Systemically suppressed isoflavonoids and their stimulating effects on nodulation and mycorrhization in alfalfa split-root systems

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Abstract In split-root systems of alfalfa (*Medicago sativa* L.), already existing nodules or arbuscular mycorrhizal roots suppress further establishment of symbiosis in other root parts, a phenomenon named autoregulation. Roots treated with rhizobial nodulation signals (Nod factors) induce a similar systemic suppression of symbiosis. In order to test the hypothesis that flavonoids play a role in this systemic suppression, split-root systems of alfalfa plants were inoculated on one side of the split-root system with *Sinorhizobium meliloti* or *Glomus mosseae* or were treated with Nod factor. HPLC-analysis of alfalfa root extracts from both sides of the split-root system revealed a persistent local and systemic accumulation pattern of some flavonoids associated with the different treatments. The two flavonoids, formononetin and ononin, could be identified to be similarly altered after rhizobial or mycorrhizal inoculation or when treated with Nod factor. Exogenous application of formononetin and ononin partially restored nodulation and mycorrhization pointing towards the involvement of these two secondary compounds in the autoregulation of both symbioses.

Keywords Arbuscular mycorrhiza ·
Autoregulation · Flavonoid · *Medicago sativa* ·
Nodulation · *Sinorhizobium meliloti*

Abbreviations
AMF   Arbuscular mycorrhizal fungi
NFs   Nod factors

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Introduction

Apart from abiotic soil factors such as nutrient and water availability, the growth of land plants is positively affected by symbiotic microorganisms. Nitrogen-fixing rhizobia (*Rhizobiaceae* bacteria) infect roots of legumes and induce the formation of nodules. More than 80% of all land plants enter symbiosis with AMF that acquire nutrients, such as phosphorus, from the soil. In the nodule

symbiosis, the infection process depends on rhizobial nodulation signals, called NFs. During the early steps of infection, the two symbiotic associations seem to share a number of signalling steps. There is increasing evidence that components required for NF signalling also play a role in establishment of the AMF symbiosis (Guinel and Geil 2002; Hirsch and Kapulnik 1998; Riely et al. 2004; Staehelin et al. 2001; Xie et al. 1995). Similarities between the two symbioses also have been suggested during later symbiotic stages (Duc et al. 1989; Vierheilig 2004a; Vierheilig and Piché 2002).

Once a plant has formed nodules, further nodulation is suppressed in other parts of the root system by a long-distance signal exchange. This regulatory mechanism has been named autoregulation of nodulation (for details see review Caetones-Anollés and Gresshoff 1991). Interestingly, autoregulation of symbiosis has also been reported for the AMF root colonization. Working with split-root systems of barley, alfalfa and soybean, it could be shown that root colonization of one side of a split-root system strongly suppressed mycorrhization of ''autoregulated roots'' on the other side (Catford et al. 2003; Meixner et al. 2005; Vierheilig 2004b; Vierheilig et al. 2000a, b). Recently, Catford et al. (2003) reported that pre-inoculation with *Sinorhizobium meliloti* of one side of alfalfa split-roots negatively affected AMF root colonization on the other side. Conversely, pre-inoculation with AMF inhibited subsequent nodulation of autoregulated roots. Moreover, application of NFs to one side of the split-root system suppressed nodulation and mycorrhization on the other side. Meixner et al. (2005) reported that the soybean supernodulating mutant *nts1007* (mutated in the receptor kinase gene *GmNARK* (Searle et al. 2003), which lacks the autoregulatory mechanism to control nodulation, did not autoregulate AMF root colonization. All these data together indicated a similar mechanism of autoregulation in both symbioses.

Flavonoids—and isoflavonoids in particular—exuded by plant roots have been reported to activate expression of rhizobial *nod* genes required for synthesis of NFs. There is some information available on the possible limitation of NF synthesis by flavonoids (Hungria and Phillips 1993; Schlaman et al. 1991; Zaat et al. 1988) and about the effect of additional flavonoid application on nodulation (Cunningham et al. 1991; Jain et al. 1990; Kapulnik et al. 1987; Kosslak et al. 1990; Zhang and Smith 1995). Most recently, Novák et al. (2002) showed that application of specific flavonoids to pea roots either increased or decreased nodulation. Flavonoids have also been suggested to be involved in the regulation of mycorrhization (Vierheilig and Piché 2002; Vierheilig 2004a). In a number of studies with various host plants, it has been shown that roots treated with certain flavonoids exhibited increased AMF root colonization (Nair et al. 1991; Scervino et al. 2005, Siqueira et al. 1991; Vierheilig et al. 1998a; Xie et al. 1995).

