DEVELOPMENT OF ARBUSCULAR MYCORRHIZAL FUNGI IN THE PRESENCE OF DIFFERENT PATTERNS OF *Trifolium repens* SHOOT FLAVONOIDS

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ABSTRACT

We tested the effects of the flavonoid 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone (NMHTV) isolated from shoots of non arbuscular mycorrhizal (AM) inoculated clover, and of the flavonoids 5,6,7,8-hydroxy-3-methoxy flavone (MH-1); 5,6,7,8-hydroxy-4'-hydroxy flavone (MH-2); and 5,7-hydroxy-3,4'-methoxy flavone (MH-3); isolated from AM clover (*Trifolium repens*) shoots, on spore germination, hyphal length, hyphal branches and the number of cluster of auxiliary cells or the number of secondary spores (Presymbiotic stage) and on the number of entry points and the percentage of AM colonized root of tomato (*Lycopersicum esculentum*) by the AM fungi *Gigaspora rosea*, *Giaspora margarita*, *Glomus mosseae* and *Glomus intraradices* (Symbiotic stage). Non significant effects of the flavonoids isolated from the shoot of mycorrhizal colonized clover on the presymbiotic and symbiotic stages of *Gigaspora* and *Glomus* endophytes were found. The flavonoid NMHTV isolated from non AM clover shoot, did not affect the percentage of germination of spores but significantly increased ($P < 0.05$) the other steps of the presymbiotic stage of *Gi. margarita* spores when 2 µM concentration was used. The symbiotic stage of *Gi. margarita* was also significantly increased when 2 µM of the flavonoid NMHTV was applied. This flavonoid had no effect on the presymbiotic development of *G. mosseae*, *G. intraradices* and *Gi. rosea* except when 8 µM concentration was used, which inhibited the hyphal length of *Gi. rosea*. These results suggest the possible implication of the flavonoid NMHTV in the susceptibility of tomato roots to the AM formation by *Gi. margarita*. The absence of stimulation of the AM presymbiotic and symbiotic stages in tomato by exogenous application of the newly synthesized flavonoids MH-1, MH-2, and MH-3, in clover shoots after AM colonization, indicated that the autorregulation of the AM symbiosis can be, at least partially, due to the disappearance of flavonoids in AM colonized plants that stimulated the AM symbiosis.

Keywords: Arbuscular mycorrhizas, flavonoids, signal molecules, symbiosis

INTRODUCTION

The colonization of plant root by arbuscular mycorrhizal (AM) fungi involves a molecular dialogue between plant and AM fungi (Harrison, 1998). This dialogue starts in the presymbiotic stage of the fungi and might be regulated by secondary metabolites from the plant before that the fungus enters into the root. Flavonoids are secondary plant metabolite presents in most plants, are components of the soil rhizosphere and play an important role in many plant microbial interactions (Phillips and Tsai, 1992; Akiyama *et al*., 2002; Larose *et al*., 2002; Vierheilig *et al*., 2002).

Most studies on the effect of AM symbiosis on plant physiology have been focused to changes produced in roots but not in shoots. However, AM symbiosis have systemic influence in several plant processes which will affect the development of the symbiosis such as signals molecules implicated in plant susceptibility to AM formation, in autoregulation of AM symbiosis or in plant defence response in AM symbiosis (Martin *et al*., 2002; Vierheilig and Piche, 2002; Taylor and Harrier, 2003; Vierheilig *et al*., 2003).

Flavonoids from plants are molecules that can be involved in the colonization of plant root by AM fungi and play an important role in the presymbiotic (Spore germination, hyphal length, hyphal branching and formation of cluster of auxiliary cells or secondary spores) and symbiotic stages (Formation of entry points and root colonization) of the AM fungi development (Morandi, 1996; Vierheilig *et al*., 1998). The establishment of the AM interaction is localized into the roots; however, the AM interaction produced physiological and molecular changes no only in the root but also in the shoot (Poulton *et al*., 2002; Taylor and Harrier, 2003). In fact, quantitative and qualitative changes in the pattern of flavonoids into the shoots of clover when these plants were inoculated with *Glomus intraradices* were observed (Ponce *et al*., 2004). In shoot of clover without mycorrhiza only the flavone 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone (NMHTV) was isolated (Ponce *et al*., 2004). When clover was colonized with *G. intraradices* the 3-methoxy-5,6,7,8-hydroxy-4'hydroxy flavone disappear from the shoot and the flavones 5,6,7,8-hydroxy-3-methoxy flavone (MH-1); 5,6,7,8-hydroxy-4'-hydroxy flavone (MH-2); and 5,7-hydroxy-3,4'-methoxy flavone (MH-3) were newly synthesized (Ponce *et al*., 2004). Formation of these plant flavonoids by mycorrhizal fungi might be involved in the enhanced resistance of mycorrhizal plants against penetration of roots by mycorrhizal fungus or soil-borne plant pathogens (Larose *et al*., 2002).

