

## Flavonoids exhibit fungal species and genus specific effects on the presymbiotic growth of *Gigaspora* and *Glomus*

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The effect of the flavonoids chrysin, isorhamnetin, kaempferol, luteolin, morin and rutin on pre-symbiotic growth, such as spore germination, hyphal length, hyphal branching and the formation of auxiliary cells and secondary spores, of the arbuscular mycorrhizal fungi *Gigaspora rosea*, *G. margarita*, *Glomus mosseae* and *G. intraradices* was studied. According to the effect on each fungal growth parameter, the tested compounds could be classified to be genus and/or species specific or specific, for a certain developmental stage of pre-symbiotic growth. A clear arbuscular mycorrhizal genus specific, and even species specific, effect of some flavonoids was observed. However, this specificity of a flavonoid could not be generalized but differs depending on the pre-symbiotic stage of the AM fungus. Moreover, our show that for a better understanding of the role of compounds in the AM symbiosis, studies should not be conducted only with one AM fungus looking at one fungal growth parameter such as spore germination or hyphal length, but should be wider, including several growth parameters and several AM fungi.

### INTRODUCTION

The establishment of the symbiotic association between plants and arbuscular mycorrhizal (AM) fungi involves a complex exchange of signals between the two partners (Gadkar *et al.* 2001, Vierheilig & Piche 2002). Flavonoids are a large group of secondary plant compounds involved in signalling in various plant-microbe interactions (Hermann 1988, Siqueira *et al.* 1991, Vierheilig *et al.* 1998). Although an essential role in the establishment of the AM has been questioned (Bécard *et al.* 1995), recent studies suggest an important role of flavonoids in the regulation of the mycorrhizal symbiosis even in the pre-symbiotic phase (Guenoune *et al.* 2001, Akiyama *et al.* 2002, Larose *et al.* 2002).

The effect of flavonoids on the different pre-symbiotic stages of AM fungi, spore germination,

hyphal length and differentiation such as hyphal branching, the formation of auxiliary cells and the formation of secondary spore can be studied in *in vitro* experiments (Morandi 1996, Vierheilig *et al.* 1998). Data from *in vitro* experiments shows that a number of flavonoids do affect the pre-symbiotic growth of AMF at all stages (Gianinazzi-Pearson *et al.* 1989, Morandi 1996, Vierheilig *et al.* 1998).

The wide distribution of the AM symbiosis in most plant species and the non-specificity of AM fungi to host plants (Smith & Read 1997) suggest that some plant compounds, such as flavonoids, are general signals for all AM fungi. However, comparing the available data on the pre-symbiotic growth of AM fungi, Vierheilig *et al.* (1998) suggested that flavonoids exhibit an AM fungus genus specific and even species-specific effect.

Unfortunately, nearly all data on the effect of flavonoids on AM fungi have been obtained under different

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experimental conditions (e.g. different *in vitro* systems, different compound concentrations), with different AM fungi, and at different stages of pre-symbiotic growth (Morandi 1996, Vierheilig *et al.* 1998). Consequently, no clear conclusion as to the fungal specificity of the tested compounds can be drawn. For example, flavonoids of the flavonol type seem to stimulate or inhibit the hyphal growth of *Gigaspora* and *Glomus*, but contradictory results have been reported on the effect of flavones on *Gigaspora* (Morandi 1996, Vierheilig *et al.* 1998).

Most studies on the effect of flavonoids on the pre-symbiotic stages of AM fungi have focused on spore germination and hyphal growth, and very few on the formation of auxiliary cells and hyphal differentiation (Morandi 1996, Vierheilig *et al.* 1998). However, some flavonoids may be involved in one step of the AM fungal development but not in the other, for example in *Gigaspora margarita* luteolin has been shown not to stimulate hyphal growth, but to stimulate the formation of auxiliary cells (Bécard, Douds & Pfeffer 1992).

