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MISE EN ÉVIDENCE DE MOLÉCULES EFFECTRICES, LES FLAVONOÏDES, IMPLIQUÉES DANS LA RÉGULATION CROISÉE DES SYMBIOSES MYCORHIZIENNE ARBUSCULAIRE ET RHIZOBIENNE CHEZ *MEDICAGO SATIVA*

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Avant-propos

En rédigeant cette thèse cela m'a permis de réfléchir sur une expérience de vie qui fut enrichissante. Il y a l'aspect scientifique et l'appentissage de connaissances qui ne sont pas à dédaigner. Mais je dirais que les aspects les plus importants sont ceux que j'ai appris au contact des autres. Je veux prendre ces quelques lignes pour remercier ceux qui ont contribué à mon avancement.

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Résumé long

Deux symbioses végétales partagent la même niche écologique, leur hôte, une légumineuse. Ces deux associations mutualistes sont la mycorhize arbusculaire (MA) et la symbiose *Rhizobium*/légumineuse. Chacune de ces relations symbiotiques, prise individuellement, peuvent contrôler leur propre colonisation par des mécanismes d'autorégulation. Ce contrôle exclusif s'effectue par rétroaction systémique et parviendrait à limiter le coût en carbone pour l'hôte.

En vue d'étudier la restriction exercée par une plante hôte sur le degré de colonisation mycorhizienne et rhizobienne, la légumineuse *Medicago sativa* a été cultivée en chambre bicompartimentée (Split-Roots). Ce dispositif expérimental a permis de séparer en deux parties distinctes le système racinaire de *M. sativa*. Le but recherché par ces expérimentations était de vérifier à distance l'autorégulation au sein d'une symbiose sur la seconde partie du système racinaire et de mettre en évidence une régulation négative croisée entre deux symbioses différentes.

En effet pour la première fois, il est démontré que l'autorégulation systémique n'est pas l'action d'une seule symbiose sur elle-même, mais que par des signaux communs entre les deux symbioses, il existe une régulation négative croisée entre elles. Cette approche permet d'examiner, sous un nouvel angle, la régulation de l'établissement d'une symbiose par une autre symbiose. Dans ces conditions, nos résultats montrent l'existence d'un lien évident de signalisation entre les deux symbioses végétales. Dans le cas précis d'une pré-colonisation de *M. sativa* par *Sinorhizobium meliloti,* les facteurs Nod se sont révélés impliqués au niveau de l'autorégulation. Dans cet exemple, les flavonoïdes exsudés par *M. sativa* sont non seulement reconnus par les *Rhizobia* mais aussi par le *Glomus mosseae.* Ces résultats représentent un acquis important pour l'interprétation de la signalisation au niveau de l'autorégulation des deux symbioses à l'étude.

Par ailleurs, l'analyse des exsudats racinaires de *M. sativa* par chromatigraphie liquide à haute performance (HPLC) montre une modification significative du patron des isoflavonoïdes lorsque cette plante est soumise à différents contrôles

dérivant d'autorégulations et de régulations négatives croisées. De plus il est loisible d'observer une variation similaire de patrons de flavonoïdes suite à l'application de facteurs Nod. En soi, d'une façon plus exacte, tous ces traitements provoquent une réduction marquée de la formononétine et de l'ononine. Ces deux isoflavonoïdes semblent être d'excellents candidats comme molécules « signal » dans la régulation à distance pour les deux symbioses végétales. De plus, l'application externe de ces deux isoflavonoïdes peut normalement restaurer la nodulation et la mycorhization.

Les flavonoïdes jouent un rôle clef dans la formation de la symbiose MA chez *M. sativa*. Lors d'expériences avec diverses souches de *G. mosseae* nous avons cherché à montrer qu'il y avait bien une régularité et une constance dans le patron de flavonoïdes considérés comme molécule signal et cela peu importe les besoins métaboliques divergents associés à ces souches de *G. mosseae*. Selon cette prémisse nous concluons que leur production n'est pas subordonnée aux besoins métaboliques des souches fongiques. La constance de l'augmentation ou la réduction du patron de ces flavonoïdes pourraient bien être le résultat du rôle de flanovoïdes impliqués pour des mécanismes de signalisations qui accompagnent nécessairement l'établissement de la symbiose MA.

Long abstract

Two symbiosis share the same ecological niche with a common legume host. These two mutualistic associations are: Arbuscular mycorrhiza (AM) and *Rhizobium*/legume symbiosis. Individually, each symbiosis could control homeostasis by negative regulatory mechanims where the main objective is to modulate carbon availability.

In perspective to study symbiosis autoregulation by both symbiosis on further colonization a split-root compartment system is our basic tool. This experimental device allows the separation of a root system in two independent parts and provides an experimental tool for understanding remote systemic signals involved in negative symbiosis regulation.

