The effect of flavones and flavonols on colonization of tomato plants by arbuscular mycorrhizal fungi of the genera *Gigaspora* and *Glomus*

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Abstract: No clear data are available on how flavonoids from different chemical groups affect root colonization by arbuscular mycorrhizal fungi (AMF) and whether flavonoids affecting the presymbiotic growth of AMF also affect root colonization by AMF. In the present work, we compared the effect of flavones (chrysin and luteolin) and flavonols (kaempferol, morin, isorhamnetin, and rutin) on root colonization (number of entry points and degree of root colonization) of tomato plants (*Lycopersicum esculentum* L.) with the effect of these flavonoids on the presymbiotic growth of these AMF, which has been reported in a recent study. With all tested AMF (*Gigaspora rosea*, *Gigaspora margarita*, *Glomus mosseae,* and *Glomus intraradices*) a correlation between the number of entry points and the percentage of root colonization was found. When the number of entry points was high, root colonization was also enhanced. Application of the flavones chrysin and luteolin and of the flavonol morin increased the number of entry points and the degree of colonization,whereas the flavonols kaempferol, isorhamnetin, and rutin showed no effect. These results show that in contrast to their effect on the presymbiotic growth of the AMF on the level of root colonization, the tested flavonoids do not exhibit a genus- and species-specificity. Moreover, comparison of our data with the data obtained by J.M. Scervino, M.A. Ponce, R. Erra-Bassells, H. Vierheilig, J.A. Ocampo, and A. Godeas. (2005*a*. J. Plant Interact. **15**: 22–30) indicates that a positive effect on the hyphal growth of AMF does not necessarily result in an enhanced AM root colonization, further indicating that the mode of action of flavonoids at the level of root colonization is more complex.

Key words: arbuscular mycorrhiza, flavonoids, flavones, flavonols, signal molecules, symbiosis.

Résumé : Il n'existe pas de données claires sur la façon dont les flavonoïdes appartenant à différents groupes chimiques affectent la colonisation racinaire par les champignons mycorhizes à arbuscule (CMA) et si les flavonoïdes qui affectent la croissance pré-symbiotique des CMA affectent aussi la colonisation racinaire par les CMA. Dans ce travail, nous avons comparé les effets exercés par des flavones (chrysine, lutéoline) et de flavonols (kaempférol, morine, isorhamnétine, rutine) sur la colonisation des racines (nombre de points d'entrée et degré de colonisation des racines) de plants de tomate (*Lycopersicum esculentum* L.) avec les effets que ces flavonoïdes exercent sur la croissance pré-symbiotique des CMA, rapportés récemment. Une corrélation entre le nombre de points d'entrée et le pourcentage de colonisation racinaire a été trouvée chez tous les CMA testés (*Gigaspora rosea*, *Gigaspora margarita*, *Glomus mosseae* et *Glomus intraradices*). Lorsque le nombre de points d'entrée était élevé, le pourcentage de colonisation des racines était aussi élevé. L'application de chrysine et de lutéoline (flavones) et de morine (flavonol) a augmenté le nombre de points d'entrée et le degré de colonisation, alors que les autres flavonols (kaempférol, isorhamnétine et rutine) n'avaient aucun effet. Ces résultats démontrent que contrairement à leurs effets sur la croissance pré-symbiotique des CMA, les flavonoïdes testés ne manifestent pas de spécificité de genre ou d'espèce en ce qui a trait au degré de colonisation des racines. De plus, la comparaison de nos résultats avec ceux obtenus par J.M. Scervino, M.A. Ponce, R. Erra-Bassells, H. Vierheilig, J.A. Ocampo, and A. Godeas (2005*a*. J. Plant Interact. **15**: 22–30), révèle qu'un effet positif des CMA sur la croissance des hyphes ne résulte pas nécessairement en une augmentation de la colonisation des racines par les MA, indiquant que le mode d'action des flavonoïdes sur la colonisation des racines est plus complexe.