Contradictory results about the positive and negative influence of flavonoids on the AM fungal development have been obtained (Tsai and Phillips, 1991; Bécard *et al*., 1992; Chabot *et al*., 1992; Baptista and Siqueira, 1994; Poulin *et al*., 1997; Vierheilig *et al*., 1998), 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).

The complex actions of the flavonoids on the AM fungi depend on the type and concentration of flavonoid, on the genera or even the specie of arbuscular endophytes and on the stage of the AM development (Vierheilig *et al*., 1998).

There is not knowledge about the effect of the flavonoids described

above on development of AM fungi. The new synthesis and suppression of some flavonoids in clover shoots colonized by the AM fungus *G. intraradices* suggests a possible role of these plant molecules in the regulation process of the AM symbiosis (Vierheilig and Piche, 2002). In this work we study the effects of flavonoids isolated from AM and non-AM inoculated clover shoot on the presymbiotic stage of the *Giaspora rosea*, *Giaspora margarita*, *Glomus mosseae* and *G. intraradices* and on the number of entry points and the percentage of root colonization of tomato by these AM fungi.

MATERIAL AND METHODS

Isolation and identification of flavonoids

The flavonoid 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone (NMHTV) was isolated from shoot of non-inoculated clover (*Trifolium repens*), and the flavonoids MH-1, MH-2 and MH-3 from AM inoculated clover shoot as described by Ponce *et al.* (2004).

Effect of flavonoids on AM spores

Flavonoids were tested on *Gigaspora rosea* (BEG 9), *Gi. margarita* (J7) from Buenos Aires Fungal Collection (BAFC), *Glomus mosseae* (BEG 12) and *G. intraradices* (DAOM 197198) spores in 9 cm diameter Petri dishes. Spores of *Gi. margarita* Becker and Hall were isolated from Ciudad Universitaria soil (Fracchia, 2002), in the province of Buenos Aires (Argentina) and morphologically identified (Bentivenga and 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 (Filtropur S, SARSTEDT), 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 concentration of 0.05% ethanol was selected because test had shown it did not affect germination and hyphal growth from *Gigaspora* and *Glomus* spores (Whereas 0.1%, 0.5 % and 1 % did). 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. Flavonoid concentrations of 0.5, 2 and 8 μM were selected for testing based of effects previously reported (Morandi, 1996; Vierheilig *et al.*, 1998). Petri dishes with 0.05 % ethanol or without ethanol were used as control.

Spores of *Gi. rosea, Gi. margarita*, *G. intraradices* and sporocarps of *G. mosseae* were isolated by wet sieving the soil (Gerdemann, 1955), from a clover pot culture (*Trifolium repens*) and were stored in water at 4°C until used. The spores of *G. mosseae* were obtained by dissecting the sporocarps. Spores of *Gi. rosea, Gi. margarita*, *G. mosseae* and *G. intraradices* were surface-sterilized (Mosse, 1962). The spores were selected with the aid of a stereomicroscope and aseptically transferred to a 5 cm diameter Petri dish with 10 ml of Gel-Gro. In the experiments ten plates replications and 10 controls of each treatment were prepared. Ten surface sterilized spores per Petri dish of *Gi. rosea, Gi. margarita, G. mosseae* or *G. intraradices* were placed onto the surface of the medium (A hundred spores per treatment).

The Petri plates were sealed to reduce dehydratation and contamination risks and were then incubated in the dark at 25°C

for 2 weeks. The percentage spore germination, hyphal length (mm), hyphal branches, the number of cluster of auxiliary cells of *Gigaspora* spores and the number of secondary spores of *G. mosseae* were determined. Hyphal length of germinated spores was assessed using the gridline intersect method (Marsh, 1971).