We discover a novelty in autoregulation, where one symbiosis is remotely acting in negatively, by restricting another symbiosis establishment. This control, accomplished via a common plant host, is clearly demonstrated. This new approach allows not only the investigation of cross-regulation but also provides the indication for of a common signalling pathway between both symbiosis. A pre-colonization of *Medicago sativa* by *Sinorhizobium meliloti* showed that Nod factors appear to play a key role in autoregulation. Furthermore, acting molecules such as flavonoids, exudated by *M. sativa roots*, are recognized by both symbionts. These results give further insights into the elucidation of autoregulatory signalling involved in both symbiosis.

Moreover, High Performance Liquid Chromatography (HPLC) analysis of roots exudates of *M. sativa* exhibits a variation for isoflavonoid patterns when these exudates are isolated from cross regulation treatments or under Nod factors application. Isoflavonoids involved in autoregulation decreases are mainly Formononetin and Ononin. These two molecules seem to be good candidates for signal molecules, which are implicated in down-regulation of symbiosis establishment. In addition exogenous application of these flavonoids has some impact in restoring symbiosis.

As those flavonoids play a pivotal role in *M. sativa* mycorrhizal symbiosis, we attempted to show a constant and reproducible pattern for different strains of *G. mosseae,* which are recognized to express variable metabolic requirements. HPLC analysis of those flavonoids confirms independent status between signalling and fungal energetic needs. The behaviour of these flavonoids is conforms to signals molecules.

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Introduction

Les symbioses qui impliquent les acteurs que sont les champignons mycorhiziens arbusculaires (MA) et le système racinaire de la majorité des plantes vasculaires constituent la plus importante association dans le monde végétal. Les protagonistes fongiques appartiennent à un groupe de champignons cœnocytiques d'environ 150 espèces. Ils appartiennent au phyllum Glomeromycota (Schüßler *et al.* 2001). Les origines de cette symbiose MA remontent à plus de 400 millions d'années selon l'étude des fossiles (Remy *et al.* 1994). Cette longue période d'adaptation a permis à plus de 200 000 espèces de plantes terrestres de co-évoluer avec les mycorhizes MA (Wang et Qiu 2006). Ces champignons MA ont développé une dépendance vitale à leur hôte et ils doivent obligatoirement s'associer aux racines de la plante pour survivre. On peut les voir facilement à l'intérieur des cellules corticales des racines où ils forment des petites structures, les arbuscules, d'où leur appellation de « champignons mycorhiziens arbusculaires » (Bonfante-Fasolo 1984 ; Smith et Smith 1997). La croissance des champignons MA ne se limite pas à l'intérieur des cellules de leur hôte. Cette croissance se manifeste aussi par un mycélium extraradical caractérisé par l'allongement d'hyphes principaux [Runner Hyphae (RH)], par la production de fines ramifications [Fine Branching (FB)] ainsi que par la formation de spores, dont la taille dépasse largement celle des autres espèces fongiques du règne des Mycota (40 à 500µm) (Juge *et al.* 2009a,b).

Le fonctionnement des symbioses végétales est basé sur des échanges bénéfiques entre les partenaires. Il est bien connu, chez la symbiose MA, que les partenaires fongiques puisent les glucides provenant de la photosynthèse de l'hôte. En retour, ils fournissent à la plante l'eau et des éléments minéraux, principalement les phosphates (Smith et Read 2008). Un type d'échange similaire domine également la symbiose *Rhizobium*-légumineuses. Dans ce dernier cas, les structures d'échanges sont des nodosités où l'azote atmosphérique est fixé par les bactéries pour être ensuite assimilé par la plante.

Les processus d'échanges physiologiques ne sont pas limités qu'aux symbioses mutualistes végétales. De nombreux champignons phytopathogènes développent des structures apparentées, les *haustoria*, qui puisent les nutriments à même les cellules hôtes (Szabo et Bushnell 2001). Cependant, comme nous le savons, ce prélèvement nutritionnel est à sens unique en faveur du parasite.

Pour comprendre les processus de reconnaissance à la base de toutes ces associations plantes-microorganismes, il faut cerner principalement le type et la séquence des signaux échangés entre les partenaires. Ces processus signalétiques conduisant à l'établissement et au maintien des relations plantes-microorganismes sont multiples et complexes, notamment chez les symbioses MA (Harrison 2005). Bécard et Piché (1989) ont observé que le dioxyde de carbone et les exsudats racinaires induisent la croissance des hyphes des champignons MA en phase pré-symbiotique. De leur côté, Gianinazzi-Pearson *et al.* (1989) ont montré que les flavonoïdes issus du métabolisme secondaire des plantes stimulaient la croissance des hyphes des champignons MA. Depuis cette étude, plusieurs travaux ont corroboré le rôle inducteur des flavonoïdes exsudés tant sur les gènes bactériens du *Rhizobium* spp. (Subramanian *et al.* 2007) que sur le signal stimulateur de croissance des partenaires fongiques MA (voir revues bibliographiques par Morandi 1996; Vierheilig *et al.* 1998a; Steinkellner *et al.* 2007). Les flavonoïdes sont des métabolites secondaires de la voie des phénylpropanoïdes des plantes (Fig. 1). Ils exercent des rôles biologiques différents avec les micro-organismes rhizosphériques selon que l'association est bénéfique ou nuisible (Peer et al. 2005). Mentionnons à titre d'exemple: la biochanine A stimule les hyphes des champignons MA, mais inhibe du même coup l'infection des champignons pathogènes.

