cm of root (Fig. 1a) and on the percentage of root length colonization (Fig. 1b) of tomato by *Gigaspora rosea*, *Gi. margarita*, *Glomus mosseae* and *G. intraradices* spores. Vertical bars are the standard deviation ($P = 0.05$). *Significantly different from control. Co = Control without flavonoids and without ethanol; Ce = Control plus 0.05% ethanol. (□) 8 μM of flavonoids, (▨) 2 μM of flavonoids and (■) 0.5 μM of flavonoids.

evidence that flavonoids isolated from AM colonized and non-colonized clover roots are active substances in the AM colonization process when exogenously applied. We observed inhibitory and stimulatory effects of flavonoids on the number

of entry points and on the percentage of root length colonization of tomato root by *Glomus* and *Gigaspora*, suggesting their implication in the regulation process of the AM symbiosis into the root.

The importance of entry points on penetration, development into roots and effectiveness of the AM fungi has been described (Buee et al., 2000). A correlation between the number of entry points and the percentage of root length colonization reached by *Gigaspora* or *Glomus* symbionts in the presence of the flavonoids tested was found. Our results suggest that these flavonoids may influence the extension of AM colonization of roots mainly through its effects on the formation of entry points.

Colonization events during the establishment of AM fungi in the root are not static and metabolic changes in the AM colonized root that regulate further colonization cannot be excluded (Larose et al., 2002). The presence of compounds inhibitory to AM fungi in AM colonized plants which are possibly involved in the regulation of posterior AM fungal development inside the root has been suggested, but the nature of these compounds is not yet known (Vierheilig and Piché, 2002). However, plant secondary metabolites such as flavonoids might act as regulators in plant–fungus interactions during the precolonization and the cell-to-cell stage of the development of the symbiosis (Siqueira et al., 1991; Akiyama et al., 2002; Larose et al., 2002; Vierheilig and Piché, 2002). We found that exogenous application the newly synthesized flavonoids acacetin and rhamnetin in clover roots after AM colonization inhibited the formation of AM penetration structures and the development of the fungus inside of the test plant root. These results point towards a possible involvement of these two flavonoids in the auto-regulation process of the AM symbiosis. Antifungal and bacteriostatic activities of rhamnetin and acacetin respectively have been reported (Serra-Bonvehi et al., 1994; Fawe et al., 1998). However, no data on the effect of acacetin and rhamnetin on AM fungi are available yet. As after application the root colonization of all AM fungal species tested was inhibited, the inhibitory effect of both compounds on AM fungi seems a general effect and not species or genus specific.

In clover root colonized with *G. intraradices* the flavonoids, NM7 (5,6,7,8,9-hydroxy chalcone) disappeared (Ponce et al., 2004). Chalcones are a family of compounds which are considered precursors in the synthesis of flavonoids (Winkel-Shirley, 2002). Enzymes involved in chalcone synthesis, such as chalcone synthase, have been related with the defence response in plants during the development of the AM symbiosis (Garcia-Garrido and Ocampo, 2002). The flavonoid NM7 inhibited the number of entry points and the percentage of root length colonization of the test plant by *Gigaspora* and *Glomus* species. Therefore,

the presence of 5,6,7,8,9-hydroxy chalcone in non-AM colonized clover roots and the disappearance of this flavonoid in mycorrhizal clover roots indicates that the AM fungus *G. intraradices* induced a mechanism, which reduces this inhibitory molecule in the mycorrhizal root.

In mycorrhizal clover roots the flavonoid quercetin was synthesized. Quercetin is the most widely distributed flavonoid in plants where mycorrhizal symbiosis is present and has a positive effect on germination or hyphal length of AM spores (Tsai and Phillips, 1991; Bécard et al., 1992; Chabot et al., 1992; Kape et al., 1992; Bel-Rhliid et al., 1993; Poulin et al., 1997). Vierheilig et al. (1998) postulated a certain specificity of the effect of quercetin on AM fungi. In an in vitro experiment it was demonstrated that the compound stimulated hyphal growth of a *Gigaspora* species, but not of a *Glomus* species. Interestingly, our results show that quercetin stimulated the penetration and development of *Gigaspora* but not of *Glomus*. However, the disappearance of the flavonoids RR4 and RR4-2 in root colonized by *G. intraradices*, which increased the development of the number of entry points and the AM colonization of roots by *Gigaspora* species indicates that the role of flavonoids in the process of regulation of AM fungal development in plant root is very complex.

The flavonoid effects depended not only on the flavonoid type but also on their concentration. In fact, a close relationship between AM root colonization and flavonoid concentration has been found (Siqueira et al., 1991). We observed that 2 and 8 μM of quercetin and RR4 increased the number of entry points and AM colonization of root by *Gi. rosea*, but only 2 μM of the quercetin and 0.5 μM of the same flavonoid were able to increase these parameters caused by *Gi. margarita*. Our results indicate that there is no relationship between flavonoid concentration and AM colonization or the number of entry point by AM fungi. However, the genus or the species of the AM fungi seems to be an important factor for the effect of a flavonoid on the development of the mycorrhizal symbiosis (Vierheilig and Piché, 2002). The penetration and development of root colonization by *Gigaspora* were stimulated by the flavonoids RR4, RR4-2 and quercetin whereas *Glomus* species were unaffected by these flavonoids. Interestingly, the flavonoids NM7, acacetin and rhamnetin were inhibitory on both genera. These results suggest that the flavonoids produced by mycorrhizal roots have a less specific effect on AM fungi than the flavonoid of the non-mycorrhizal root. Our results also suggest that there was not relationship between the fact that the clover roots were colonized with *G.*

intraradices and the effect of the individual flavonoids on the different AM fungi tested.

In conclusion, different flavonoids seem to be involved differently in the regulation process of the AM symbiosis. Their mode of action appears complex, and depends not only on flavonoid type but also on AM fungal symbiont.

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