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Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus

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Summary

Abundant data on the effect of flavonoids on spore germination, hyphal growth and root colonization by AMF are available. Moreover, the flavonoid pattern in mycorrhizal roots changes, thus flavonoids have been suggested as arbuscular mycorrhizal signalling compounds. In our work we studied the accumulation of flavonoids in roots of *Medicago sativa* i) after the exposure of uncolonized roots to sterile solutions containing *Glomus intraradices* tissue, ii) at three different stages of colonization by *G. mosseae*, iii) colonized by *G. mosseae, G. intraradices* or *Gigaspora rosea*.

We could show that flavonoid accumulation in *M. sativa* roots i) is induced before root colonization, pointing towards the presence of a fungal-derived signal, ii) depends on the developmental stage of the symbiosis and iii) depends on the root-colonizing arbuscular mycorrhizal fungus. The data presented indicate not only a time-specificity of the flavonoid accumulation during the mycorrhizal association, but also an arbuscular mycorrhizal fungal-specificity. The possible functions of the flavonoid pattern changes are discussed.

Key words: arbuscular mycorrhiza – flavonoid – Glomales – *Medicago sativa* – signalling – specificity

Abbreviations: AMF = arbuscular mycorrhizal fungus (fungi)

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Introduction

The arbuscular mycorrhizal symbiosis is a complex exchange of signals between the host plant and arbuscular mycorrhizal fungi (AMF), starting during the pre-colonization phase, continuing during the formation of appressoria, the fungal penetration into the root, the intraradical phase with the arbuscule formation, and finalizing with the degradation of the fungal structures (collapsing arbuscules) in the root (Smith and Read 1997). For the complexity of the different steps it seems not too daring to hypothesize that at each stage differing signalling events occur.

Abundant data show an effect of flavonoids on spore germination, hyphal growth and root colonization by AMF (reviewed by Morandi 1996, Vierheilig et al. 1998 a). Moreover, starting from appressoria formation until a later stage of the symbiosis, when the AMF is well established, the flavonoid pattern in the root changes, thus flavonoids have been suggested as signalling compounds during root colonization by AMF (Harrison and Dixon 1993, Volpin et al. 1994, 1995, Morandi 1996, Vierheilig et al. 1998 a). Working with roots which are thought to be flavonoid-free, but still form the AM association, Bécard et al. (1995) suggested that flavonoids «… are not part of a specific plant-fungus relationship in mycorrhizae». New reports on flavonoids and AM reopen the discussion about the involvement of flavonoids as regulatory compounds during mycorrhization (Guenoune et al. 2001, Akiyama et al. 2002). Vierheilig and Piché (2002) presented a complex model for the role of flavonoids during the AM association. As proposed by Bécard et al. (1995), flavonoids might not be essential for the actual root colonization process, however, as different stages of the AM symbiosis may be triggered by different plant signals, specific for each stage, flavonoids might play a role during later stages of the AM symbiosis. Interestingly two recent reports show a role of flavonoids as regulatory signals for the susceptiblity of roots to AMF at the beginning of the formation of the symbiosis (Guenoune et al. 2001, Akiyama et al. 2002). Guenoune et al. (2001) demonstrated that the flavonoid medicarpin is accumulated in roots with high phosphate (P) levels. Medicarpin exhibits a strong inhibitory effect on hyphal growth of *G. intraradices* (Guenoune et al. 2001) and is known to possess antifungal activity towards other fungi (Higgins 1978), thus probably preventing the roots with a high P status from being colonized by AMF. Akiyama et al. (2002) detected the flavonoid isovitexin 2″-*O*-β-glucoside in non-mycorrhizal, P-deficient melon roots but not in roots with a high P status or in mycorrhizal roots. Application of the flavonoid to AM plants not only enhanced root colonization in plants grown under low P conditions, but also in plants grown under high P conditions, thus clearly showing that a high P-status or the mycorrhizal status of a plant, can reduce the accumulation of a flavonoid in roots which stimulates mycorrhization.

AMF are found in several genera such as *Glomus, Gigaspora* and *Sclerocystis* (Walker and Trappe 1993). Recently, after reviewing the literature on secondary plant compounds e.g. flavonoids and AMF, Vierheilig et al. (1998 a) suggested that AMF might have genus- or even species-specific signaling requirements during the AM symbiosis. Looking at the *in vitro* effect of various flavonoids on AMF, a certain specificity seems obvious. Whereas some flavonoids such as quercetin exhibit a general stimulatory effect on the hyphal growth of different AMF genera (Tsai and Phillips 1991, Bécard et al. 1992, Chabot et al. 1992, Kape et al. 1992, Baptista and Siqueira 1994), data with biochanin A showed a stimulation of *Glomus* (Nair et al. 1991, Vierheilig et al. 1998 a) but not *Gigaspora* species (Bécard et al. 1992, Chabot et al. 1992, Baptista and Siqueira 1994).