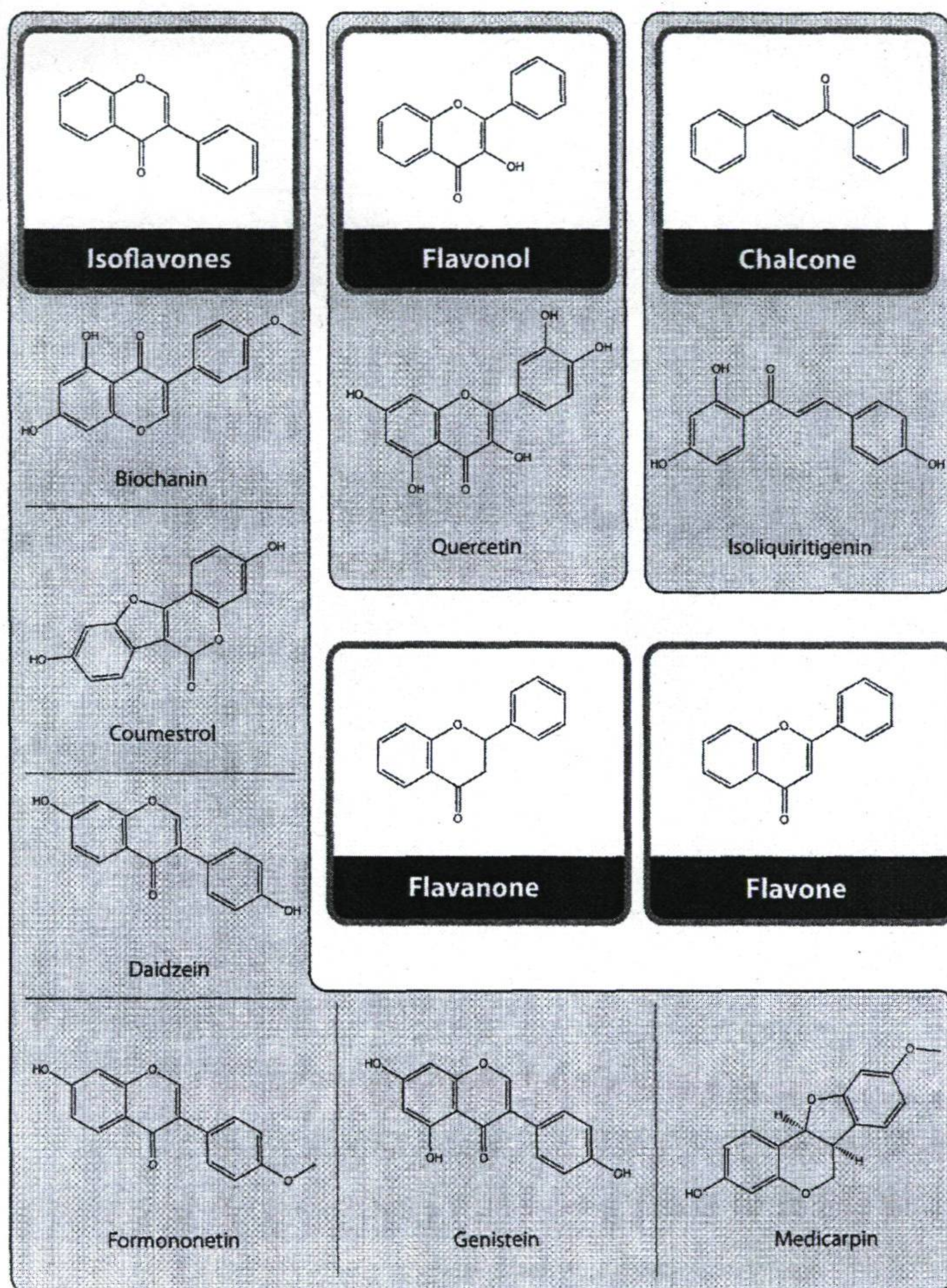

Figure 1: Structure chimique des molécules utilisées dans cette étude.