To our knowledge, no data are available yet whether flavonoids are involved in autoregulation of symbiosis. In this study, we report on alterations of isoflavonoid levels in split-root systems of alfalfa plants, which were inoculated on one side either with *S. meliloti* or with the AM fungus *Glomus mosseae*. In both symbioses, formononetin and its glycoside ononin were locally and systemically down-regulated. In a further series of experiments, we applied these isoflavonoids to split-roots and studied their effects on nodulation and symbiosis.

Materials and methods

Biological material, growing conditions and experimental design

Alfalfa (*Medicago sativa* L. cv. Sitel) plants were inoculated with *G. mosseae* (Nicolson & Gerdemann) Gerd. & Trappe (BEG12; European Bank for the Glomales) or for nodulation experiments with *S. meliloti* strain 1021. Corresponding control plants were grown under the same conditions. Alfalfa plants were grown in a growth chamber [day/night cycle: 16/8 h, 23/19°C; PAR (photosynthetically active radiation): 300 $\mu mol/m^{-2}$ s^{-1}; relative humidity 50%]. All experiments were repeated twice with five replicates per treatment.

Split-root systems

Alfalfa seeds 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 (30 min, 121°C) mixture of silicate sand, TurFace (Applied Industrial Materials Corp.; Buffalo Grove, Illinois, USA) and soil (1:1:1 by vol). To induce the development of lateral roots, tips of main roots from 10 days old plantlets were excised. Plantlets were then placed into the same substrate as described above. Twenty-five days later, split-root systems were established as described previously (Vierheilig et al. 2000a). The split-root system consists of two units, each containing one half of the alfalfa root system. The two compartments are separated by an imper-meable PVC membrane in order to prevent any flow of molecules or root growth from one side to the other. Thus, symbiosis can be established in split-root systems on one side without any contact on the other side. Control plants were grown under the same growth conditions as the inocu-lated plants.

Isoflavonoid levels in a split-root system of alfalfa with one side inoculated with S. meliloti

Sinorhizobium meliloti strain 1021 was cultivated at 25°C in a TY–streptomycin (50 μg/ml) medium (Beringer 1974) on a rotary shaker (200 rpm) to reach an $OD_{600} = 0.25$. Bacteria were then harvested by centrifugation ($6000g \times 10$ min) and resuspended in sterile H_2O. For inoculation with *S. meliloti*, one side of a split-root system was inoculated with 10 ml of this bacterial suspension (one day after the transfer of plants into the split-root system). After 15 days, the split-root system and non-inoculated control plants were harvested. The nodulated side of the split-root formed 168 ± 11 nodules per 100 mg root (FW), whereas no nodules were seen in the non-nodulated side. The harvested plant material was then frozen (-80°C) and lyophilized. Flavonoid analysis was performed with samples of all three different treatments (control roots; nodulated roots from the inoculated side of the split-root system; roots from the non-inoculated side of the split-root system).

Isoflavonoid levels in a split-root system of alfalfa with one side treated with NFs

The tetrameric Nod factor NodSm-IV(C16:2, S) was purified from *S. meliloti* strain 1021(pEK327) (Schultze et al. 1992; kindly provided by Eva Kondorosi, CNRS, Gif-sur-Yvette, France). Supernatants of bacterial cultures were extracted with *n*-butanol and fractionated by reverse-phase HPLC (Waters C18 column) with 35% acetoni-trile/water, 40 mM ammonium acetate as the mobile phase. The fraction containing NodSm-IV(C16:2, S) was collected and desalted as described by Staehelin et al. (2000).

Eight days after establishment of the split-root system, 4 ml of a 10^{-8} M NF solution (dissolved in water) were daily applied to one side (volume 65 ml) of the system. After 8 days, the NF-treated side, the roots from the other side of the split-root system and non-treated control plants were harvested. Lyophilized root material was used for flavonoid analysis.