Species- or genus-specific signalling requirements of AMF in roots should lead to specific accumulation patterns of the signalling compounds. There are some data available on the accumulation of secondary plant compounds in roots colonized by one AMF (reviewed by Morandi 1996, Vierheilig et al. 1998 a), however, there is only scarce information comparing the accumulation pattern of secondary plant compounds in roots induced by various AMF. Cyclohexenone derivatives are secondary plant compounds exclusively found in roots colonized by AMF (Maier et al. 1997, 2000, Vierheilig et al. 2000 a, b). A comparison of the accumulation pattern of cyclohexenone derivatives in different plants colonized by various AMF showed no qualitatively different accumulation pattern, but a quantitatively different, AMF-specific accumulation could be detected (Vierheilig et al. 2000 a). Soybean roots colonized by various AMF showed no AMF species- or genus-specific accumulation of the phytoalexin glyceollin I (Morandi et al. 1984, Scharf et al. 1997). However, certain AMF-specific changes of the (iso)-flavonoids coumestrol and daidzein could be observed when roots were colonized by *Glomus mosseae* or *G. fasciculatum* (Morandi et al. 1984).

The presence of AMF-derived signals acting on the plant before the formation of appressoria is more and more a matter of debate (Salzer and Boller 2000, Vierheilig and Piché 2002). Plants in the AMF non-host familiy *Brassicaceae* are characterized by the absence of fungal structures in the root or even on the root surface. However, in roots of *Brassicaceae* inoculated with AMF changes of the β-1,3-glucanase and chitinase activity (Vierheilig et al. 1994) and changes of the glucosinolate levels could be observed (Vierheilig et al. 2000 c). Volpin et al. (1994) studied the formononetin accumulation in *M. sativa* plants with a high-phosphate status, inoculated with *G. intraradices*. In the rhizosphere of these plants the AMF was present, but no root colonization could be observed, however, formononetin levels were increased. In another study, in roots of Ri T-DNA-transformed tomato plants challenged with a spore extract of *G. intraradices* new polypeptides appeared, which were not detected in *G. intraradices* colonized roots (Simoneau et al. 1994). These data point strongly towards the action of AMF-derived signal(s) on plant roots before root colonization.

In our work we studied the accumulation of flavonoids in roots of *Medicago sativa*: i) after the exposure of uncolonized roots to sterile solutions containing *Glomus intraradices* tissue (as an indicator for AMF-derived signals before appressoria formation); ii) at three different stages of colonization (from the beginning, indicated by first fungal structures in the root, until a late stage of the AM symbiosis indicated by abundant collapsed arbuscules) by *G. mosseae* to test the hypothesis of a time-specificity of the flavonoid accumulation; iii) colonized by the AMF *G. mosseae, G. intraradices* or *Gigaspora rosea* to test the hypothesis of a AMF species- or genus-specific accumulation pattern.

Materials and Methods

Biological material and growing conditions

Seeds of alfalfa (*Medicago sativa* L. cv. Sitel) were surface-sterilized by soaking in 0.75 % sodium hypochlorite for 5 min, rinsed with tap water and germinated in pots in a steam-sterilized (40 min, 120 °C) mixture of silicate sand, TurFace (Applied Industrial Materials, Corp.; Buffalo Grove, Illinois; USA), and soil (v : v : v/1 : 1 : 1).

Plants were grown in the above described growth substrate in a growth chamber (day/night cycle: 16 h; 23 °C/8 h; 19 °C; rel. humidity 50 %). Three different AMF were used: *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe (BEG 12; La Banque Européenne des Glomales; International Institute of Biotechnology; Kent; GB); *Glomus intraradices* Smith & Schenck (DAOM 197198; Department of Agriculture, Ottawa, Canada); *Gigaspora rosea* Nicolson & Schenck (DAOM 194757).

Flavonoid accumulation in *M. sativa* roots exposed to fungal tissue of *G. intraradices*

After 29 d in pots, roots were carefully rinsed with water. Roots of intact non-mycorrhizal plants (shoot and root) were placed in small dishes (diameter 15 mm) and immersed in one of the following solutions: i) 1.5 mL dist. water; ii) 1.5 mL dist. water + 1 mg fungal tissue (dry weight). The fungal tissue, obtained in a sterile water solution (kindly provided by Premier Tech, Rivière de Loups, Québec, Canada), consisted of spores and fragments of hyphae of *G. intraradices* isolated from axenic carrot AMF cultures. Before use the fungal tissue was rinsed several times with sterile dist. water.

Dishes with test plants were covered with aluminium foil to protect roots from light. Shoots of the treated plants were exposed to the same growing conditions as plants in the other two experiments (see above). Twice a day 0.8 mL of distilled water were added to the dishes to assure that roots were always covered by the solutions. After 50 h roots (plant age 31 d) were harvested and the concentration of flavonoids in lyophilized roots was determined (see below).

Inoculation experiments

Inocula of the three AMF were either produced with AMF colonized Ri T-DNA transformed carrot roots and hyphae and spores formed in these monoxenic root-AMF cultures (*G. intraradices*) or from surface sterilized spores of *G. mosseae* or *Gi. rosea*. Thus soil microorganisms associated with AMF in natural stands should be absent.

To produce inocula of the tested fungi, several spores of each fungus (in the case of *G. intraradices*, spores and infected root pieces) were placed in close vicinity to roots of beans (*Phaseolus vulgaris* L. cv. Sun Gold) growing in pots in the above described autoclaved substrate. Control mock inoculum was prepared identically as fungal inoculum, except in absence of fungi and with a water filtrate of a mixture of the three inoculi (McAllister et al. 1997).