Note: les noms des molecules sont données en anglais

Les flavonoïdes ne sont pas les seules molécules qui participent au développement des mycorhizes MA ou qui influencent le développement des agents pathogènes. D'autres molécules, connues sous le nom générique de strigolactones induiraient non seulement la germination de graines chez les plantes parasites (*Striga* spp. et *Orobanche* spp.), mais elles stimuleraient la formation de fines ramifications chez les hyphes MA (Yoneyama *et al.* 2008)

La caractérisation des voies de signalisation entre les plantes et les microorganismes symbiotiques est un sujet de recherche fort complexe en soi. Et ce, à juste titre, puisque l'élucidation des mécanismes de reconnaissance et de maintien qui président à la formation des mycorhizes MA demeure un défi d'envergure.

Les informations pertinentes à la signalisation ont été obtenues par la recherche approfondie des phénomènes entourant la symbiose avec des plantes mutantes de légumineuses. Ces études ont permis, de caractériser une partie importante de la signalisation entre les partenaires symbiotiques de l'association *Rhizobium*-légumineuses (Blaylock *et al.* 1997; Sagan *et al.* 1995). Nous observons chez plusieurs plantes mutantes, une inhibition de formation des nodosités. Cette inhibition serait provoquée par des blocages métaboliques qui empêchent la synthèse de facteurs Nod chez *Rhizobium* spp. (Dénarié *et al.* 1996; Long 1996). Xie *et al.* (1995) ont réussi à stimuler la colonisation mycorhizienne chez *Medicago sativa* par application de facteurs Nod. Ces auteurs imputent aux flavonoïdes le rôle de médiation vis-à-vis des facteurs Nod responsables potentiels du contrôle de la mycorhization chez les symbioses MA. Par extrapolation, on peut penser que des facteurs Myc, semblables aux facteurs Nod, seraient aussi relâchés par un partenaire fongique lors de l'établissement de la symbiose MA. Même si la nature de ces facteurs Myc n'est pas encore connue, leur existence est pressentie suite à l'observation de la variation de la concentration cytoplasmique du calcium induit dans les cellules hôtes (Parniske 2008). Ces pics calciques suggèrent l'existence d'un maintien du sentier des signaux pour les deux types molécules de signalisation, Nod et Myc (Navazio *et al.* 2007)

L'homologie des signalisations chez *Rhizobia* spp. et chez les champignons MA est la prémisse à l'élaboration de la trame de fond de la présente thèse. De plus nous ne pouvons pas ignorer l'avancement rapide des travaux de recherche et les connaissances sur la régulation de la symbiose *Rhizobium*-légumineuses. La comparaison des voies de la régulation commune entre les deux symbioses végétales est prometteuse et c'est dans cet esprit que nous avons abordé l'étude de la signalisation chez la symbiose mycorhizienne arbusculaire.

Nous avons choisi de présenter la portion centrale de cette thèse sous forme d'articles scientifiques. Les deux premiers chapitres traitent de la régulation croisée interspécifique lorsque les racines de *Medicago sativa* sont colonisées par *Sinorhizobium meliloti* et le *Glomus mosseae*. Nous avons utilisé un système à deux compartiments « Split-roots » pour séparer le système racinaire de *M. sativa* en deux de façon à étudier la signalisation à distance de la plante hôte en fonction du type de colonisation ou de l'addition de substances régulatrices. Les résultats du chapitre 1 démontrent clairement que la formation et le maintien des nodosités et des mycorhizes MA sont soumis à des mécanismes communs de régulation et qu'ils ne sont vraisemblablement pas sous l'influence des hydrates de carbone. Au chapitre 2, nous avons suivi la composition des flavonoïdes en relation avec les processus d'autorégulation et de régulation négative croisées induits par l'ajout de facteurs Nod ou en fonction de la rivalité des symbiotes bactériens ou fongiques à coloniser les racines de *M. sativa*.

Le troisième chapitre traite de la récurrence des flavonoïdes en fonction de l'inoculation des racines de *M. sativa* par trois souches de la même espèce de *G. mosseae*. Il s'agit de vérifier le comportement des flavonoïdes par rapport aux forces de puits de carbone de chacune des souches de *G. mosseae* inoculées individuellement sur les racines de la légumineuse.

Nous tenterons donc de montrer au cours de ce travail le rôle de certains flavonoïdes comme molécules « signal » chez le champignon *G. mosseae*. Nous avons accordé une grande importance à l'uniformité du comportement de ces signaux probables au niveau intraspécifique et ce indépendamment des

divergences métaboliques entre souches. Nous verrons que les flavonoïdes s'imbriquent dans un modèle signalétique pour la symbiose MA et seraient associés aux facteurs Myc.

Chapter 1: Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors

Auteurs: Jean Guy Catford, Christian Staehelin, Sylvain Lerat, Yves Piché and Horst Vierheilig

Avant-Propos

Le contenu de ce chapitre a fait l'objet d'une publication dans le journal scientifique : Journal of Experimental Biology, Vol. 54, No 386, pp 1481-1487, May 2003. La répartition des contributions des co-auteurs est la suivante : Christian Staehelin a fourni les facteurs Nod. Sylvain Lerat a participé aux expériences de marquage radioactif. Yves Piché et Horst Vierheilig ont dirigé le travail.