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Introduction

The arbuscular mycorrhiza (AM) is the most prevalent symbiosis in the plant kingdom. This symbiosis between soil-borne fungi of the phylum Glomeromycota (Schüßler et al. 2001) and their host plants is the result of a complex molecular dialogue between the host and the arbuscular mycorrhizal fungus (AMF) before and after the penetration of the root by the fungus (Vierheilig and Piché 2002; Vierheilig 2004), resulting in various metabolic changes such as the alteration of the flavonoid pattern in the roots of the host plants (Harrison and Dixon 1993; Akiyama et al. 2002; Larose et al. 2002). Thus, flavonoids, which are known as key signals in the rhizobial symbiosis, have been suggested to be also involved in signalling during AM symbiosis (Phillips and Tsai 1992; Vierheilig et al. 1998*b*; Vierheilig and Piché 2002; Vierheilig 2004).

A clear effect of a number of flavonoids on the presymbiotic AM fungal development has been reported (Akiyama et al. 2002; Guenoune et al. 2002; Scervino et al. 2005*a*, 2005*c*; see review by Vierheilig et al. 1998*b*).

Depending on the chemical group flavonoids exhibit stimulatory or inhibitory effects or no effect on AM hyphal growth. Flavonols, which have a hydroxyl group on position 3 of the C aromatic ring and at least one hydroxyl group on the β-aromatic ring, stimulate AM hyphal growth. In contrast, flavones, with no hydroxyl group on position 3 of the C aromatic ring or no hydroxyl group on the β-aromatic ring, act similarly to luteolin and chysin in displaying no effect on AM hyphal growth (Bécard et al. 1992; Chabot et al. 1992; Morandi et al. 1992; Vierheilig et al. 1998*b*; Scervino et al. 2006).

Owing to differing experimental conditions that include different growth conditions, application of compounds at different times, and different compound concentrations, the available data are difficult to compare, and it is not clear whether the observed differences are due to differing experimental conditions, to an AM genera and (or) species specificity, or to flavonoids from different chemical groups.

Most recently, Scervino et al. (2005*a*) reported that the flavones chrysin and luteolin and the flavonol morin show a stimulatory effect on the presymbiotic hyphal growth of several *Glomus* and *Gigaspora* species, whereas the flavonols kaempferol, isorhamnetin, and rutin stimulate hyphal growth of *Gigaspora* but not *Glomus* species.

Although the effect of compounds on the presymbiotic hyphal growth of AMF is interesting, for the establishment of a successful AM symbiosis their effect on root colonization is of importance. However, only few studies have been performed on the effect of flavonoids on root colonization by AMF. In these studies it has been shown that exogenous application of flavonoids to plants can enhance the level of root colonization by AMF (Nair et al. 1991; Siqueira et al. 1991*a*, 1991*b*; Xie et al. 1995; Vierheilig et al. 1998*b*; Scervino et al. 2005*b*); results of the latter study indicated that certain flavonoids identified in mycorrhizal and nonmycorrhizal roots of clover plants are involved in the regulation of the AM symbiosis. However, no clear data are available yet on how flavonoids from different chemical groups affect AMF. As well, it is unresolved whether the stimulatory or inhibitory effects reported from studies on presymbiotic hyphal growth of AMF are clear indicators for similar stimulatory or inhibitory effects of these compounds on AM root colonization.

To better understand these aspects, we presently studied the effect of flavonoids with different chemical structure (the flavones chrysin and luteolin, and the flavonols kaempferol, morin, isorhamnetin, and rutin), whose effect on presymbiotic growth of the AMF types *Gigaspora rosea*, *Gigaspora margarita*, *Glomus mosseae*, and *Glomus intraradices* was recently examined (Scervino et al. 2005*a*) on AM root colonization by these four AMF types.

Materials and methods

The effect of the flavonoids chrysin, isorhamnetin, kaempferol, luteolin, morin, and rutin on the number of AMF entry points and the degree of root length colonization of tomato (*Lycopersicum esculentum* L. 'Marglobe') by four different AMF was tested. The AMF types used were *Gigaspora rosea* Nicolson & Schenck (BEG 9; La Banque Européenne des Glomales; International Institute of Biotechnology; Kent; Great Britain), *Gigaspora margarita* Becker & Hall (J7) from the Buenos Aires Fungal Collection (BAFC), *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe (BEG 12), and *Glomus intraradices* Smith & Schenck (DAOM 197198; Agriculture and Agri-Food Canada, Ottawa, Canada).