To obtain a rapid, homogenous root colonization in the timecourse experiment with *G. mosseae* and in the inoculation experiment with the three different AMF, a compartment system consisting of one central inoculum compartment which is joined with two lateral test plant compartments was used for inoculation of the test plants (for more details see Wyss et al. 1991). The central inoculum compartments contained beans with an inoculum of each fungus (production of the inoculum in pots see above). Inoculum compartments were prepared by growing beans in the silicate sand, TurFace, soil substrate described above in presence of fungal inoculum or in the control treatment, in presence of mock inoculum. After 1 month the AM symbiosis was well established in the inoculum compartments and the system was ready for inoculation. Pre-germinated *M. sativa* plants were transferred from pots into the lateral compartments and lateral compartments were joined with the inoculum compartments. As the central inoculum compartments and the lateral compartments are equiped on the side facing each other with a nylon screen (60 μm mesh), fungal hyphae can pass from the central inoculum compartment into the lateral test plant compartments and colonize roots of the test plants, however, roots from the central inoculum compartment could not pass.

As a rhizobium-free experimental set-up was essential, harvested roots were thouroughly checked for nodule formation. As first signs of nodule formation can be observed after 10–14 d in presence of rhizobia, the absence of nodules at the end of the experiment (28 – 32 d) was a strong indicator that our system was rhizobia-free.

Neither during inoculum production nor during the experiments plants were fertilized. In each separate experiment all treatments were watered similarly. All data are the means ± standard deviation of four plants per treatment.

Timecourse of flavonoid accumulation in *M. sativa* roots colonized by *G. mosseae*

After growing for 20 d in pots (where surface sterilized seeds were germinated), *M. sativa* plants were transferred to lateral compartments of the compartment system described by Wyss et al. (1991) and 4 d later inoculated with the AMF *G. mosseae*. Plants were harvested 7 d, 18 d and 32 d after inoculation and the percentage of root colonization and the concentration of flavonoids in lyophilized roots were determined (see below).

Flavonoid accumulation in *M. sativa* colonized by three different AMF

After 14 d in pots (where surface sterilized seeds were germinated), *M. sativa* plants were transferred to lateral compartments of the compartment system described by Wyss et al. (1991) and immediately inoculated with one of the three AMF (*G. mosseae, G. intraradices, Gi. rosea*). Two weeks after inoculation (plant age 28 d) plants were har-

vested and the percentage of root colonization and the concentration of flavonoids in lyophilized roots were determined (see below).

Estimation of root colonization and detection of autofluorescing spots

For the determination of root colonization several fresh roots from each plant were cleared by boiling in 10 % KOH and stained according to the method of Vierheilig et al. (1998 b) by boiling in a 5 % ink (Shaeffer; black)/household vinegar (= 5 % acetic acid) solution. Stained roots were observed with a light microscope and the percentage of root colonization was determined according to a modified method of Newman (1966).

Fluorescence microscopy observations for the detection of autofluorescing collapsed arbuscules of *G. mosseae* were made in the time course experiment on living roots using a Reichert-Jung Microscope equipped with a fluorescence device. Fluorescence was excited by a band pass blue filter combination (450 – 495 nm).

Root extraction and high-performance liquid chromatography

At harvest roots were rinsed with tap water and root fresh weight (FW) was determined. Thereafter roots were stored at – 20 °C and finally lyophilized. The freeze-dried roots (0.5 – 2.00 g) were crushed and flavonoids were extracted in 100 % acetone overnight at 4 °C as described by Edwards and Kessmann (1992). The mixture was filtered and the extract was evaporated. The residue was dissolved in 0.5 mL of methanol.

HPLC analysis was performed on a reverse-phase analytical column (Kingsorb C_{18}, 150 mm × 4.6 mm id., 3 µm) employing a diode array detector. The flavonoids were separated by a 45 min linear gradient from 20 to 75 % solvent B (solvent A = H_2O + 0.1 % TFA, solvent B = acetonitrile) with a flow rate of 0.5 mL/min. The chromatograms were recorded at 210, 250, 260 and 350 nm depending on the UV absorption maximum of the compounds. The compound identification was based on the peak retention times and the comparison with the UV spectra of the standards.

Retention times: Ononin (Formononetin-7-O-glucoside) 14.74 min (Sequoia Research Products), 7,4′-dihydroxyflavone 15.50 min (Indofine), daidzein (4′,7-dihydroxyisoflavone) 17.20 min (Indofine), 7,4′-dihydroxyflavanone 18.30 min, 4,4′-dihydroxy-2′-methoxychalcone 20.30 min (kindly provided by D. A. Phillips, University of California, Davis, USA), genistein (4′,5,7-trihydroxyisoflavone) 23.11 min (Indofine), coumestrol 23.21 min (Fluka) isoliquiritigein (2′,4,4′-trihydroxychalcone) 26.30 min (Indofine), formononetin (7-hydroxy-4′-methoxyisoflavone) 27.14 min (Indofine), medicarpin 31.44 min (Sequoia Research Products), biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) 33.76 min (Sigma-Aldrich).