Isoflavonoid levels in a split-root system of alfalfa inoculated with G. mosseae

The outer side of each split-root compartment was equipped with a Nylon screen (Nitex mono-filament,100 μm mesh), which can be penetrated by hyphae but not by roots. To inoculate alfalfa with *G. mosseae*, one outer side of the split-root compartment was joined at the day of transfer of plants into the split-root system with a donor compartment, which on its sides was also equip-ped with the Nylon screen. This donor compart-ment contained beans (*Phaseolus vulgaris* L. cv. Sun Gold) colonized by *G. mosseae*. Hyphae of the AMF penetrated the Nylon screen and colo-nized one side of the split-root system, however, the second side of the split-root system remained without root colonization by the AMF. After 20 days the mycorrhizal and non-mycorrhizal roots of the split-root system and the roots of the control plants were harvested and frozen (-80°C). After lyophilization, roots were used for flavo-noid analysis. In all samples, the degree of AMF root colonization was determined at the time of harvest.

Effects of isoflavonoid application on
nodulation in split-root systems pre-inoculated
with S. meliloti

One side of the split-root system of alfalfa plants
was pre-inoculated with *S. meliloti* as described
above. After 15 days, when nodules had formed
on the first side of the split-root system (168 ± 11
nodules per 100 mg root FW), the second side was
inoculated with *S. meliloti*. At the same time for-
mononetin or ononin were applied to the second
side of the split-root system. The application of
isoflavonoids was repeated every second day
during 15 days. Thereafter the root material was
harvested and the number of nodules determined.

Effects of isoflavonoid application on
mycorrhizal colonization in split-root systems
pre-colonized by G. mosseae

One side of the split-root system was pre-inocu-
lated with *G. mosseae* as described above. After
20 days, when the AMF symbiosis was well estab-
lished on the first side of the split-root system
(54 ± 3% root colonization), the second side was
inoculated with *G. mosseae*. At the same time
formononetin or its glycoside ononin were applied
to the second side of the split-root system and this
treatment was repeated every second day during
20 days. Thereafter plants were harvested and the
degree of AMF root colonization was determined.

Application of isoflavonoids to roots

Formononetin (1.34 mg) or ononin (2.15 mg)
were dissolved in 4 ml ethanol and diluted with
H_2O to a final volume of 1,000 ml. In a pre-
experiment, it was found that 0.4% ethanol did
not exhibit any effect on AMF root colonization.
Volumes of 10 ml of the diluted isoflavonoid
solutions (with a final concentration of 5 μM)
were applied every second day to the second side
of the split-root system.

Determination of nodule number and AMF
root colonization

At the time of harvest, roots were carefully rinsed
with water and the root fresh weight and the

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

Root extraction and high-performance liquid
chromatography

At harvest roots were rinsed with tap water and
root fresh weight (FW) was determined. There-
after roots were stored at −20°C and finally
lyophilized. The freeze-dried roots (0.5–2.0 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 × 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 Prod-
ucts), 7,4′-dihydroxyflavone 15.50 min (Indofine),
7,4′-dihydroxyflavanone 18.30 min, 4,4′-dihydr-
oxy-2′-methoxychalcone 20.30 min (kindly pro-
vided by D.A. Phillips, University of California,
Davis, USA), daidzein (4′,7-dihydroxyisoflavone)
17.20 min (Indofine), genistein (4′,5,7-trihydr-
oxyisoflavone) 23.11 min (Indofine), coumestrol
23.21 min (Fluka), formononetin (7-hydroxy-4′-
methoxyisoflavone) 27.14 min (Indofine), medi-
carpin 31.44 min (Sequoia Research Products).

Results

Analysis of isoflavonoids in alfalfa roots indicated for each treatment a specific isoflavonoid pattern. Levels of certain isoflavonoids remained similar in all samples, whereas others varied in response to *S. meliloti* or *G. mosseae*. The data presented in this study are restricted to those isoflavonoids that exhibited similar altered levels during symbiosis and Nod factor treatment, namely formononetin and its glycoside ononin, medicarpin and daidzein. The levels of 7,4′-dihydroxyflavone, 7,4′-dihydroxyflavanone, 4,4′-dihydroxy-2′-methoxychal cone and genistein were not changed in any of the treatments (data not shown), whereas only in mycorrhizal plants coumestrol levels were locally and systemically enhanced (data not shown).