Ayant pour titre:

Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors

Résumé

Les plantes légumineuses établissent des symbioses avec les champignons mycorhiziens arbusculaires (MA) et avec les protéo-bactéries rhizobiales. Ces bactéries forment des nodosités sur les racines et lorsque la symbiose est bien établie, il peut y avoir suppression systémique de formation de nouveaux nodules sur les nouvelles racines. Ce phénomène se nomme l'autorégulation. Les champignons MA affichent un comportement similaire en réprimant l'établissement de nouvelles structures fongiques sur d'autres parties du système racinaire lorsque la symbiose est bien établie. Dans ce chapitre, nous utiliserons le système de bicompartiments « Split-Roots » avec des plants de *Medicago sativa* (Patel) pour

comparer les autorégulations des symbioses avec *Sinorhizobium meliloti* et avec le champignon le *Glomus mosseae*. Nous démontrerons que la nodulation présente sur une partie du système influence par signalisation systémique la colonisation d'une autre partie du système racinaire et que cette autorégulation est réciproque. Les nodules présents sur une moitié du système racinaire dans un bicompartiment vont supprimer la colonisation subséquente par le champignon MA dans le second compartiment. De manière similaire, un système racinaire ayant une symbiose MA bien établie dans un premier compartiment causera la répression subséquente de formation de nodules dans un second compartiment. Cette régulation croisée à distance sera aussi causée par l'application de facteurs abiotiques Nod (lipo-chito-oligosaccharides) sur une partie du système racinaire. Les facteurs Nod-IV(C16:2S) purifiés à partir de *Sinorhizobium meliloti* n'ont pas influencé l'allocation du carbone (^{14}C) au niveau des deux parties du système racinaire en bicompartiments. Ces résultats permettent d'exclure la possibilité d'une compétition pour le carbone comme mécanisme de régulation. Nos résultats indiquent la présence de mécanismes de régulation systémique semblables pour les symbioses rhizobienne et mycorhizienne arbusculaire.

Abstract

Roots of legumes establish symbiosis with arbuscular mycorrhizal fungi (AMF) and nodule-inducing rhizobia. The existing nodules systemically suppress subsequent nodule formation in other parts of the root, a phenomenon termed autoregulation. Similarly, mycorrhizal roots reduce further AMF colonization on other parts of the root system. In this work, split-root systems of alfalfa (*Medicago sativa*) were used to study the autoregulation of symbiosis with *Sinorhizobium meliloti* and the mycorrhizal fungus le *Glomus mosseae*. It is shown that nodulation systemically influences AMF root colonization and vice-versa. Nodules on one half of the split-root system suppressed subsequent AMF colonization on the other half. Conversely, root systems pre-colonized on one side by AMF exhibited reduced nodule formation on the other side. An inhibition effect was also observed with Nod

factors (lipo-chito-oligosaccharides). NodSm-IV (C16:2, S) purified from *S. meliloti* systemically suppressed both nodule formation and AMF colonization. The application of Nod factors, however, did not influence the allocation of ^{14}C within the split-root system, excluding competition for carbohydrates as the regulatory mechanism. These results indicate a systemic regulatory mechanism in the rhizobial and the arbuscular mycorrhizal association, which is similar in both symbioses.

1.1 Introduction

Legumes are hosts for two different types of root symbionts, nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi (AMF). The establishment of symbiosis is the result of a complex series of interactions between the symbiont and the host plant (reviewed by Hirsch and Kapulnik, 1998; Albrecht *et al.*, 1999). For the infection process, the exchange of symbiotic signal molecules is required. Rhizobial bacteria generally enter roots of legumes via root-hairs and induce the formation of root nodules. One important group of rhizobial signals is Nod factors (lipo-chito-oligosaccharides) (Perret *et al.*, 2000). AMF hyphae colonize the root cortex and form highly branched, bush-like structures within a host cell, the so-called arbuscules. Via its extraradical mycelium, the AMF provide the plant with nutrients, mainly phosphorus (Smith and Read, 1997). There is growing evidence that processes leading to nodule initiation and mycorrhiza are similarly regulated (reviewed by Hirsch and Kapulnik, 1998; Albrecht *et al.*, 1999; Guinel and Geil, 2002; Stracke *et al.*, 2002; Staehelin *et al.*, 2001; Vierheilig and Piché, 2002). From the plant's perspective, the development of a symbiotic association is a beneficial, but also a costly process. To control the number of nodules, legumes have developed negative regulatory systems to maintain homeostasis of nodulation. The phytohormone ethylene, for example, inhibits rhizobial infection (Penmetsa and Cook, 1997). Similarly, the existing nodules systemically inhibit subsequent nodulation in other parts of the root system. This feedback control is termed autoregulation. In split-root systems, rhizobial inoculation of one half of the root