In order to test the hypothesis of a genus specific, or even species specific, effect of flavonoids during the different pre-symbiotic growth phases of AM fungi, we studied the effect of two flavones and four flavonols on spore germination, hyphal length and hyphal differentiation of the AM fungi *Gigaspora rosea*, *G. margarita*, *Glomus mosseae*, and *G. intraradices*. Data on the effect of the flavones chrysin and luteolin, and of the flavonols kaempferol, morin and rutin, on the pre-symbiotic growth of AM fungi are available, but no studies on the effect of isorhamnetin on AM fungi have previously been performed. Isorhamnetin is a flavonol present in plants of several different families and has been proposed as a plant taxonomic tool (Santos & Salatino 2000, Schieber *et al.* 2002).

## MATERIAL AND METHODS

The effect of the flavonoids, chrysin, isorhamnetin, kaempferol, luteolin, morin and rutin on *Gigaspora rosea* from the International Bank for *Glomeromycota* (BEG 9), *G. margarita* (J7) from Buenos Aires Fungal Collection (BAFC), and, *Glomus mosseae* (BEG 12) and *G. intraradices* from the National Mycological Herbarium of Canada (DAOM 197198) spores were tested in 9 cm diam Petri dishes. Spores of *Gigaspora margarita* were isolated from Ciudad Universitaria soil (Fracchia 2002), in the province of Buenos Aires (Argentina) and identified according to Bentivenga & Morton (1995).

All flavonoids tested were purchased from Sigma (Buenos Aires) and dissolved in absolute ethanol to provide 4 mM stock solutions. The flavonoid solutions were filtered through filter paper and sterilized twice by filtration through a 0.20 µm Millipore membrane and thereafter 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, OH). In a pre-experiment the effect of 0.05, 0.1, 0.5, and 1% ethanol-water solutions on the percentage of germination and hyphal length of *Gigaspora* and *Glomus* spores was tested. The concentration of 0.05% ethanol for the flavonoid solutions was selected because it did not affect the percentage of spore germination and hyphal length. The flavonoids dissolved in absolute ethanol were added to 10 ml of Gel-Gro at a final concentration of 0.5 and 2 µM in 0.05% ethanol. Petri dishes with 0.05% ethanol or without ethanol were used as control.

Spores of *Gigaspora rosea*, *G. margarita*, *Glomus intraradices* and sporocarps of *G. mosseae* were isolated by wet sieving (Gerdemann 1955) soil from a leek pot culture (*Trifolium repens*) and stored in water at 4 °C until used. The spores of *G. mosseae* were obtained by dissecting the sporocarps. All spores were surface-sterilized as described by Mosse (1962). The spores were selected with the aid of a stereomicroscope and aseptically transferred to plates with 10 ml of Gel-Gro. In the experiment, ten replicates and ten controls of each treatment were prepared. Ten surface sterilized spores of *Gigaspora rosea*, *G. margarita*, *Glomus mosseae* or *G. intraradices* were placed onto the surface of the medium. The Petri dishes were sealed to reduce dehydration and contamination risks and were then incubated in the dark at 25 ° for 2 wk. The percentage spore germination, hyphal length, hyphal branches, the number of clusters of auxiliary cells of *Gigaspora*, and the number of secondary spores of *Glomus* were determined. The number of secondary spores of *G. intraradices* was not determined. The 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 with similar results.