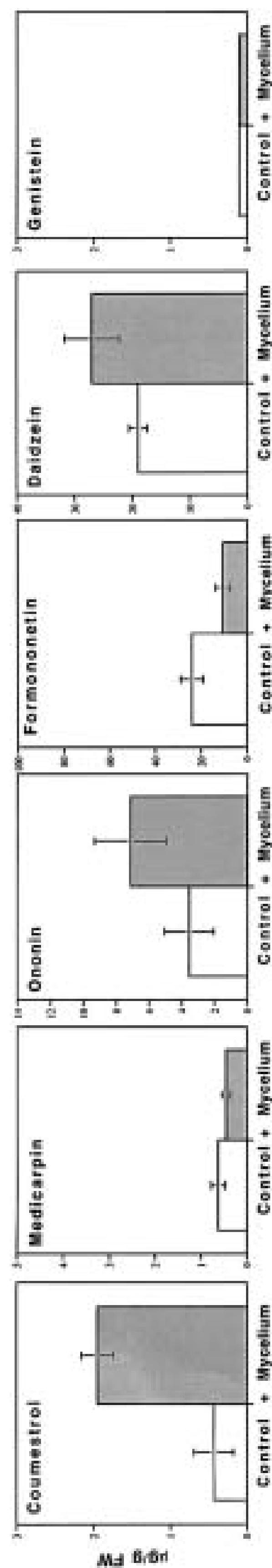

Figure 1. Flavonoid accumulation in *Medicago sativa* roots 50 h after the application of fungal material (a mixture of hyphae and spores) of *Glomus intraradices* to the roots. Data are the means ± standard deviation of four replicates from four individual plants.

Results

Detection of flavonoids

Coumestrol, medicarpin, ononin, formononetin, daidzein, genistein, biochanin A and 4′,7-dihydroxyflavanone, 4,4′-dihydroxy-2′-methoxychalcone could be detected in AMF and non-AMF roots. Isoliquiritigenin and 7,4′-dihydroxyflavone could not be detected in roots in any of the experiments. As no changes in the level of 4′,7-dihydroxyflavanone, 4,4′-dihydroxy-2′-methoxychalcone could be observed, no further data are provided.

Flavonoid accumulation in *M. sativa* roots exposed to fungal tissue of *G. intraradices*

The application of fungal tissue consisting of spores and fragments of hyphae of the AMF *Glomus intraradices* to roots of *M. sativa* resulted in an accumulation of coumestrol and daidzein, whereas the accumulation of ononin was less clear. Formononetin levels were reduced and no effect on medicarpin and genistein could be observed (Fig. 1).

Timecourse of flavonoid accumulation in *M. sativa* roots inoculated with *G. mosseae*

After 7 days of inoculation abundant hyphae on the root surface and several arbuscules could be observed (Fig. 2). The percentage of colonized root length was (10.5 % ± 3.5). No autofluorescing spots, an indicator for collapsed arbuscules, were detectable. Abundant arbuscles were visible after 18 days in colonized roots (50 % ± 8) and several autofluorescing spots could be observed. At the end of the experiment root colonization reached (78 % ± 3) and abundant arbuscules and autofluorescing spots (collapsed arbuscules) could be observed.

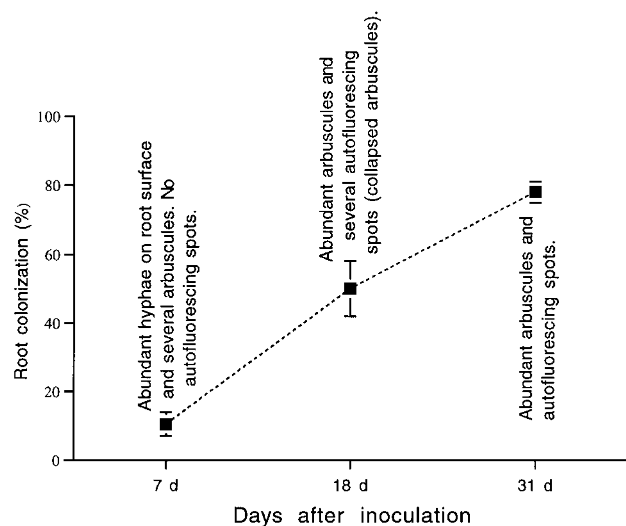

Figure 2. Timecourse of root colonization by *Glomus mosseae* in *Medicago sativa* plants. Data are the means ± standard deviation of four replicates from four individual plants.

Coumestrol was accumulated in mycorrhizal roots throughout the experiment (Fig. 3). Medicarpin accumulation was increased in mycorrhizal roots at the beginning (7 d) of the experiment and showed highest levels at the end of the experiment (32 d). At 18 d no difference in the medicarpin accumulation could be observed in mycorrhizal and non-mycorrhizal roots.

A higher accumulation of ononin could be observed in mycorrhizal roots until 18 d. Thereafter levels were similar in AMF and non-AMF roots.

Formononetin levels changed throughout the experiment. Formononetin levels at 1 d were lower in mycorrhizal roots, whereas an increase in mycorrhizal roots was detected at 32 d. Accumulation of daidzein was slightly higher in mycorrhizal roots at the beginning of the experiment but not at later stages. Genistein was only increased in non-mycorrhizal roots at the end (32 d) of the experiment.