Spores of *Gigaspora margarita* (J7) were isolated from Ciudad Universitaria soil (Fracchia 2002) in the province of Buenos Aires, Argentina, and identified as described previously by Bentivenga and Morton (1995). Spores of *Gigaspora rosea*, *Gigaspora margarita*, *Glomus intraradices*, and sporocarps of *Glomus mosseae* were isolated by wet sieving (Gerdemann 1955) soil from a sorghum pot culture (*Sorghum vulgare*) and were stored in water at 4 °C for at least 4 weeks before use. The spores of *Glomus mosseae* were obtained by dissecting the sporocarps. All spores were surface-sterilized for 20 min with an aqueous solution (2% *m*/*v*) of chloramine T plus 200 μg/mL streptomycin and a trace of surfactant and thoroughly washed afterwards (Mosse 1962).

Tomatoes were inoculated using the monosporic culture technique previously described (Fracchia et al. 2001). Briefly, five surface sterilized spores were transferred with a sterilized Pasteur capillary pipette to a 5 cm diameter Petri dish with an autoclaved solution of 10 mL of 10 mol/L 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (pH 7) plus 0.04 g of Gel-Gro™ (ICN Biochemicals, Aurora, Ohio, 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 for further experimentation.

Tomato seedlings were grown in 5 cm diameter Petri dishes with 10 mL of an 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 in each Petri dish. Plants were grown in a chamber with illumination provided by an incandescent and cool-white lamp (Sylvania, Danvers, Massachusetts, USA) operating at 400 nmol/(m^2·s), 400–700 nm, with a 16 h light : 8 dark cycle at 25 °C : 19 °C and 50% relative humidity.

All flavonoids were dissolved in absolute ethanol to obtain 4 mmol/L stock solutions. The flavonoid solutions were filtered (filter paper) and sterilized twice by passing through a 0.20 μm membrane (Millipore, Billerica, Massachusetts, USA). The effect of 0.05%, 0.1%, 0.5%, and 1% ethanol–water (*v*/*v*) 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 highest one 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™ (4 g/L; MP Biomedicals, Solon, Ohio, USA) at a final concentration of 0.5 and 2 μmol/L in 0.05% ethanol. These concentrations of the flavonoids were selected because most of them were shown previously to have a significant effect on different steps of AM fungal development (Morandi et al. 1992; Vierheilig et al. 1998*b*). Petri dishes with 0.05% ethanol or without ethanol were used as controls.

The flavonoids and the content of a vermiculite–perlite dish with a 2-week-old tomato seedling were transferred at the same time onto the Gel-Gro™ medium with the germinated spores. The hyphal development was observed by binocular microscopy every 2 days through the bottom of the Petri dish. All plants inoculated with single germinated spores developed mycorrhizas. Nondestructive observation of hyphal development and hyphal contact of AM fungi with the plant root was possible with this system (Fracchia et al. 2001).

In the experiment 20 replicates per treatment 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. Ten replicates were harvested 6 weeks after transplanting and the percentage of root colonization was measured.

After harvesting the root system was cleared and stained (Phillips and Hayman 1970). The roots from each replicate were cut into 1 cm segments that were mixed and repeatedly subdivided to yield random samples of 30 root segments. These were mounted on slides and examined using a compound microscope at 160× magnification and the number of entry points (appressoria) per 30 cm of root was determined (Ocampo et al. 1980). The percentage of root colonization was measured by the line intersect method (Giovannetti and Mosse 1980).

Experimental data were statistically analyzed by a one-way analysis of variance and Tukey's test (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

The water control treatment and the 0.05% ethanol exhibited the same effect on all fungal parameters studied (data not shown). Thus, we included only the values of the water control treatment in the figures. In addition, the dry weight of tomato plants grown in Petri dishes was similar in all treatments (data not shown).

The number of entry points of *Gigaspora rosea* and *Gigaspora margarita* in tomato roots in the presence of 0.5 and 2 μmol/L of isorhamnetin and kaempferol was similar to the control without flavonoids (Figs. 1A and 1B). The application of 0.5 and 2 μmol/L chrysin, luteolin, and morin clearly increased the number of entry points of *Gigaspora rosea* and *Gigaspora margarita*. Rutin increased the number of entry points of *Gigaspora margarita* but not those of *Gigaspora rosea*. The application of 0.5 μmol/L of chrysin and morin significantly increased the number of entry points of *Glomus mosseae*, while luteolin increased the number of entry points of this endophyte when applied at 2 μmol/L. Chrysin increased the number of entry points of *Glomus intraradices* when applied at 0.5 and 2 μmol/L, luteolin when applied at 2 μmol/L, and morin when applied at 0.5 μmol/L.