Effect of flavonoids on AM root colonization

The effect of the flavonoids of clover plants shoots on the number of AM entry points and percentage of root colonization of tomato (*Lycopersicum esculentum*) by *Gi. rosea*, *Gi. margarita*, *G. mosseae* and *G. intraradices* spores were tested in 5 cm diameter Petri dishes using a monosporic culture technique (Fracchia *et al.*, 2001). 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 MES buffer (pH 7) plus 0.04 g of Gel-Gro. Petri dishes were incubated at 25°C for 8 d 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 diameter 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 sterilised with 10% sodium hypochlorite for 2 min and 1 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. The flavonoids and the content of a vermiculite-perlite dish with 2 week-old tomato seedling was transferred at the same time onto the Gel-Gro medium with the germinated spore. The hyphal development was observed every 2 d 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 x160 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.

Statistical treatments

Experimental data were statistically analysed by ANOVA and Tukey´s test

(P<0.05). Percentage data were subjected to arcsine transformation before analysis. Each experiment was repeated at least twice. Statistical analyses were conducted in SPSS software, version 11.0 (SPSS Inc., 1989–2001).

RESULTS

Microscopic observations of stained roots showed AM fungi only in AM inoculated clover plants, where 80 ± 9 % of root were colonized.

Under our experimental conditions the dry weights of tomato shoots grown in Petri dishes were similar (125 ± 18 mg) in all treatments. There were no significant effects of the flavonoids MH-1, MH-2 and MH-3, isolated from the shoot of clover colonized by AM fungi, on percentage of spore germination, hyphal length and number of hyphal branches of *Gi. rosea, Gi. margarita, G. mosseae* and *G. intraradices* at any of the concentrations tested. No significant effect of these flavonoids on number of clusters of auxiliary cells of *Gi. rosea* and *Gi. margarita*, and on the number of secondary spores of *G. mosseae* and *G. intraradices* were also found.

In presence of the flavonoids MH-1, MH-2 and MH-3, the values for percentage of spore germination were 46 ± 5 % for *Gigaspora* and 60 ± 5 % for *Glomus*. The hyphal length was 30 ± 5 mm for *Gigaspora* and 13 ± 2 mm for *Glomus*. The number of hyphal branches was 5 ± 1 for *Gigaspora* and 17 ± 3 for *Glomus*. The number of clusters of auxiliary cells was 0.6 ± 0.1 for *Gigaspora* and the number of secondary spores was 3.5 ± 0.4 for *Glomus*.

There were non significant effects of the flavonoids MH-1, MH-2 and MH-3,

on the number of entry points and the percentage of tomato root colonized by these endophytes. The values for the number of entry points were 9 ± 2 for *Gigaspora* and 30 ± 5 for *Glomus* and for the percentage of AM root colonization were 12 ± 2 % for *Gigaspora* and 35 ± 6 % for *Glomus*.

The flavonoid NMHTV isolated from shoot of clover noninoculated with AM fungi did not affect the percentage of germination of *Gi. rosea, Gi. margarita, G. mosseae* and *G. intraradices* spores (Data not shown). The concentration of 8 µM of NMHTV inhibited the hyphal length of *Gi. rosea*. However, the presence of 2 µM of NMHTV significantly increased the hyphal length of *Gi. margarita*. The hyphal length of *G. mosseae* and *G. intraradices* was not affected by NMHTV at any of the concentrations tested (Fig 1).

The flavonoid NMHTV did not affect on the number of hyphal branches of *Gi. rosea, G. mosseae* or *G. intraradices* spores, while the number of hyphal branches of *Gi. margarita* was significantly increased when 2 µM of NMHTV was applied (Fig. 2). The application of 2 µM of the flavonoid NMHTV increased the number of clusters of auxiliary cells of *Gi. margarita*, while this flavonoid did not have effects on the number of clusters of auxiliary cells of *Gi. rosea* (Fig. 3).