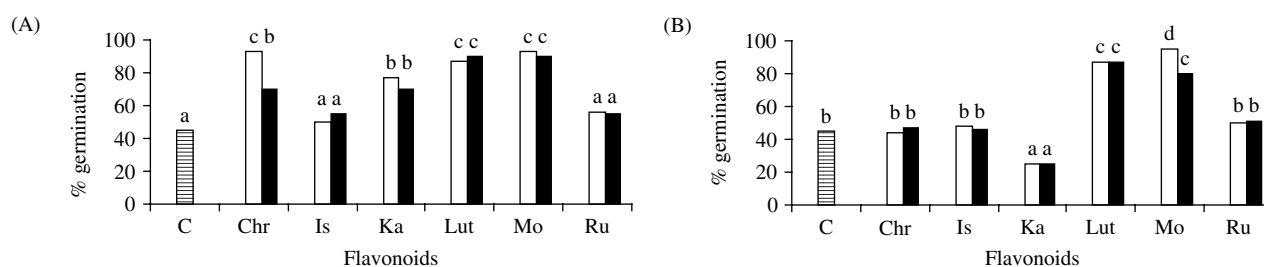
## RESULTS

The water control and the 0.05% ethanol treatments in all cases had 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.

Fig. 1 shows that both tested concentrations of the flavonoids luteolin and morin increased spore germination of *Gigaspora rosea* and *G. margarita*. Chrysin stimulated spore germination of *G. rosea*, but showed no effect on *G. margarita*, whereas kaempferol stimulated spore germination of *G. rosea*, but inhibited spore germination of *G. margarita*. Isorhamnetin and rutin did not significantly affect the percentage of germination of both *Gigaspora* species spores. None of the tested flavonoids affected the spore germination of the two *Glomus* species tested (data not shown). Data are summarized in Table 1.

**Table 1.** Spore germination (A), hyphal length (B), hyphal branching (C) and clusters of auxiliary cells or secondary spores (D) of AM fungi in the presence of different flavonoids. When one concentration of the tested compound affected the spore germination it was marked (+) for a stimulatory and (–) for an inhibitory effect. When both concentrations showed no effect it was marked (0).

	Chrysin	Isorhamnetin	Kaempferol	Luteolin	Morin	Rutin
<b>(A)</b>						
<i>Gigaspora rosea</i>	+	0	+	+	+	0
<i>G. margarita</i>	0	0	–	+	+	0
<i>Glomus mosseae</i>	0	0	0	0	0	0
<i>G. intraradices</i>	0	0	0	0	0	0
<b>(B)</b>						
<i>Gigaspora rosea</i>	+	+	+	+	+	0
<i>G. margarita</i>	+	+	+	+	+	+
<i>Glomus mosseae</i>	+	0	0	+	+	0
<i>G. intraradices</i>	+	0	0	+	+	0
<b>(C)</b>						
<i>Gigaspora rosea</i>	+	0	0	+	+	0
<i>G. margarita</i>	+	0	0	+	+	+
<i>Glomus mosseae</i>	+	0	0	0	+	0
<i>G. intraradices</i>	+	0	0	0	+	0
<b>(D)</b>						
<i>Gigaspora rosea</i>	+	+	+	+	+	+
<i>G. margarita</i>	0	+	+	+	+	+
<i>Glomus mosseae</i>	+	0	0	0	+	0



**Fig. 1.** Effect of chrysin (Chr), isorhamnetin (Is), kaempferol (Ka), luteolin (Lut), morin (Mo) and rutin (Ru) on the percentage of germination of (A) *Gigaspora rosea* and (B) *G. margarita* spores. 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; and ■, 2 µM of flavonoids.