Looking at the effect of the tested flavonoids on root colonization we observed a similar pattern. The application of 0.5 and 2 μmol/L of chrysin, luteolin, and morin increased the colonization of tomato roots by *Gigaspora rosea* and *Gigaspora margarita* (Fig. 2). However, the percentage of roots colonized by *Gigaspora rosea* and *Gigaspora margarita* in the presence of isorhamnetin and kaempferol was similar to the control plants. Rutin increased the AM colonization of tomato by *Gigaspora margarita* but not by *Gigaspora rosea*.

The AM colonization of tomato roots by *Glomus mosseae* or by *Glomus intraradices* in the presence of isorhamnetin, kaempferol, and rutin was similar to the control (without flavonoids). Chrysin and morin increased the colonization of tomato root by *Glomus mosseae* when applied at 0.5 μmol/L, while luteolin increased root colonization when applied at 2 μmol/L. Chrysin increased the AM colonization of tomato root by *Glomus intraradices* when applied at 0.5 and 2 μmol/L, whereas the application of 2 μmol/L of luteolin and 0.5 μmol/L of morin increased the colonization tomato root by this endophyte.

Discussion

After spore germination the AMF grows towards the host plant (Vierheilig et al. 1998*a*; Sbrana and Giovannetti 2005), finally penetrating the root and forming its intraradical structures. The importance of entry points for penetration, formation of the AM, and effectiveness of the AM fungi has been described previously (Smith and Read 1997). Most recently, a close relationship between the number of entry points and the degree of colonization was reported (Scervino et al. 2005*b*). In the latter study, after flavonoid application to to-

Fig. 1. Effect of chrysin, luteolin, isorhamnetin, kaempferol, morin, and rutin on the number of entry points for colonization by *Gigaspora rosea* (A), *Gigaspora margarita* (B), *Glomus mosseae* (C), and *Glomus intraradices* (D) on tomato roots. Bars with the same letter above them indicate column values that are not significantly different, as determined by Tukey's test ($P = 0.05$). Control (hatched bars), 2 μmol/L of flavonoids (white bars), 0.5 μmol/L of flavonoids (black bars).