Flavonoid accumulation in *M. sativa* colonized by three different AMF

Root colonization in AMF plants reached high levels (*Glomus mosseae* 71 ± 9; *Glomus intraradices* 87 ± 1; *Gigaspora rosea* 88 ± 7). Coumestrol, daidzein, genistein and biochanin A were accumulated in all mycorrhizal plants (Fig. 4); however, the accumulation levels varied depending on the root-colonizing AMF. For example, coumestrol was higher accumulated in roots colonized by *G. intraradices* and *Gi. rosea*, but less accumulated in roots colonized by *G. mosseae* and daidzein was higher accumulated in roots colonized by *G. intraradices* and *G. mosseae*, but less accumulated in roots colonized by *Gi. rosea*. Medicarpin, ononin, daidzein and formononetin accumulated higher with the two *Glomus* species and was not or only to a low level accumulated with *Gi. rosea* (Fig. 4).

Discussion

Several flavonoids have been described in mycorrhizal and non-mycorrhizal roots of *M. sativa*, such as coumestrol (Harrison and Dixon 1993), medicarpin (Harrison and Dixon 1993), ononin (Volpin et al. 1994, 1995), formononetin (Harrison and Dixon 1993, Volpin et al. 1994, 1995), daidzein (Harrison and Dixon 1993), genistein (Volpin et al. 1995), biochanin A (Volpin et al. 1995, Douds et al. 1998), 4′,7-dihydroxyflavanone (Maxwell et al. 1989), 4,4′-dihydroxy-2′-methoxychalcone (Maxwell et al. 1989), isoliquiritigenin and 4′,7-dihydroxyflavone (Maxwell et al. 1989, Harrison and Dixon 1993).

In none of our experiments were any changes observed in the level of 4′,7-dihydroxyflavanone, 4,4′-dihydroxy-2′-methoxychalcone. Contrary to other reports (Maxwell et al. 1989,

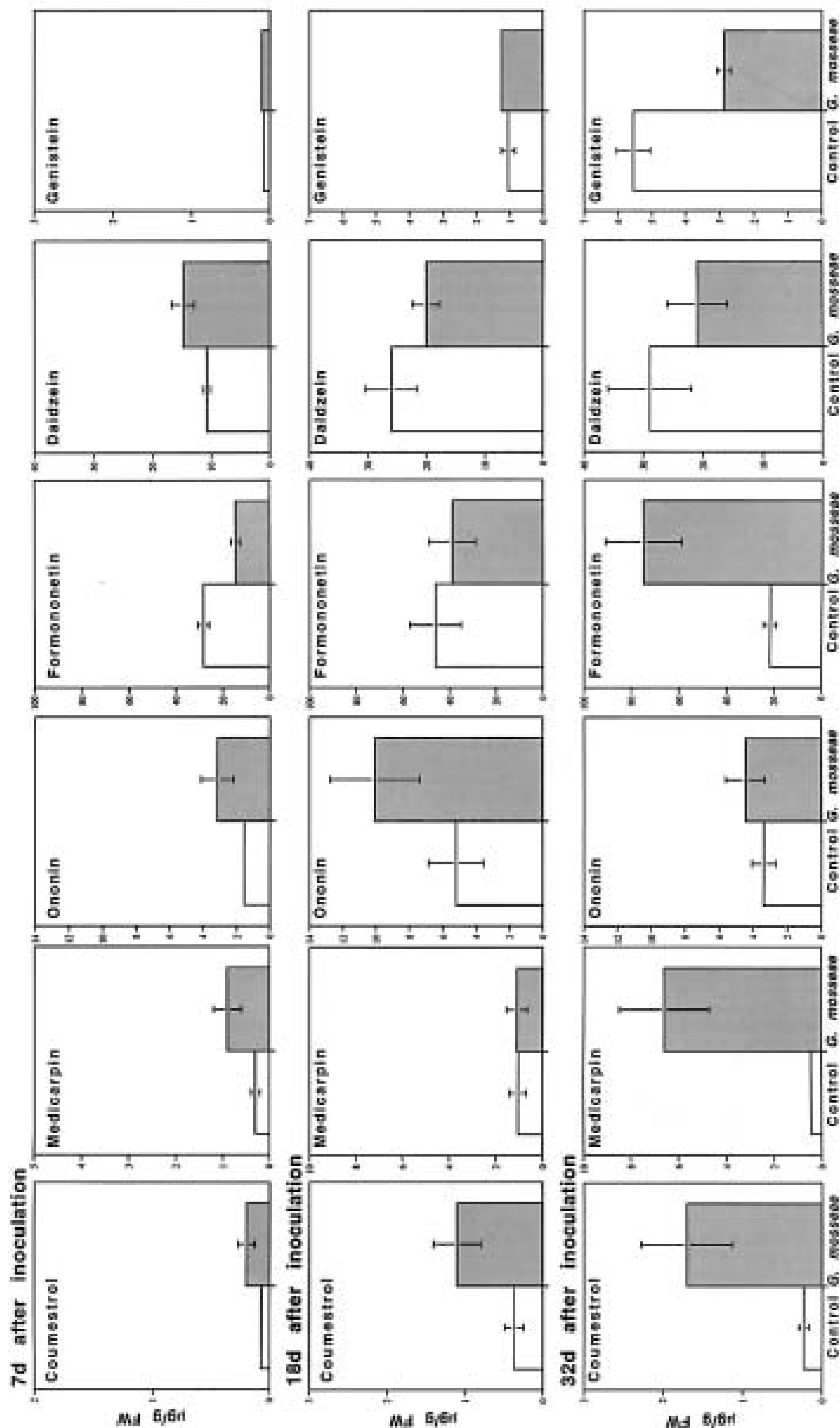

Figure 3. Timecourse of flavonoid accumulation in *Medicago sativa* roots colonized by *Glomus mosseae*. Data are the means ± standard deviation of four replicates from four individual plants.