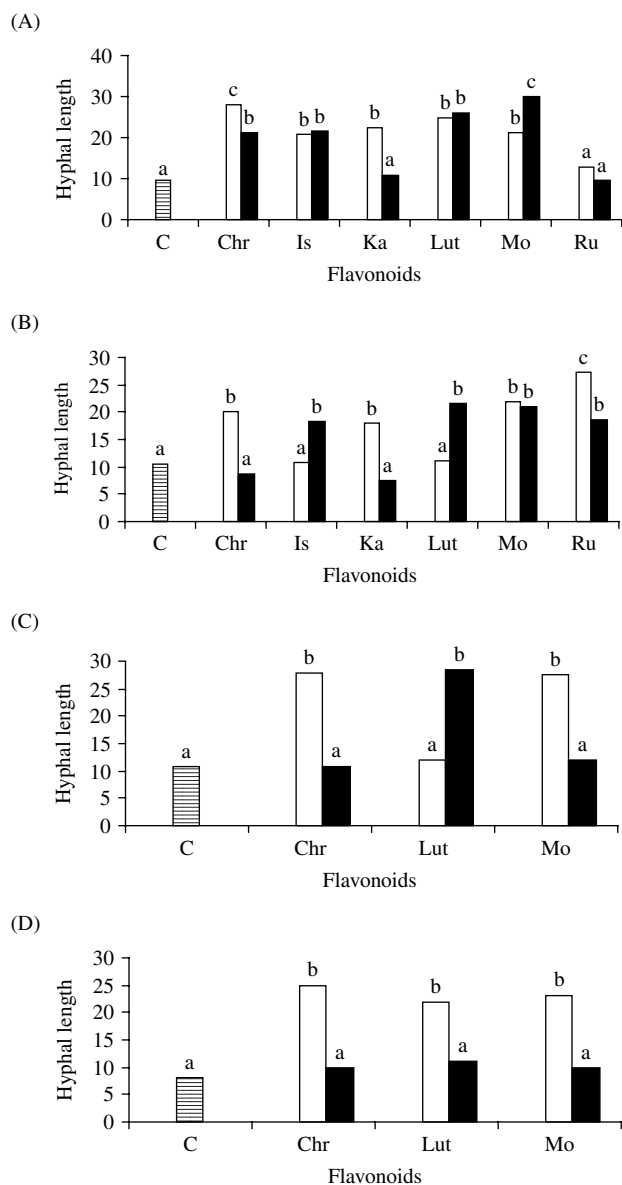
All flavonoids tested increased, at least at one concentration, the hyphal length of the two *Gigaspora* species tested, except rutin which had no effect on the hyphal length of *G. rosea*, but stimulated hyphal length in *G. margarita*. Chrysin, luteolin and morin stimulated, at least with one of the tested concentrations, the hyphal length of the two *Glomus* species, whereas neither isorhamnetin and rutin, nor kaempferol, showed any effect (Fig. 2A, B). Data are summarized in Table 1.

As Fig. 3 shows, the flavonoids chrysin, luteolin and morin increased the number of hyphal branches of the two *Gigaspora* species, whereas rutin stimulated branching of *G. margarita*, but showed no effect on branching of *G. rosea*. Neither isorhamnetin nor kaempferol showed any effect on branching on the two *Gigaspora* species. Only chrysin and morin increased the number hyphal branches of *Glomus mosseae* and *G. intraradices*. All other tested compounds showed no effect (data not shown). Data are summarized in Table 1.