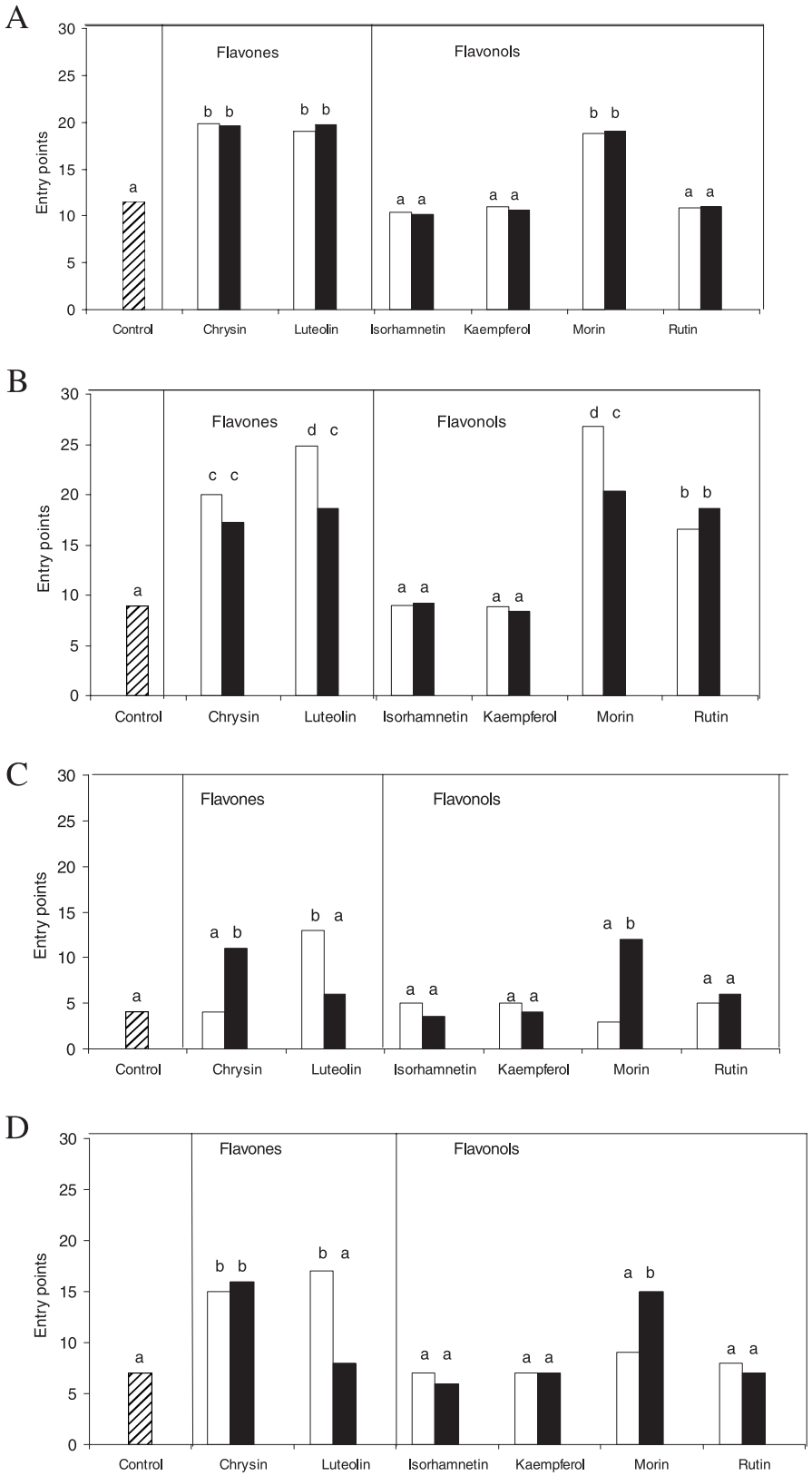
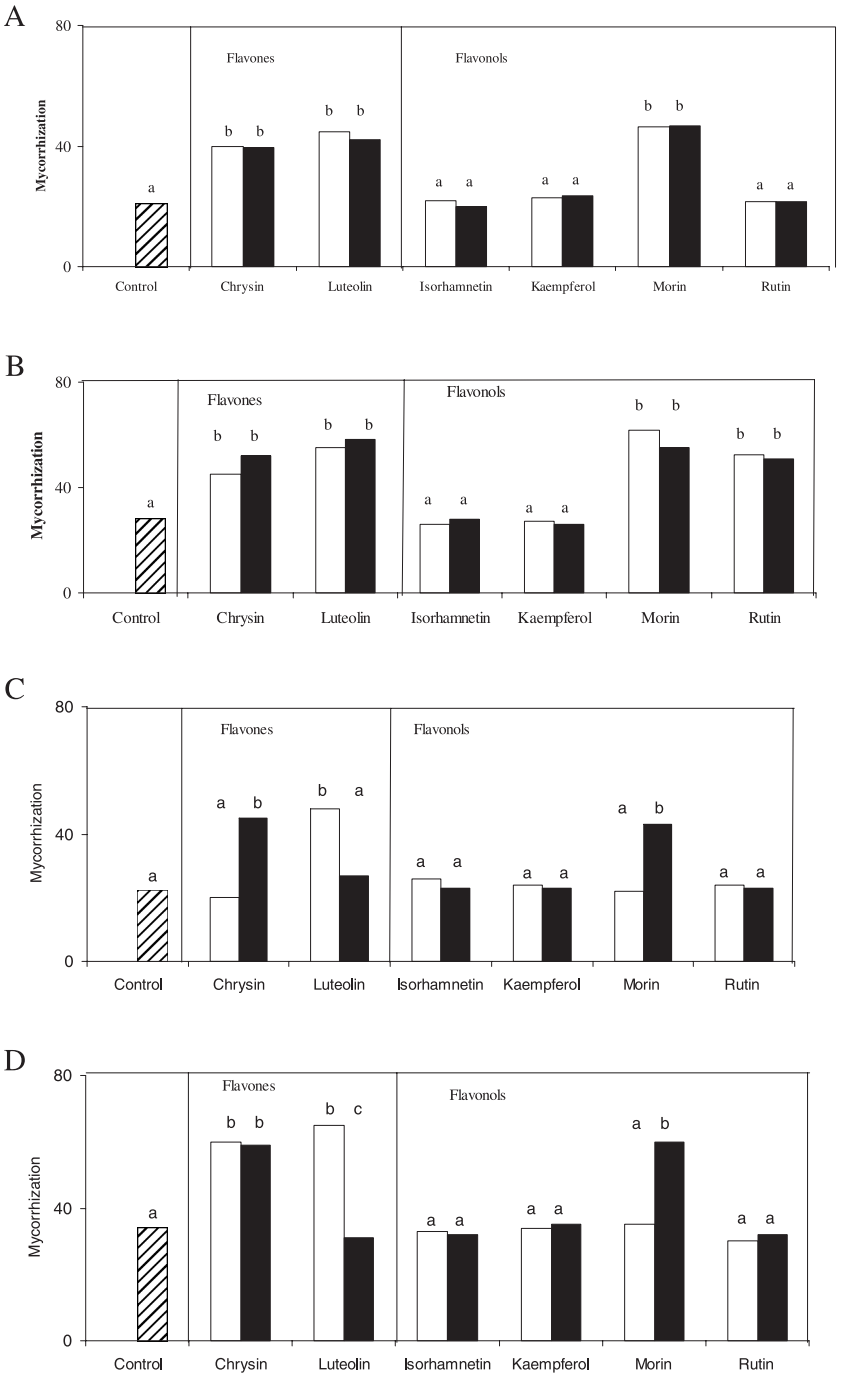