The number of secondary spores of *G. mosseae* was unaffected by the application of NMHTV (Fig. 3). The number of entry points and the percentage of tomato root colonized by *Gi. margarita* significantly increased when 2 µM of the flavonoid NMHTV was applied (Fig. 4 and 5).
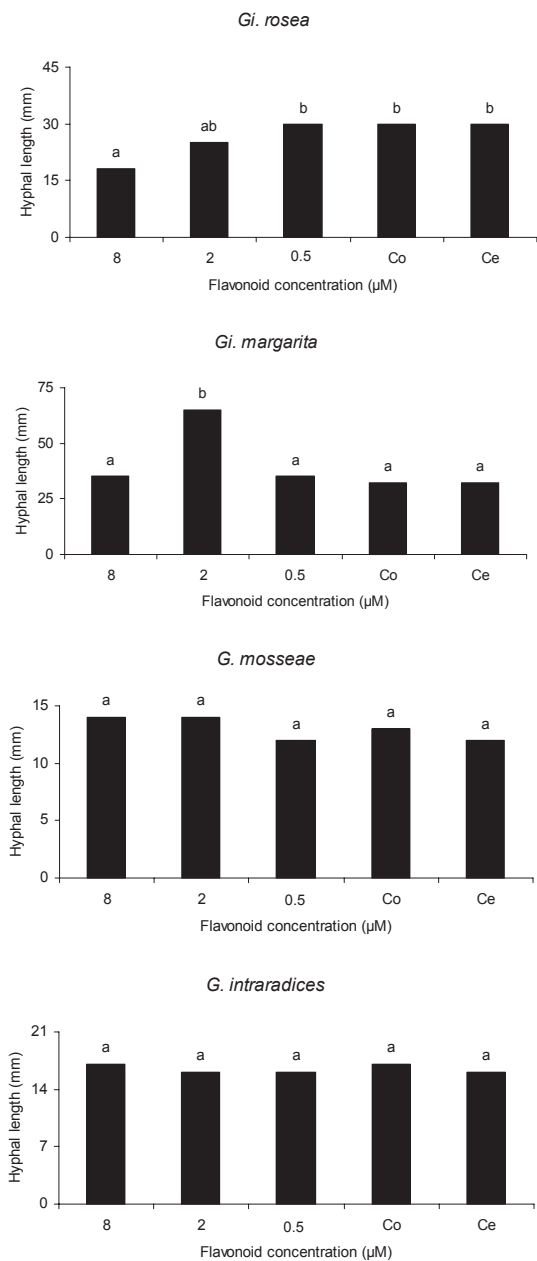
Figure 1: Effect of 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone extracted from *Trifolium repens*, on the hyphal length (mm) of *Gi. rosea*, *Gi. margarita*, *G. mosseae* and *G. intraradices* spores. Co = Control without flavonoids; Ce = Control plus 0.05% ethanol. Values with the same letter are not significantly different as determined by Tukey's test (P < 0.05)
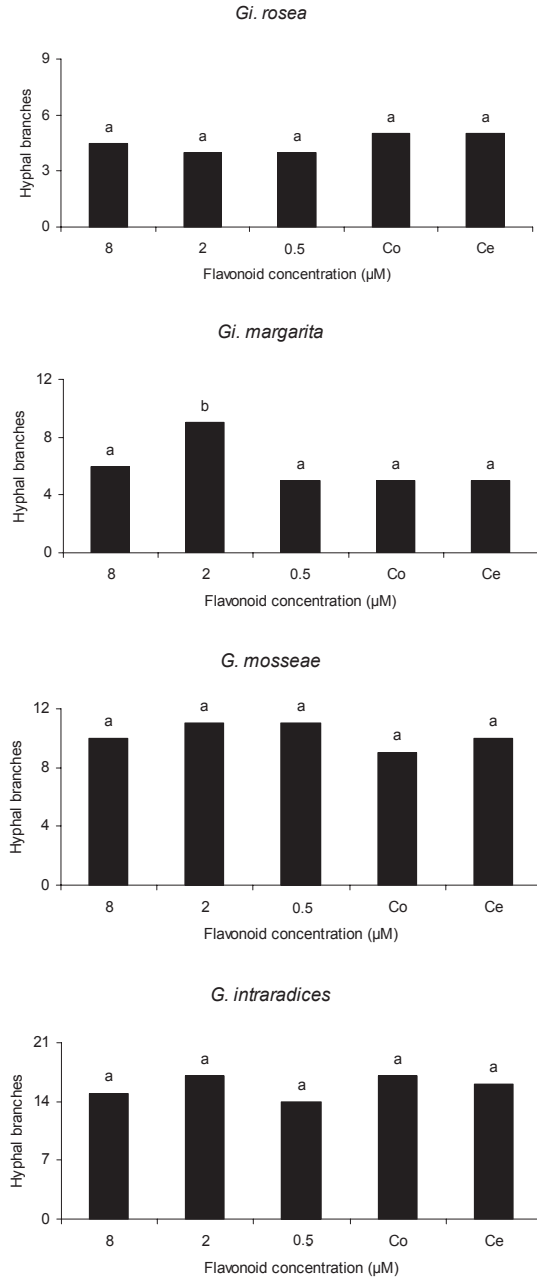
Figure 2: Effect of 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone extracted from *Trifolium repens*, on the number of hyphal branches of *Gi. rosea*, *Gi. margarita*, *G. mosseae* and *G. intraradices* spores. Co = Control without flavonoids; Ce = Control plus 0.05% ethanol. Values with the same letter are not significantly different as determined by Tukey's test (P < 0.05)