Compared to non-inoculated control plants, inoculation with *S. meliloti* on one side of a split-root system resulted in decreased levels of formononetin and ononin in the inoculated part of the split-root (nodulated roots). Interestingly, levels of formononetin and ononin were also low in the non-inoculated side of the split-root, indicating a systemic effect from inoculated to non-inoculated root parts. Medicarpin levels were low

in non-inoculated control plants and slightly reduced in both parts of the split-root system. Daidzein levels in nodulated roots were elevated, whereas the non-inculated part of the split-root system exhibited levels comparable to those in non-inoculated control plants (Fig. 1). Application of NFs to one side of a split-root system strongly reduced the levels of formononetin, ononin and medicarpin. Lowest levels were found for formononetin, which were about 10-fold lower than in non-inoculated control plants. Levels of these isoflavonoids were also systemically down-regulated in the half of the split-root, which has been not treated with NFs. In contrast to the infection with *S. meliloti* resulting in nodulated roots, application of NFs to roots did not affect the daidzein levels in our split-root experiments (Fig. 2).

When one side of the split-root system was inoculated with the AMF *G. mosseae*, the levels of formononetin and ononin in mycorrhizal roots were not altered compared to non-inoculated control plants. The non-inoculated side of the split-root however, exhibited low levels of formononetin and ononin, indicating a systemic regulation of these isoflavonoids. Medicarpin and
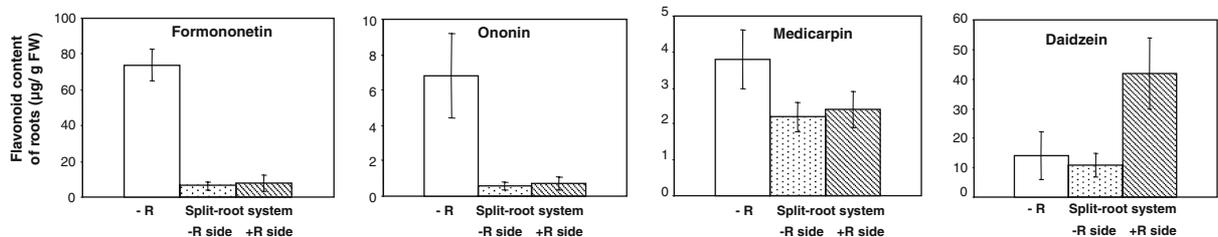

Fig. 1 Flavonoid pattern in a split-root system of alfalfa with one side inoculated with *Sinorhizobium meliloti* (+R side) and the other side without inoculation (−R side) and in roots of non-inoculated control plants (−R). Data represent mean ± SE (*n* = 5)
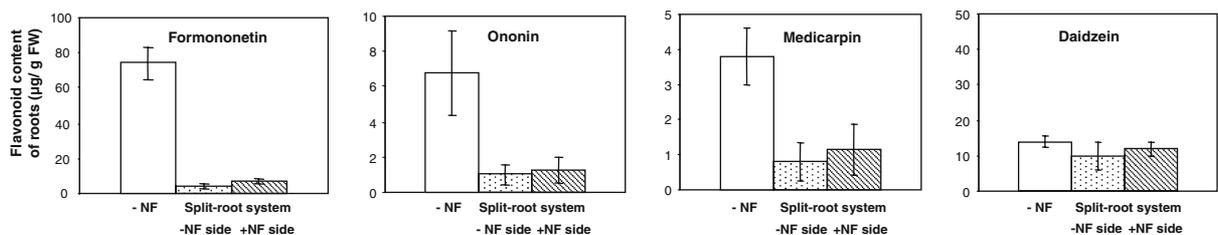

Fig. 2 Flavonoid pattern in a split-root system of alfalfa with one side treated with nod factor (+NF side) and the other side without NF treatment (−NF side) and in roots of non-treated control plants (−NF). Data represent mean ± SE (*n* = 5)

daidzein levels were stimulated in the side with mycorrhizal roots, when compared to non-mycorrhizal control plants. Daidzein was also strongly accumulated in the non-mycorrhizal part of the split-root system (Fig. 3).