Fig. 2. Effect of chrysin, luteolin, isorhamnetin, kaempferol, morin, and rutin on the percentage of root length colonization of tomato by *Gigaspora rosea* (A), *Gigaspora margarita* (B), *Glomus mosseae* (C), and *Glomus intraradices* (D). Bars with the same letter above them indicate column values that are not significantly different, as determined by Tukey's test ($P = 0.05$). Control (hatched bars), 2 µmol/L of flavonoids (white bars), 0.5 µmol/L of flavonoids (black bars).



mato plants inoculated with *Gigaspora* or *Glomus* species an increased number of entry points always resulted in an enhanced degree of root colonization. We found an identical pattern. A higher number of entry points in the presence of the flavonoids crysin, luteolin, morin, and rutin always resulted in an enhanced colonization rate by the tested *Gigaspora* or *Glomus* species. Thus, the data previously presented (Scervino et al. 2005*b*) and our results strongly suggest that flavonoids influence the extension of AM colonization of roots mainly through effects on the formation of entry points.

The establishment of the AM symbiosis is the result of a complex exchange of signals between the host and the AMF. In analogy to the rhizobium–legume interaction, in the AM association flavonoids may be signalling compounds (Phillips and Tsai 1992; Vierheilig et al. 1998*b*). Several chemical groups within the flavonoids such as the isoflavones, flavones, flavonols, and flavanones affect AMF dif-

ferently; whereas flavonols in general have been reported to stimulate AM hyphal growth, reports on the effect of flavones on the presymbiotic AM hyphal growth are contradictory, ranging from a stimulating to an inhibitory effect on AMF (Morandi et al. 1992; Vierheilig et al. 1998*b*).

Little information is available on the effect of flavones and flavonols on AM root colonization. Chrysin exhibits a stimulatory effect on root colonization by a *Glomus* species (Siqueira et al. 1991*a*). This stimulatory effect of the flavone chrysin on root colonization was confirmed in our study with both *Glomus* and *Gigaspora* species. Moreover, we observed that the flavone luteolin and the flavonol morin are also stimulating substances for the AM colonization with both tested AM genera when exogenously applied, whereas the flavonol rutin exhibited a stimulatory effect only with *Gigaspora margarita*, and the flavonols kaempferol and isorhamnetin did not affect root colonization.

These data on the effect of flavones and flavonols on AM root colonization show a completely different pattern then data on the effect of the two flavonoid groups on the presymbiotic hyphal growth of AMF. The effect of flavones on the presymbiotic growth of AMF ranges from stimulation to negligable effect to inhibition (Vierheilig et al. 1998*b*; Scervino et al. 2005*a*), whereas we found a clear stimulatory effect on root colonization of the tested flavones. The picture was less clear with the tested flavonols, which showed no effect or stimulation of AM root colonization. From the obtained results we can conclude that the effect of flavones and flavonols on AMF differs on the level of presymbiotic hyphal growth and on the level of arbuscular mycorrhizal root colonization.