DISCUSSION

Plant flavonoids are metabolites implicated in the presymbiotic development of the AM fungi in soil and in the recognition process of plant-AM fungal interaction (Bécard and Piche, 1989; Paula and Siqueira, 1990; Vierheilig and Piche, 2002).

In the presymbiotic stage of the AM fungi, spore germination seems to be very sensitive to the action of flavonoids, especially those belonging to *Gigaspora* genus (Bécard *et al*., 1992; Chabot *et al*., 1992). None of the flavonoids isolated from clover shoot inhibited the percentage of germination of *Gigaspora* or *Glomus* species tested. It been reported that flavones had variable and even contradictory effects on the hyphal growth of *Gigaspora* spores (Vierheilig *et al*., 1998). The fact that the flavone NMHTV increased the hyphal length of *Gi. margarita*, while have inhibited that of *Gi. rosea* reinforce this idea.

The concentrations of flavonoids seems to be important in their effect on AM fungi. It has been suggested that low concentration of flavonoids was more effective on the AM fungal growth (Tsai and Phillips, 1991; Bécard *et al*., 1992; Baptista and Siqueira, 1994). However, our results do not allow establishing a close relationship between the concentration of the flavonoids used and their effects on any of the steps of the presymbiotic stage of the *Glomus* and *Gigaspora* species tested.

The influence of flavonoids on the AM fungi seems to be different at genera level (Vierheilig *et al*., 1998). In our experiment we found that NMHTV had different effect not only at genera but also at the species level. None of the *Glomus* species tested were affected by any of the flavonoids assayed. However, the flavonoid NMHTV increased hyphal length, number of hyphal branches, number of clusters of auxiliary cells, number of entry points and the percentage of tomato root colonized by *Gi. margarita*, but did not affect these steps of development of *Gi. Rosea*.