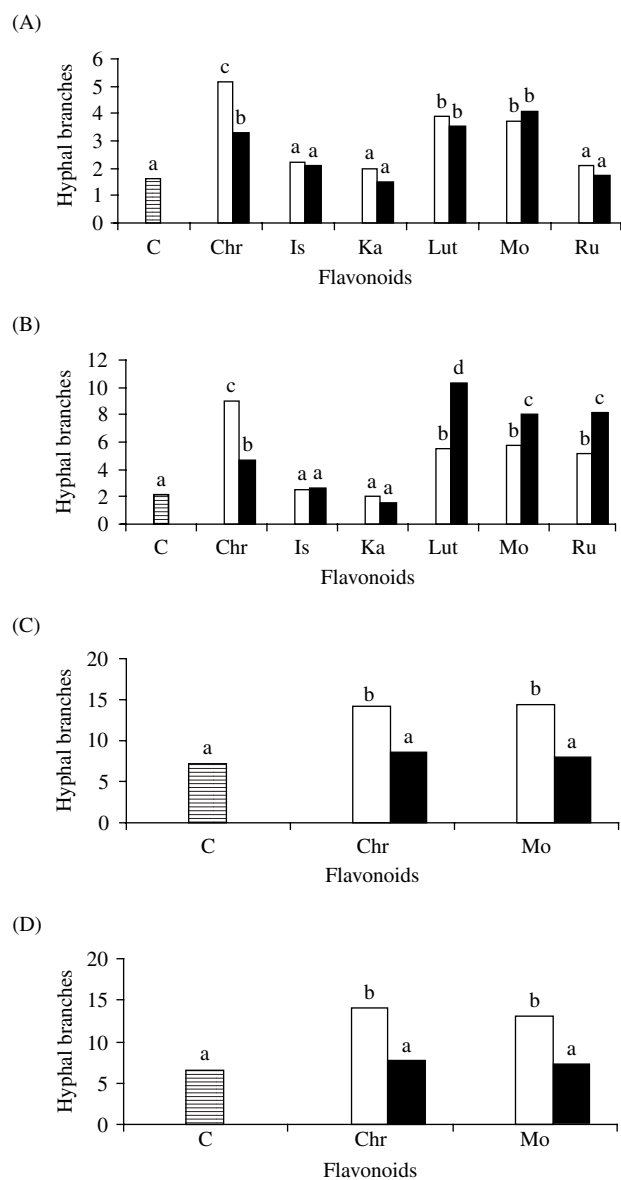
At least one of the tested concentrations of the flavonoids isorhamnetin, kaempferol, luteolin, morin and rutin increased the number of clusters of auxiliary cells of the two *Gigaspora* species. Chrysin stimulated the formation of auxiliary cells of *G. rosea*, but had no effect on *G. margarita* (Fig. 4A, B). The number of secondary spores of *Glomus mosseae* was enhanced at least with one concentration of chrysin and morin. Isorhamnetin, kaempferol, luteolin and rutin did not have effect on the number of secondary spores of *G. mosseae* (Fig. 4C, D). Data are summarized in Table 1.

## DISCUSSION

Abundant data on the effect of flavonoids on different stages of pre-symbiotic growth are available. Most studies have been performed with AM fungi of the genus *Gigaspora*, far less with *Glomus* species, and virtually nothing with other genera (Morandi 1996, Vierheilig *et al.* 1998). Most data are available on the



**Fig. 2.** Hyphal length (mm) of (A) *Gigaspora rosea* and (B) *G. margarita* spores in presence of chrysin (Chr), isorhamnetin (Is), kaempferol (Ka), luteolin (Lut), morin (Mo) and rutin (Ru), and (C) *Glomus mosseae* and (D) *G. intraradices* spores in presence of Chr, Lut and Mo. See Fig. 1 for further explanation and key.