No literature on the effect of exogenously applied morin on root colonization is available. However, luteolin applied at higher concentrations than in our experiment is inactive in combination with *Glomus intraradices* (Siqueira et al. 1991*a*). A closer look at these apparently contradictory results shows that while we found a stimulatory effect at all tested concentrations of luteolin with the two *Gigaspora* species, the stimulatory effect on the two *Glomus* species depended on the concentration. This indicates that the experimental set-up could be an important factor for studies on the bioactivity of compounds and results obtained with differing experimental set-ups are not always comparable.

The flavonol rutin increased the number of entry points and the percentage of colonization of tomato by *Gigaspora margarita* but not those of *Gigaspora rosea*, *Glomus mosseae*, or *Glomus intraradices*. Presymbiotic stimulation of *Gigaspora* development by kaempferol has been reported (Bécard et al. 1992; Chabot et al. 1992), but we observed that kaempferol as well as isorhamnetin had no effect on the number of entry points and percentage of colonization of tomato by *Gigaspora* or *Glomus*.

An effect on AM fungi not only depends on the flavonoid but also on the tested concentration (Vierheilig et al. 1998*b*). Different concentrations of flavonoids can affect AM fungal development differently (Vierheilig et al. 1998*b*; Siqueira et al. 1991*a*). Looking at the different concentrations applied we observed a certain genus- and species-specificity. Chrysin applied at 0.5 and 2 μmol/L increased the number of entry points and AM colonization of roots by *Gigaspora rosea*, *Gigaspora margarita*, and *Glomus intraradices* but only 0.5 μmol/L of the same flavonoid increased these parameters with *Glomus mosseae*. Moreover, root penetration and development of colonization by *Gigaspora* were stimulated by all doses of the flavonoid morin whereas *Glomus* species were increased only in the 0.5 μmol/L treatment. A clear specificity could be observed with rutin because it increased the number of entry points and colonization formed by *Gigaspora margarita* only.

The stimulatory effect of certain flavonoids on root colonization might be simply explained by a stimulation of the presymbiotic growth of the tested fungi resulting in an enhanced number of entry points, which finally results in a higher root colonization. Looking at morin and rutin, this hypothesis seems confirmed. Morin, which stimulates presymbiotic growth of AMF (Chabot et al. 1992; Baptista and Siqueira 1994; Scervino et al. 2005*a*), clearly enhanced root colonization in our experiment. Rutin, which exclusively stimulates presymbiotic growth of *Gigaspora margarita* but not of the other AMF such as *Gigaspora rosea*, *Glomus mosseae*, and *Glomus intraradices* (Scervino et al. 2005*a*), in our experiment also enhanced exclusively the number of entry points and AM colonization by *Gigaspora margarita*.

Interestingly, looking at the data obtained with the other flavonoids we saw a different pattern. Kaempferol has been reported to stimulate the presymbiotic growth of *Gigaspora* species (Bécard et al. 1992; Chabot et al. 1992; Scervino et al. 2005*a*), but not of *Glomus* species (Scervino et al. 2005*a*). If the enhanced root colonization we observed is because of a simple stimulatory effect on hyphal growth, we would expect that kaempferol would enhance the root colonization by *Gigaspora* species but not by *Glomus* species. However, kaempferol showed no effect on root colonization by the two *Gigaspora* species, or by the two *Glomus* species tested. The same pattern as with kaempferol was observed with isorhamnetin. Moreover, chrysin, which in our experiment stimulated root colonization, inhibits the presymbiotic growth of AMF (Bécard et al. 1992; Chabot et al. 1992). This means that an enhanced or reduced AM root colonization in presence of a flavonoid is not because of a simple stimulatory or inhibitory effect of the flavonoid on the hyphal growth of AMF.

To summarize, our data show that the effect of flavones and flavonols on AMF differs on the level of presymbiotic hyphal growth and on the level of arbuscular mycorrhizal root colonization. Moreover, in contrast to the reported genus- and species-specific effect of the flavonoids on the presymbiotic hyphal growth, we do not observe a clear specificity when looking at their effect on root colonization. Interestingly, our data exclude a simple hyphal growth stimulating effect as being responsible for the observed enhanced root colonization in presence of certain flavonoids, indicating that the mode of action of flavonoids at the level of root colonization is more complex.

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