Hyphal branching of the AM fungi has been described as one of the first event in host root recognition by the fungus during the presymbiotic phase (Giovannetti *et al*., 1996). The fact that 8 μM of NMHTV inhibited hyphal length but not hyphal branching of *Gi. rosea* indicated that hyphal branching, at least of *Gi. rosea* possibly requires a different stimulatory mechanisms or induction that does hyphal growth (Vierheilig *et al*., 1998). We found that NMHTV, which exhibit a hyphal length stimulating effect of *Gi. margarita*, also enhanced hyphal branching and number of auxiliary cells of spores. These results suggest that this flavonoid have a more specific role on hyphal branching of *Gigaspora* independently on their effect on hyphal length. The flavonoid NMHTV also increased the number of entry points and the percentage of tomato root colonized by *Gi. margarita*. The importance of entry points on penetration, development into roots and effectiveness of the AM fungi has been described (Buee *et al*., 2000). A close relationship between the number of entry points and the percentage of root colonization reached by *Gigaspora* or *Glomus* symbionts in presence of the flavonoids tested was found. Our results suggest that the flavonoid NMHTV may influence the extension of AM colonization of roots mainly through its effects on the formation of entry points.
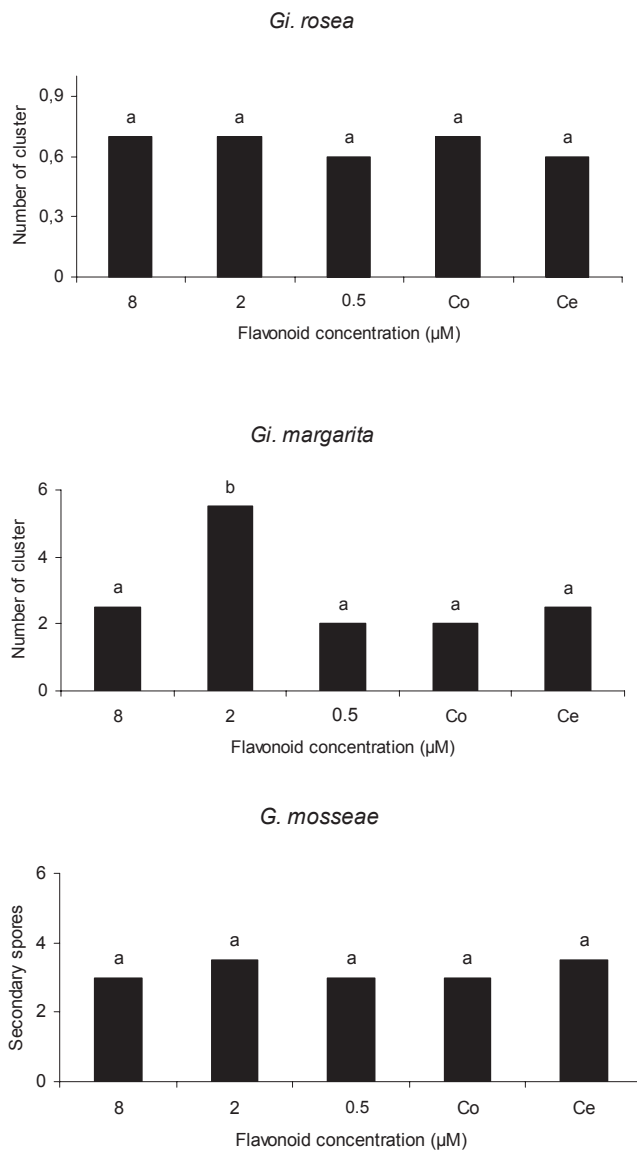
Gi. rosea