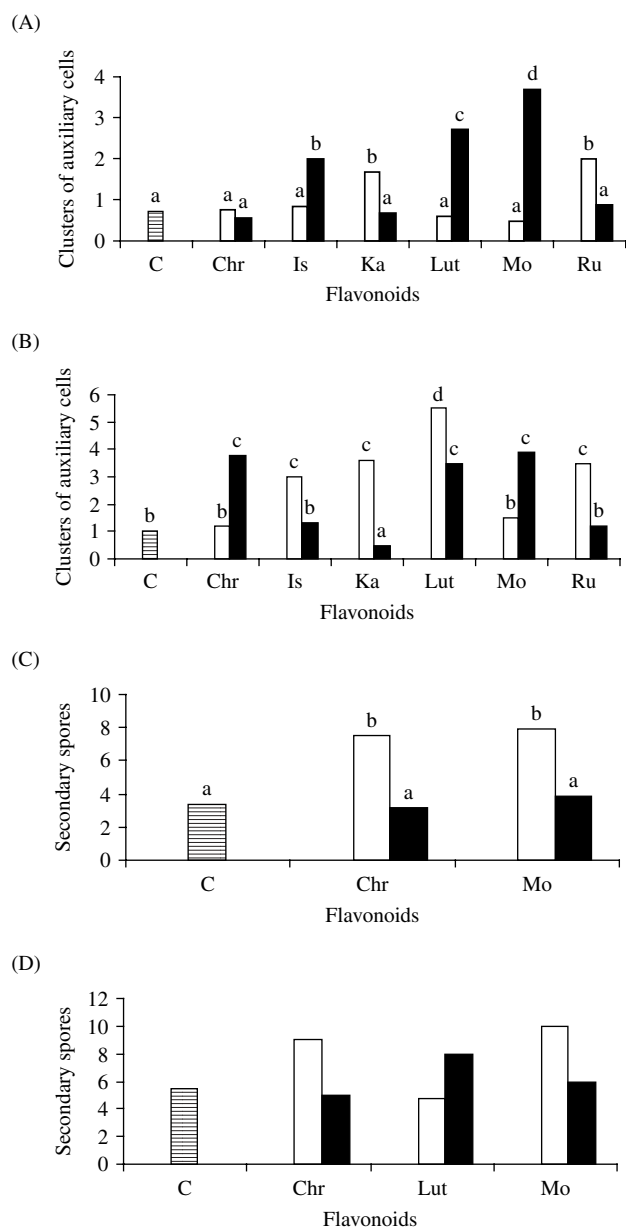
**Fig. 3.** Number of hyphal branches of (A) *Gigaspora rosea* and (B) *G. margarita* spores in presence of chrysin (Chr), isorhamnetin (Is), kaempferol (Ka), luteolin (Lut), morin (Mo) and rutin (Ru), and (C) *Glomus mosseae* and (D) *G. intraradices* spores in presence of Chr and Mo. See Fig. 1 for further explanation and key.

effect of compounds on hyphal growth, less on spore germination, and few on other parameters such as fungal differentiation.

Although the comparison of data from different studies strongly point towards a genus or even species specific effect of flavonoids on the pre-symbiotic growth of AM fungi (Vierheilig *et al.* 1998), the hypothesis of AM fungal genus specificity, or even species specificity, of flavonoids on different pre-symbiotic fungal stages has never been systematically tested.

In most studies only one parameter of pre-symbiotic growth of one AM fungus was recorded. We tested the effect of flavonoids on all parameters of pre-symbiotic growth such as spore germination, hyphal length,

hyphal branching, and other hyphal differentiation such as the formation of secondary spores and auxiliary cells in two species each of the genera *Gigaspora* and *Glomus*. Fungal differentiation events, for example the formation of secondary spores and auxiliary cells, can differ between the genera *Gigaspora* and *Glomus* and even between the two *Glomus* species tested. We therefore compared the effect of flavonoids on the growth parameters of spore germination, hyphal length, and hyphal branching, between different AM fungal genera and species. Comparing spore germination in the genera *Gigaspora* and *Glomus*, a difference was observed. Whereas none of the tested compounds affected the germination of the two *Glomus* species tested, chrysin,



**Fig. 4.** Number of clusters of auxiliary cells of (A) *Gigaspora rosea* and (B) *G. margarita* spores in presence of chrysin (Chr), isorhamnetin (Is), kaempferol (Ka), luteolin (Lut), morin (Mo) and rutin (Ru), and secondary spores of (C) *Glomus mosseae* and (D) *G. intraradices* spores in presence of Chr, Lut and Mo. See Fig. 1 for further explanation and key.

luteolin and morin stimulated the spore germination of the two *Gigaspora*, pointing towards a genus specific effect of these compounds on spore germination. An indication for a species specific effect was observed with kaempferol. Whereas it stimulated spore germination of *Gigaspora rosea*, spore germination of *G. margarita* was reduced. Other authors have reported that most of the flavonoids we tested had negative effects on the germination of spores of *Gigaspora margarita* (Bécard, Douds & Pfeffer 1992, Chabot *et al.* 1992). These partially contradictory results might be due to the differing experimental conditions the studies utilized.