Taken these data together, they indicate that formononetin and its glycoside ononin are systemically down-regulated in response to *S. meliloti* infection, treatment with NFs and root colonization by *G. mosseae*. Hence, low levels of formononetin and ononin in non-infected roots are metabolic markers for autoregulated roots. We therefore tested in a second series of experiments whether an application of formo-nonetin or ononin to autoregulated roots can stimulate nodulation and mycorrhization. To induce autoregulation signalling, a split-root system was inoculated on one side. As reported previously (Catford et al. 2003), pre-inoculation

with *S. meliloti* on one side of the split-root system resulted in a drastic suppression of nodule formation on the later inoculated side of the split-root system. As shown in Fig. 4, only few nodules were counted on the second half of the split-root system, whereas plants, which have been not pre-inoculated, formed about 10-fold more nodules. When formononetin was applied to the second side of the split-root system of pre-inoculated plants, nodule formation on this autoregulated side was strongly increased. Nodule numbers reached values that were similar to those from not pre-inoculated plants. This finding indicates that formononetin counteracts autoregulation of nodulation. Similar to formo-nonetin, its glycoside ononin also promoted nodulation on the autoregulated side of split-roots, albeit to a lower extent than the aglycone (Fig. 4).
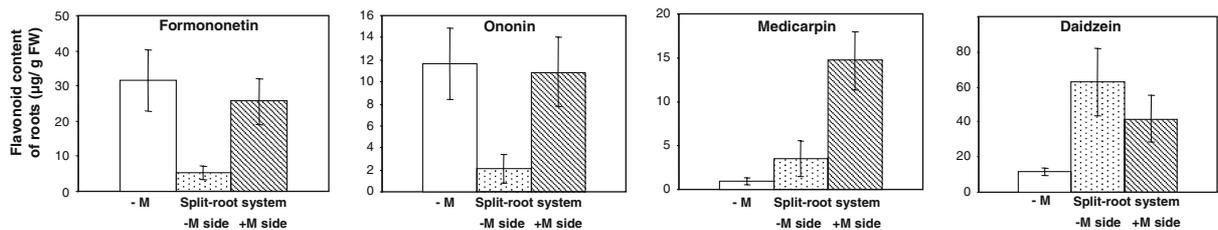

Fig. 3 Flavonoid pattern in a split-root system of alfalfa with one side inoculated (+M side) with *Glomus mosseae* and the other side without inoculation (−M side) and in roots of non-inoculated control plants (−M). Data represent mean ± SE ($n = 5$)

Fig. 4 Effect of rhizobial pre-inoculation of one half of the split-root system of alfalfa on subsequent nodule formation on the second half of the split-root system with or without application of formononetin or ononin (10 ml of a 5 µM solution every second day) to the second half. Data represent mean ± SE ($n = 5$)
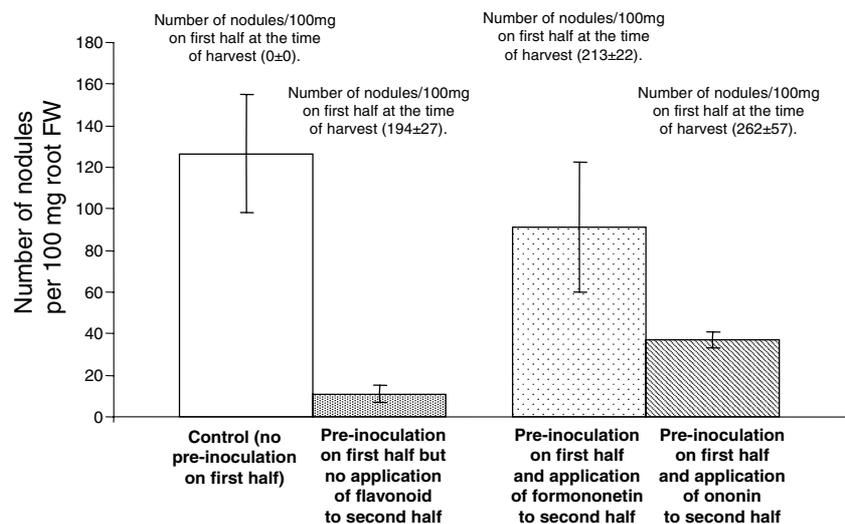
Pre-inoculation with *G. mosseae* on one side of the split-root system resulted in a drastic suppression of AMF root colonization on the second side of the split-root system, indicating autoregulation of mycorrhization (Catford et al. 2003; Fig. 5). When the glycoside ononin was applied to the autoregulated side of the split-root, AMF root colonization by *G. mosseae* was enhanced on this side. AMF root colonization, however, did not reach the degree of those plants that were not pre-inoculated with *G. mosseae*. In contrast to ononin, application of the aglycone formononetin did not affect mycorrhization in our split-root experiments (Fig. 5).