system partially blocks subsequent nodulation of the other half. Grafting experiments revealed that some forms of autoregulation are controlled by the shoot (for details see review by Caetano-Anollés and Gresshoff, 1991a). Recently, it has been shown that nodulation is suppressed when one side of a split-root system was pretreated with Nod factors, indicating that the observed autoregulatory effect is triggered by a pre-nodulation event (van Brussel *et al.*, 2002) and a first gene involved in this autoregulation has been identified. Har1 and Nts mutants of *Lotus japonicus* and soybean, respectively, lost their ability to regulate the nodule number and thus display a supernodulating phenotype. The mutated genes encode putative receptor kinases with similarities to CLAVATA1 of Arabidopsis, which negatively regulates tissue differentiation. It has been hypothesized that the receptor kinase interacts with a small peptide involved in systemic shoot controlled regulation of nodule development (Krusell *et al.*, 2002; Nishimura *et al.*, 2002; Searle *et al.*, 2003). An autoregulatory mechanism has been also described for the AMF symbiosis. In barley plants, prior mycorrhizal colonization of one side of a split-root system resulted in a suppression of further root colonization on the other side (Vierheilig *et al.*, 2000a, b). Thus, systemic autoregulatory mechanisms seem to control both nodule formation and AMF colonization. In this study, it was demonstrated that autoregulation of one symbiosis systemically affects the outcome of the other symbiosis.

1.2 Materials and methods

1.2.1 Biological material, growing conditions and experimental design

Alfalfa (Medicago sativa L. cv. Sitel) plants were infected with *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe (BEG 12; European Bank for the Glomales). For nodulation experiments, *Sinorhizobium meliloti* strain 1021 was used (http://sequence. toulouse.inra.fr/meliloti.html). 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; relative humidity 50%; light: 300 µmol m^{-2} s^{-1} PAR (photosynthetically active radiation)). All experiments were repeated twice with five replicates per treatment.

1.2.2 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 pot in a steam-sterilized (40 min, 120 °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 11-d-old plantlets were cut off. Plantlets were then placed into the same substrate as described above. Four weeks later, split-root systems were established as described previously (Vierheilig *et al.*, 2000a). Briefly, the split-root system consists of two units, each containing one half of the alfalfa root system. The two compartments are separated by an impermeable PVC membrane in order to prevent any flow of molecules or root growth from one side to the other. Thus one side of the split-root system can be inoculated with one of the symbionts or treated with Nod factors without any contact with the other side.

1.2.3 Inoculation with AM fungi.

The outer side of each split-root compartment is equipped with a nylon screen (60 µm mesh), which can be penetrated by hyphae but not by roots. To inoculate alfafa with *G. mosseae*, this outer side was joined to a similarly designed compartment. This donor compartment contained beans (*Phaseolus vulgaris* L. cv. Sun Gold) colonized by *G. mosseae*. To avoid contamination of the beans in the donor compartments by rhizobia, plants were fertilized three times a week with 5 ml of a KNO_3 (0.808 g l^{-1}), $Ca(NO_3)_2.4H_2O$ (1.808 g l^{-1}) solution until 3 weeks before the experiment. No nodulation of beans could be observed throughout the experiment.

After 19 d, 53±8% of the alfalfa roots in the neighbouring compartment were colonized by the fungus. No nodules could be detected. The other half of the split-root system remained noninfected.

1.2.4 Inoculation with *Sinorhizobium meliloti*

Rhizobium strain 1021 was cultivated on TY-streptomycin (50 mg ml^{-1}) media at 25 °C on a rotary shaker (200 rpm) to reach an OD_{600}=0.2. Cells were then harvested by centrifugation (6000 g for 10 min) and resuspended in sterile H_2O. For inoculation, 5 ml of this bacterial suspension was added to a selected compartment of the split-root system. After 15 d, alfafa roots formed 48±9 nodules (per 100 mg root FW), whereas the other compartment of the split-root system remained without nodules.

1.2.5 Application of Nod factors to roots

The tetrameric Nod factor NodSm-IV(C16:2, S) was purified from *S. meliloti* strain 1021(pEK327) (Schultze *et al.*, 1992). Supernatants of bacterial cultures were extracted with n-butanol and fractionated by reverse-phase HPLC (Waters C18 column), using 35% acetonitrile/water and 40 mM ammonium acetate as the mobile phase. The fraction containing NodSm-IV(C16:2, S) was desalted as described by Staehelin *et al.* (2000). Four ml of a 10^{-8} M solution (dissolved in water) were daily applied to one side of the split-root system.