It has been suggested that flavonoids of the flavonol type (in our study: isorhamnetin, kaempferol morin and rutin) stimulate hyphal length in *Gigaspora* and *Glomus*, but contradictory results have been reported on the effect of flavones (in our study: chrysin and luteolin) on *Gigaspora* (Vierheilig *et al.* 1998).

In our experiment, looking at the hyphal length in both *Glomus* species, a stimulatory effect of chrysin and luteolin could be observed. These compounds also exhibited a stimulatory effect on the two *Gigaspora* species tested, indicating the non-specificity of flavones in respect to AM fungal pre-symbiotic hyphal length. Morin, a flavonol, seemed to be similarly non-specific as the flavones chrysin and luteolin. No data were available yet on the effect of isorhamnetin on AM fungi, and nothing was known on the effect of kaempferol and rutin on *Glomus* species. Our results show that all three compounds do not affect hyphal length of the *Glomus* species, but stimulated the fungi of the genus *Gigaspora*, indicating the genus specificity of these flavonols. Interestingly, in the *Gigaspora* rutin showed even a species specific effect, showing no effect on *G. rosea*, but stimulating the hyphal length of *G. margarita*.

Hyphal branching of AM fungi has been described as one of the first events in host root recognition by the fungus during the pre-symbiotic phase (Giovannetti *et al.* 1996). A partly similar pattern of the tested compounds on hyphal length was observed on hyphal branching. Whereas chrysin and morin seemed to be general signalling compounds for AM fungi, enhancing the branching in all genera and species tested, luteolin showed a clear genus specific effect. Rutin exhibited a species specific effect in *Gigaspora*, whereas kaempferol and isorhamnetin seemed to play no role in the hyphal branching of any of the tested fungi.

The formation of auxiliary cells occurs in the genus *Gigaspora* (Smith & Read 1997) but not in the genus *Glomus*. All tested compounds, except chrysin, stimulated the formation of auxiliary cells in the two *Gigaspora* species, showing that they do not exhibit a species specific effect on the formation of these structures. However, chrysin, stimulating the formation of auxiliary cells in *G. rosea*, showed no effect with *G. margarita*, thus exhibiting a species specific effect.

The formation of secondary spores of *Glomus mosseae* could not be compared to a similar phenomenon of the other AM fungi tested, however, partially a similar trend was observed as with the other fungal parameter. Chrysin and morin, which have been shown to stimulate hyphal length and hyphal branching, also stimulated the formation of secondary spores. Interestingly, kaempferol, with no effect on hyphal length and hyphal branching of the two *Glomus* species, stimulated the formation of secondary spores of *G. mosseae*, showing the importance of observing all fungal parameters to determine the specificity of compounds to pre-symbiotic growth stages. The effect of flavonoids on different stages of the pre-symbiotic

growth was particularly clear with the two *Glomus* species. In contrast to the *Gigaspora* species, none of the tested flavonoids showed any effect on the spore germination of the *Glomus* species. All other pre-symbiotic stages of *Glomus* were affected by chrysin and morin, similar to the tested *Gigaspora* species.

It has been reported that flavonoids stimulate AM fungal development at low concentrations were used (Tsai & Phillips 1991, Bécard *et al.* 1992, Baptista & Siquiera 1994). Our results showed the importance of testing different compound concentrations. In many cases an effect could be observed only with one concentration, but not with the other.

To summarize, we provide for the first time clear data on a genus specific and even species specific effect of flavonoids on AM fungi. However, the specificity of a flavonoid can not be generalized, but differs depending on the pre-symbiotic stage of the AM fungus, possibly due to its differing role during the pre-symbiotic growth phase. Moreover, our data show that for a better understanding of the role of compounds in the AM symbiosis, studies should not be conducted only with one AM fungus looking at one fungal growth parameter such as spore germination or hyphal length, but should be wider, including several fungal growth parameter and several AM fungi.

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