Discussion

Inoculation of one side of an alfalfa split-root system with *S. meliloti*, *G. mosseae* or the application of NF to one side of an alfalfa split-root system has been reported to suppress nodulation or mycorrhization on the other side (Catford et al. 2003). In this study, we have used split-root systems in order to test whether isoflavonoid levels in alfalfa roots are systemically altered in response to *S. meliloti* and *G. mosseae*. We could demonstrate that infection with either *S. meliloti* or *G. mosseae* influenced the levels of isoflavonoids in roots, which were not in contact with the invading symbionts. Hence, specific isoflavonoids in roots are systemically regulated upon infection.

Our study shows significant symbiosis-related alterations for four isoflavonoids: formononetin, its glycoside ononin, medicarpin and daidzein. In the pathway for biosynthesis of alfalfa isoflavonoids, formononetin is a precursor of the phytoalexin medicarpin. Daidzein however, is most likely not an intermediate in medicarpin synthesis. It has been proposed that 4'-O-methyltransferase is the key enzyme at the branching point for medicarpin synthesis (Liu and Dixon 2001). In this view, our data indicate that root colonization by *G. mosseae* activates the medicarpin synthesis pathway as reported previously (Larose et al. 2002), whereas infection with *S. meliloti* downregulates this pathway. Daidzein levels accumulated in roots in reponse to both symbionts. Similar to the infection with *S. meliloti*, application of NFs resulted in reduced levels of formononetin, ononin and medicarpin, indicating a suppression of this synthetic pathway. A similar, albeit weaker, suppression of formononetin occurred in alfalfa roots that were challenged with Nod factors for 50 h (C.S., G.L. and H.V., unpublished results; Vierheilig et al. 2004b). The suppression effect of NFs on specific isoflavonoids involved in medicarpin synthesis is remarkable, as various genes involved in isoflavonoid biosynthesis have been reported to be up-regulated in response to NFs (i.e., in *M. truncatula*; Lohar et al. 2006).

In the split-root experiments of this study, inoculation with *S. meliloti* on one side also

Fig. 5 Effect of AM pre-inoculation of one half of the split-root system of alfalfa on subsequent AMF root colonization of the second half of the split-root system with or without application of formononetin or ononin (10 ml of a 5 μM solution every second day) to the second half. Data represent mean ± SE ($n = 5$)
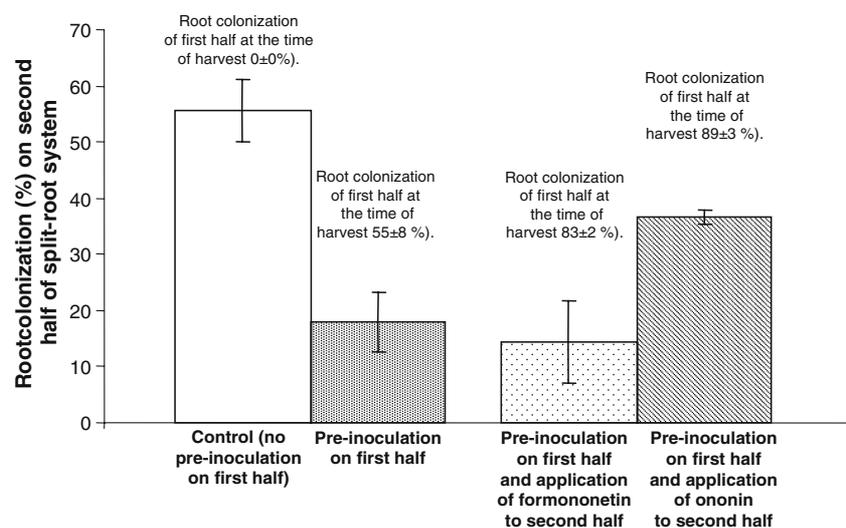
altered isoflavonoids of the medicarpin synthesis pathway on the other side (Fig. 1). We conclude that levels of formononetin and its glycoside ononin are not only locally reduced in response to *S. meliloti*, but also systemically down-regulated in parts of the root system that were not in contact with *S. meliloti*. When NFs were used for the experiment, we observed a similar systemic reduction for formononetin, ononin as well as medicarpin (Fig. 2). As NFs are able to induce autoregulation of symbiosis in alfalfa split-roots (Catford et al. 2003), we suggest that systemic down-regulation of formononetin and ononin levels in non-treated root parts is a metabolic reponse to the putative autoregulation signals that mediate suppression of symbiosis. Root colonization by *G. mosseae* also can elicit suppression of symbiosis in other parts of the root system (Catford et al. 2003) and we therefore expected a systemic down-regulation of formononetin and ononin also during the mycorrhizal symbiosis. Indeed, our data indicate very low levels of these isoflavonoids in the non-inoculated side of the split-root (Fig. 3). Thus, all known treatments eliciting autoregulation signaling—i.e., inoculation with *S. meliloti*, application of NFs, and inoculation with *G. mosseae*—suppressed accumulation of formononetin and ononin in the whole root system.