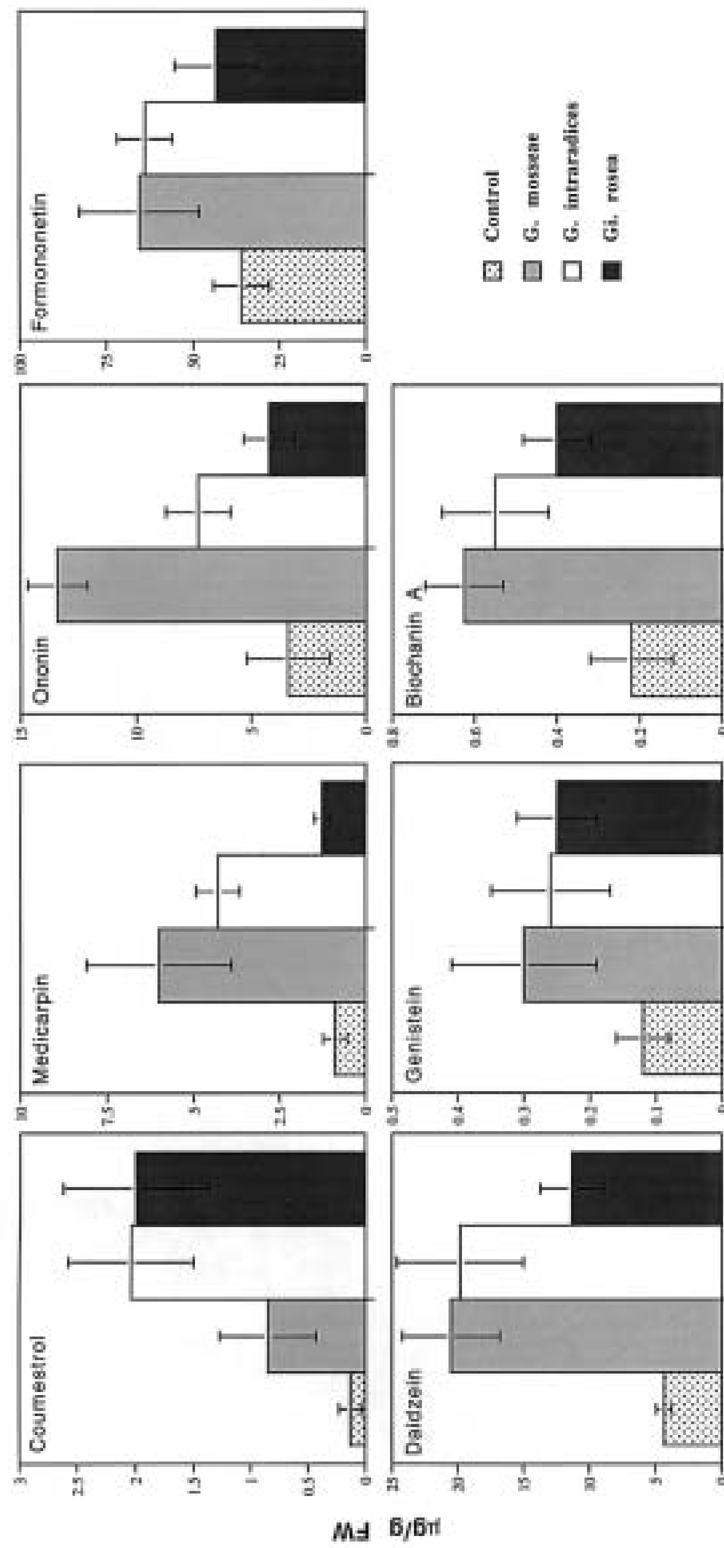

Figure 4. Flavonoid accumulation in *Medicago sativa* roots colonized by the AMF *Glomus mosseae, G. intraradices* or *Gigaspora rosea.* Data are the means ± standard deviation of four replicates from four individual plants.

Harrison and Dixon 1993), neither in roots of non-mycorrhizal nor in roots of mycorrhizal *M. sativa* plants did we detect isoliquiritigenin and 7,4′-dihydroxyflavone. These differences are possibly due to the different *M. sativa* varieties and/or growing conditions.

The presence of AMF-derived signals acting on the plant before the formation of appressoria is more and more a matter of debate (Salzer and Boller 2000, Vierheilig and Piché 2002). In our experiment we tested whether AMF-derived signals can change the flavonoid pattern in roots before appressoria formation. After an application of a mixture of spores and extraradical hyphae of *G. intraradices* to *M. sativa* roots, coumestrol, ononin, and daidzein levels were increased, whereas formononetin was detected at lower levels and no effect on medicarpin and genistein could be observed. Interestingly, there are data available showing a stimulatory effect of coumestrol on AMF-hyphal growth (Morandi et al. 1992) and a stimulation of root colonization by AMF after an application of coumestrol or daidzein to the growth substrate (Xie et al. 1995). Our data are first indications that at this stage of the communication chain between the AMF and a plant, the AMF, with signals released by its mycelium, is actually «asking for» a change of the concentration of certain flavonoids, which exhibit stimulatory effects on AMF.