1.2.6 Treatment with $^{14}CO_2$

For the $^{14}CO_2$ labelling experiment, plant shoots were placed inside a 945 ml (18x20 cm) transparent freezer bag (Ziplock) together with a 29.5 ml cup containing a basic solution of 37 kBq (1 mCi) $NaH^{14}CO_3$ (Amersham Pharmacia

Biotech). The plastic bag was then closed and a sealing compound placed around the shoot stem. Gaseous $^{14}CO_2$ was produced by injecting 1 ml lactic acid (85%) into the cup.

1.2.7 Determination of 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 microscope to determine the percentage of root colonization according to a modified method of Newman (1966).

1.2.8 AMF colonization followed by subsequent AMF colonization and nodulation.

After the transfer of plants into a split-root system, one compartment was immediately inoculated with the AMF. After 19 d, when the AMF was well established (Vierheilig *et al.*, 2000a, b), the second compartment was inoculated either with AMF or with *S. meliloti*. Plants colonized by AMF were harvested 11 d. later. Plants inoculated with *S. meliloti* were harvested 14 days post-inoculation (dpi).

1.2.9 Prior nodulation followed by subsequent nodulation and AMF Colonization

Four days after establishment of the split-root system, the first compartment was inoculated with *S. meliloti*. After 15 d, the second compartment was inoculated either with *S. meliloti* or with AMF. The plants inoculated with AMF were harvested

11 d later. Plants infected with *S. meliloti* were harvested 14 d after inoculation of the second compartment.

1.2.10 Effect of Nod factors on nodulation and AMF colonization

Eleven days after establishment of the split-root system, Nod factors were applied to one half of the split-root system. This treatment was repeated daily until the time of harvest. Eight days after the first treatment, the second compartment was inoculated either with AMF or with *S. meliloti.* The mycorrhizal roots were harvested 11 dpi. and the nodule number was determined 14 dpi.

1.2.11 ^{14}C allocation in a split-root system after treatment with Nod factors.

Eleven days after establishing the split-root system, Nod factors were applied to one half of the root system. Eight days later, plant shoots were exposed to $^{14}CO_2$ (see above) and incubated for 2 h in the growth chamber. After this pulse period, the bags were removed under a venting fume-hood and plants were returned to the growth chamber. After a 24 h chase period, the two halves of the split-root system were harvested and their fresh weights recorded. The root systems were then oven-dried (24 h at 65 °C), weighed, and used to determine the level of incorporated radioactivity. Roots were ground in liquid nitrogen and digested according to the technique described by Clifford *et al.* (1973) with the tissue solubilizer NCS. Radioactivity was assessed by liquid scintillation spectrometry. Counts were standardized with a quench curve and expressed in dpm. Data were indicated as percentage of ^{14}C in the total root.

1.3 Results

1.3.1 Effects of either AMF or Sinorhizobium on subsequent AMF colonization

Split-root systems are a useful tool to study autoregulation, i.e. systemic suppression of subsequent root colonization by an already colonized part of the root. In this work, alfalfa plants were used, which have been reported to exhibit strong autoregulation of nodule formation (Caetano-Anollés and Bauer, 1988; Caetano-Anollés and Gresshoff, 1991b). After cutting off the tip of the main root, plants developed lateral roots within a relatively short time and formed a homogeneous root system, which was separated into two equal parts. It was investigated in a first experiment whether pre-inoculation of one side of the root system influenced subsequent AMF colonization on the other part. The first half of the split-root system was either inoculated with the mycorrhizal fungus *G. mosseae* or with nodule inducing *S. meliloti*. Control plants were not noculated on this side of the split-root system. After 19 d, all plants were inoculated with *G. mosseae* on the second half of the split-root system. Eleven days later, AMF colonization was investigated on harvested roots. AMF colonization in the pre-inoculated side was 57 ±6% and plants infected with *S. meliloti* formed 107±11 nodules (24±8 nodules per 100 mg root FW) on this half of the root system. As seen in Fig. 2, the degree of AMF colonization in the second side of the split-root system depended on the treatment of the first side. Compared to control plants without pre-inoculation, subsequent AMF colonization was reduced in plants already colonized by *G. mosseae*. Interestingly, a similar reduction of AMF colonization was observed when the first half of the split-root system was infected with *S. meliloti*. These data show that already formed nodules on one part of the root system inhibit subsequent AMF colonization of other parts of the root system.