Nodule formation and mycorrhization is suppressed in autoregulated parts of the root system. Our isoflavonoid analyses indicate low levels of formononetin and ononin in all autoregulated roots. We therefore wondered whether these isoflavonoids are limiting factors for establishment of symbiosis in autoregulated roots. Our data point to this direction, as application of formononetin or ononin to autoregulated roots promoted nodule formation. Ononin showed a similar stimulating effect on establishment of the AMF symbiosis. With other words, application of isoflavonoids to roots inactivated or compensated the symbiosis-suppressing effects induced by long-distance autoregulation signals.

In the rhizobial symbiosis, flavonoids have been proposed to play a limiting factor for NF synthesis and optimal establishment of symbiosis (Zaat et al. 1988; Schlaman et al. 1991; Hungria and Phillips 1993). Interestingly, exogenous application of flavonoids to legumes has been reported to increase or decrease nodulation (Cunningham et al. 1991; Jain et al. 1990; Kapulnik et al. 1987; Kosslak et al. 1990; Novák et al. 2002; Zhang and Smith 1995;). One possible explanation is that specific flavonoids induce rhizobial *nod* gene expression required for synthesis of NFs, whereas others do not or even repress NF synthesis. Formononetin however, did exhibit *nod* gene inducing activity on a *S. meliloti* strain (Zuanazzi et al. 1998). It is therefore unlikely that the promoting effect on nodulation induced by formononetin (Fig. 4) is related to NF synthesis of *S. meliloti* strain 1021.

Flavonoids have also been suggested to be involved in the regulation of mycorrhization (Vierheilig and Piché 2002; Vierheilig 2004a). A number of studies with various host plants showed that roots challenged with specific flavonoids increased AM root colonization (Nair et al. 1991; Scervino et al. 2005; Siqueira et al. 1991; Vierheilig et al. 1998a; Xie et al. 1995). Ononin promoted AMF root colonization in our experiments (Fig. 5), indicating that certain isoflavonoids have antagonistic effects on autoregulation of AMF root colonization. In contrast to the nodule symbiosis, formononetin did not stimulate mycorrhization in autoregulated parts of the root system. Hence, although autoregulation of nodulation and mycorrhization seem to share some common signalling events (Catford et al. 2003; Meixner et al. 2005), the effects of systemically regulated isoflavonoids on establishment of symbiosis are different.

Application of isoflavonoids to roots might induce multiple responses. One possibility is that flavonoids and NFs interfere with the polar auxin transport in roots (Boot et al. 1999; Jacobs and Rubery 1988; Mathesius et al. 1998). Treatment of roots with auxin transport inhibitors induced the formation of nodule-like structures in alfalfa (Hirsch et al. 1989) and promoted mycorrhizal colonization in various plants, including alfalfa (Xie et al. 1996, 1998). Moreover, analysis of phytohormones in soybean roots suggested that auxin levels might be involved in autoregulation of symbiosis (Caba et al. 2000; Meixner et al. 2005). Future split-root experiments are required to test whether formononetin and ononin alter

auxin transport in the autoregulated part of alfalfa roots and whether other isoflavonoids have similar effects on roots.

To summarize, our study shows that levels of specific isoflavonoids are systemically suppressed in response to *S. meliloti*, NFs and *G. mosseae*. Comparing the systemic isoflavonoid accumulation pattern in all three treatments, we propose that formononetin and ononin are possibly involved in the systemic suppression of nodulation and mycorrhization. Application of formononetin and ononin to autoregulated roots promoted nodulation, whereas ononin stimulated mycorrhization. Future studies with plant mutants lacking autoregulation are required to demonstrate that the long-distance signals controlling autoregulation of symbiosis are identical to those signals that systemically regulate isoflavonoid levels.

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