Some striking differences can be observed when we compare changes of the flavonoid pattern induced by AMF-derived signals with changes in *M. sativa* at the stage of appressoria formation (Harrison and Dixon 1993). At the stage of appressoria formation an increase of daidzein has been reported (Harrison and Dixon 1993), similar to the increase we found with AMF-derived signals. However, appressoria formation increased medicarpin and formononetin levels and showed no effect on coumestrol (Harrison and Dixon 1993), whereas we found no effect of AMF-derived signals on medicarpin and formononetin and increase of the coumestrol levels. From these results we can conclude i) the presence of a signal(s) released by the fungal tissue of *G. intraradices* which is perceived by the root and ii) a different accumulation pattern induced by these fungus-derived signal(s) before appressoria formation and at the stage of appressoria formation. However, for the second conclusion we have to keep in mind that the experimental conditions were different in the two experiments (this report and Harrison and Dixon 1993) e.g. different *M. sativa* cultivars and different *Glomus* species, thus results have to be compared with caution.

In the timecourse experiment at the very beginning of root colonization of *M. sativa* by *G. mosseae* (with abundant hyphae on the root surface and several arbuscules), the flavonoid accumulation pattern was reminiscent of the accumulation pattern we obtained with fungal tissue of *G. intraradices* applied to *M. sativa* roots, except for medicarpin which was now accumulated in the mycorrhizal roots. This similar accumulation pattern indicates a possible role of the flavonoids before appressoria formation and after fungal root penetration. Of specific interest is the accumulation pattern of medicarpin. The accumulation of medicarpin is not induced by AMF-derived signals, but at the stage of appressoria formation (Harrison and Dixon 1993) and when the fungus has formed its first structures in the root (our results at 7 d). This could mean that once the AMF has formed its appressoria it is perceived in a similar way as a pathogen, with an accumulation of medicarpin, the major phytoalexin in the initial stage of a defense response (Dixon et al. 1992). Later the perception of the AMF by the plant seems to change. It has been reported that AMF root colonization suppresses medicarpin accumulation and that «… root colonization of alfalfa roots by AMF probably depends upon suppression of production of the isoflavonoid …» (Guenoune et al. 2001). This was confirmed at 18 d in our experiment. We found a no altered medicarpin accumulation at this stage of intense AM root colonization, when enhanced levels of medicarpin might negatively the AMF.

Comparing the accumulation pattern throughout the timecourse of colonization with *G. mosseae* we found some interesting variations. Whereas coumestrol and medicarpin were accumulated at the beginning of root colonization and at the end of the experiment, in mycorrhizal plants the accumulation levels of ononin and daidzein were only higher at the beginning of root colonization, whereas genistein showed no changes at the beginning of root colonization, but decreased levels at the end of the experiment. This could mean that the different flavonoids, could be involved in signalling at different stages of root colonization. Whereas coumestrol seems to play a role at any stage of root colonization, medicarpin probably plays a role at the beginning of root colonization and at a later stage. Ononin and daidzein seem to play a certain role at the beginning of root colonization, but not at a later stage, whereas genistein probably plays a specific role only at a later stage.

The most variable accumulation pattern was found for formononetin. At the beginning of root colonization we found reduced levels in the mycorrhizal roots, whereas levels increased at later stages. A similar increase at a later stage has been observed in *M. sativa* plants colonized by *G. intraradices* (Volpin et al. 1995). Interestingly, the levels of the formononetin precursor daidzein were altered inversely. When formononetin was reduced in mycorrhizal roots, daidzein was increased, and at the end of the experiment, when formononetin increased, daidzein levels seemed to decrease. This could mean that the role of formononetin and/or daidzein is changing during the symbiosis and that the formononetin/daidzein levels are linked inversely.

Until now there are no data on the function of the different flavonoids during the AM symbiosis, however, in our experiment for coumestrol, medicarpin and/or formononetin there is a possible link between their accumulation at the end of the experiment and the autoregulation of mycorrhization.

Recently, an autoregulatory mechanism of mycorrhization has been suggested. When roots are highly AMF-colonized, further root colonization by AMF is reduced in order to reduce

the costs of the symbiosis for the plant as a carbon source (Vierheilig et al. 2000 b, d, Vierheilig and Piché 2002). In 1999 Pinior et al. showed that root exudates of mycorrhizal plants lose their AMF hyphal growth- and root colonization-stimulating effect compared with root exudates from non-mycorrhizal plants. A recent study by Akiyama et al. (2002) strongly indicated that the flavonoid pattern is responsible for this effect. Whereas in non-mycorrhizal phosphate-deficient plants a root colonization-stimulating flavonoid is accumulated, no such accumulation occurs in mycorrhizal plants.

Another possible mechanism involved in the mycorrhizal autoregulation, apart from the suppressed/not induced accumulation of a stimulatory compound (Akiyama et al. 2002), is the accumulation of an inhibitory compound.