Gi. margarita

G. mosseae

Figure 3: Effect of 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone extracted from *Trifolium repens*, on the number of clusters of auxiliary cells of *Gi. rosea*, *Gi. margarita*, and on the number of secondary spores of *G. mosseae* spores. Co = Control without flavonoids; Ce = Control plus 0.05% ethanol. Values with the same letter are not significantly different as determined by Tukey's test ($P < 0.05$)
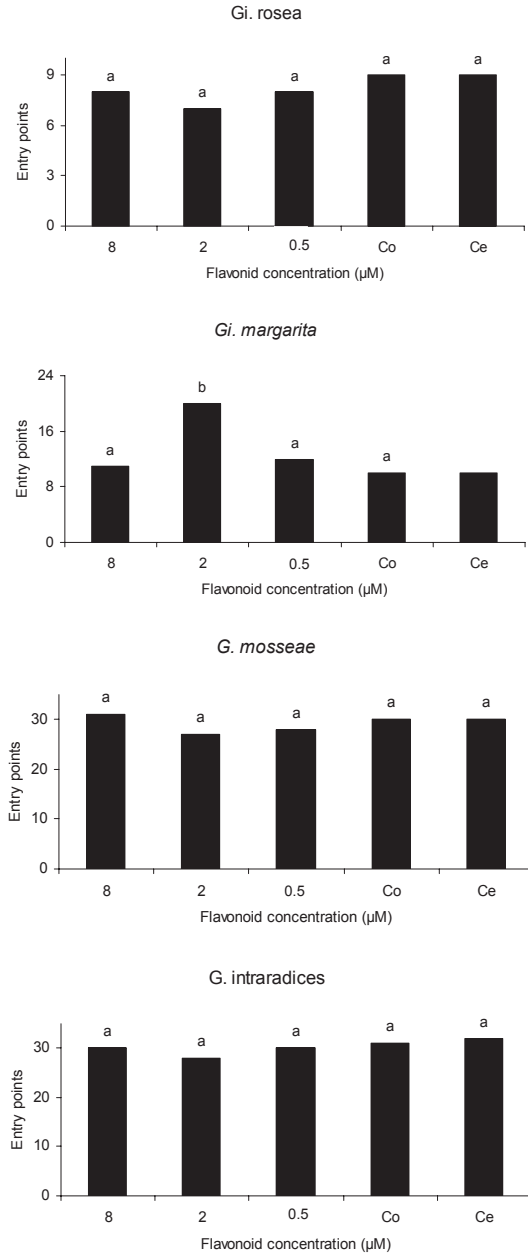
Figure 4: Effect of 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone on the number of entry points per 30 cm of tomato root of *Gi. rosea*, *Gi. margarita*, *G. mosseae* and *G. intraradices* endophytes. Co = Control without flavonoids; Ce = Control plus 0.05% ethanol. Values with the same letter are not significantly different as determined by Tukey's test ($P < 0.05$)
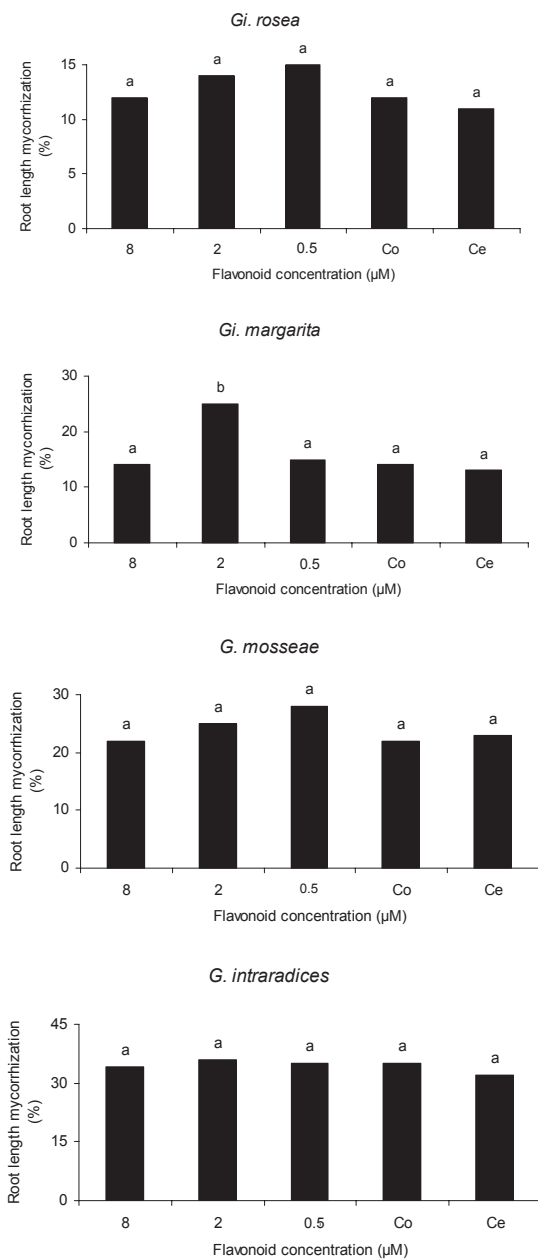
Figure 5: Effect of 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone extracted from *Trifolium repens*, on the percentage of root colonization of tomato by *Gi. rosea*, *Gi. margarita*, *G. mosseae* and *G. intraradices* endophytes. Co = Control without flavonoids; Ce = Control plus 0.05% ethanol. Values with the same letter are not significantly different as determined by Tukey's test (P < 0.05)

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 *et al.*, 2002). AM symbiosis have systemic influence in signals molecules implicated in plant susceptibility to AM formation (Vierheilig and Piche, 2002; Taylor and Harrier, 2003; Vierheilig *et al.*, 2003). We observed stimulatory effects of the flavonoid NMHTV from shoot of non AM colonized plants not only on the presymbiotic stage of the fungus before that enters into the root but also during the penetration and development of the fungus *Gi. margarita* inside the roots. These results suggest the possible implication of this flavonoid in the susceptibility of tomato plant to the formation of AM symbiosis with this endophyte. This flavonoid synthesized in non AM colonized plant was not detected in plant colonized with the AM fungus and we also observed no synthesis of flavonoids in the shoots of clover that stimulated the AM symbiosis of tomato. On the other hand, exogenous application of the newly synthesized flavonoids in clover shoots after AM colonization did not stimulate the presymbiotic stages, the formation of AM penetration structures and the development of the fungus inside of the test plant root. This could mean that once the AM symbiosis is well established the observed suppression of further root colonization by AM fungi can be at least partially due to the disappearance of flavonoids that stimulated the AM symbiosis and to the formation of flavonoids which did not stimulate the formation and development of the AM symbiosis.

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