In our experiment the advanced stage of the symbiosis was characterized by its high root colonization levels and the presence of abundant collapsed arbuscules. Collapsed arbuscules exhibit a strong autofluorescence (Vierheilig et al. 1999, 2001 b), which has been suggested to be due to the synthesis of phenolics elicited by cell-wall components released by the arbuscule which is disintegrated by plant hydrolases (Vierheilig et al. 2001 a, b). A high accumulation of a certain compound at the time when arbuscules collapse could point towards a specific role of this compound at this stage. It is well documented that depending on the flavonoid and its concentration, flavonoids can exhibit an inhibitory and/or a stimulatory effect on fungi (Van Etten 1976, Wyman and Van Etten 1978, Weidenbörner and Jha 1994, Dakora and Phillips 1996, Vierheilig et al. 1998 a). Coumestrol at lower levels, as found at the beginning of the establishment of the AM, might exhibit a stimulatory effect. At higher levels, as found when plants were highly colonized, it might be inhibitory to further colonizing. Such a concentration – dependent effect of coumestrol could be observed on the vesicle cluster formation of *Gigaspora margarita* (Morandi et al. 1992). Thus, the high levels of coumestrol, medicarpin and formononetin in plants which are highly colonized, might be linked to the suppression of further root colonization by AMF of already mycorrizal plants and even might be involved in the enhanced resistance of mycorrhizal plants against soil-borne pathogens.

It would not make wonder that mycorrhizal plants, while trying to limit their costs in the AM symbiosis also acquire bioprotection against pathogenic fungi. It seems plausible that an already mycorrhizal plant develops only one mechanisms to repulse colonization by fungi, not discriminating between AMF and soil-borne pathogenic fungi (Vierheilig and Piché 2002). Interestingly, medicarpin was highly accumulated in alfalfa plants which, because of their high P-status, were not colonized by the fungus (Guenoune et al. 2001). Thus enhanced medicarpin levels accumulated in roots might affect the susceptibility of plants to the AMF and/or soil-borne pathogens. Medicarpin has been reported to exhibit a strong inhibitory effect on hyphal growth of *G. intraradices* (Guenoune et al. 2001) and is known to possess antifungal activity towards pathogenic fungi (Higgins 1978).

In the rhizobium-legume symbiosis, roots secrete (iso)flavonoids which induce bacterial genes involved in the synthesis of nodulation signals (lipo-chitooligosaccharides) that allow rhizobia to enter the root through infection threads (Perret et al. 2000). Rhizobial strains differ in their specificity for the secreted (iso)flavonoids and this may determine the specificity of a given rhizobium-legume interaction (Phillips and Tsai 1992, Dakora and Phillips 1996). Recently the hypothesis was brought forward that in mycorrhizal roots the flavonoid pattern might change depending on the AMF genus- or even species-specific requirements (Vierheilig et al. 1998 a). In our experiment in roots of *M. sativa*, no indication for a qualitative species-specific accumulation could be observed, as flavonoids which accumulated in roots colonized by *G. mosseae* also accumulated in roots colonized by *G. intradices*. However, some flavonoids accumulated to different amounts depending on the *Glomus* spp., confirming a certain quantitative species-specific accumulation of secondary plant compounds (Morandi et al. 1984, Vierheilig et al. 2000 a). Ononin for example, was highly accumulated in roots colonized by *G. mosseae,* but was only weakly accumulated in roots colonized by *G. intradices* and coumestrol was highly accumulated in roots colonized by *G. intradices* and only a low accumulation could be detected in roots colonized by *G. mosseae*. From these data a simple effect of different colonization levels (*G. mosseae* 71 ± 9; *G. intraradices* 87 ± 1) on the flavonoid accumulation level can not be excluded, however, looking at the coumestrol levels found in soybean roots colonized by *G. mosseae* or *G. fasciculatum* with a high accumulation with *G. fasciculatum* and a low accumulation with *G. mosseae* (Morandi et al. 1984), a similar pattern as found in our study, we rather suggest that the low coumestrol accumulation is *G. mosseae* specific.

For the hypothesis of a genus-specific accumulation of flavonoids the picture looked different. As shown for the species-specific accumulation, with some compounds, daidzein and biochanin A, a certain quantitative genus-specific flavonoid accumulation in roots colonized by one of the two *Glomus* spp. or by *Gi. rosea* could be observed.

However, there were also strong indications for a qualititative genus-specific accumulation. Ononin, Medicarpin and to a certain degree formononetin (the data are less clear with formononetin) were only accumulated in the interaction with the two *Glomus* species, but not with *Gi. rosea*, pointing to possibly different genus-specific signalling requirements of AMF for these compounds.

The only compound in our study that accumulated to similar levels in all AMF-colonized roots was genistein. Few data are available on the *in vitro* effect of genistein on AMF. Genistein has been shown to inhibit hyphal growth of *Gi. margarita* (Chabot et al. 1992), but had no effect on hyphal growth of *Gi. gigantea* (Baptista and Siqueira 1994) and on root colonization of *Glomus* spp. inoculated plants (Siqueira et al. 1991, Xie et al. 1995). The similar accumulation pattern of genistein in mycorrhizal roots independent of the root-colonizing AMF

could indicate a general, non-species- or non-genus-specific regulatory role of this compound during mycorrhization. However, the contradictory results on its *in vitro* effects on AMF of different species and genera give no indication for a general function (Siqueira et al. 1991, Chabot et al. 1992, Baptista and Siqueira 1994, Xie et al. 1995).

To summarize, we could show a modulation of the accumulation of certain flavonoids in mycorrhizal plants depending not only on the developmental stage of the symbiosis but also on the root-colonizing AMF. This strongly indicates not only a time-specificity of the flavonoid accumulation, but also an AMF-specificity. Further studies are needed to identify the exact role of the changing compound accumulations and the role of each compound during mycorrhization.

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