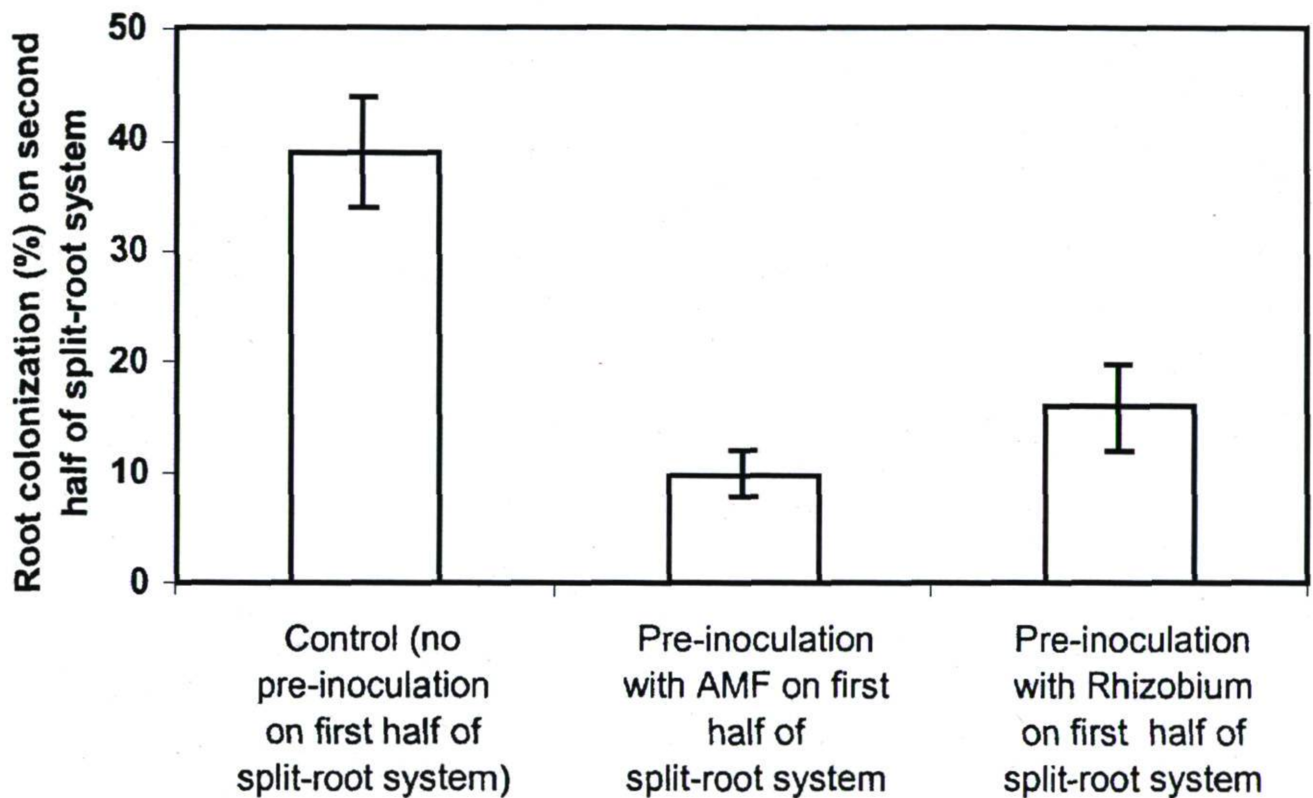

Figure 2: Effect of pre-inoculation on subsequent AMF colonization in alfalfa roots. Data (means ±SE) indicate the degree of mycorrhizal colonization (expressed as % of total root length) in the second compartment of the split-root system. The first compartment was preinoculated either with *G. mosseae* (root colonization at the time of harvest 57±6%) or with S. meliloti (107±11 nodules). Control plants were not pre-inoculated.

1.3.2 Effects of either AMF or *Sinorhizobium* on subsequent nodule formation.

In a reciprocal experiment, it was tested whether the establishment of symbiosis on one compartment of the split-root system inhibits subsequent nodule formation in the other compartment (Fig. 3). Control plants were not pre-inoculated and formed 263±33 nodules on the inoculated part of the split-root system. Nodules in the first compartment strongly suppressed subsequent total nodulation (Fig. 3A) and number of nodules per 100 mg root FW (Fig. 3B), indicating autoregulation of nodule formation. A suppression of nodule development was also observed when plants were inoculated with AMF on one side of the root system and subsequently infected with *S. meliloti* on the other side. When looking at the total nodule number these plants formed approximately 4-fold fewer nodules compared with control plants which were not preinoculated on the first half of the root system (Fig. 3A). Similar data were obtained when root FW was used as the basis. The nodule number per 100 mg root FW was suppressed by an already established symbiotic interaction (Fig. 3B). These data indicate that AMF colonization of one side of the root system inhibited nodulation on the other side.

1.3.3 Systemic effects of Nod factors on nodule formation and AMF colonization

S. meliloti produces a number of Nod factors, which trigger early host plant responses that enable bacterial entry into root-hairs. To test the effect of purified Nod factors on the subsequent establishment of symbiosis, the first half of the split-root system was treated with 10±8 M NodSm-IV(C16 :2, S), a tetrameric sulphated Nod factor from *S. meliloti*. The application was repeated daily until plants were harvested.

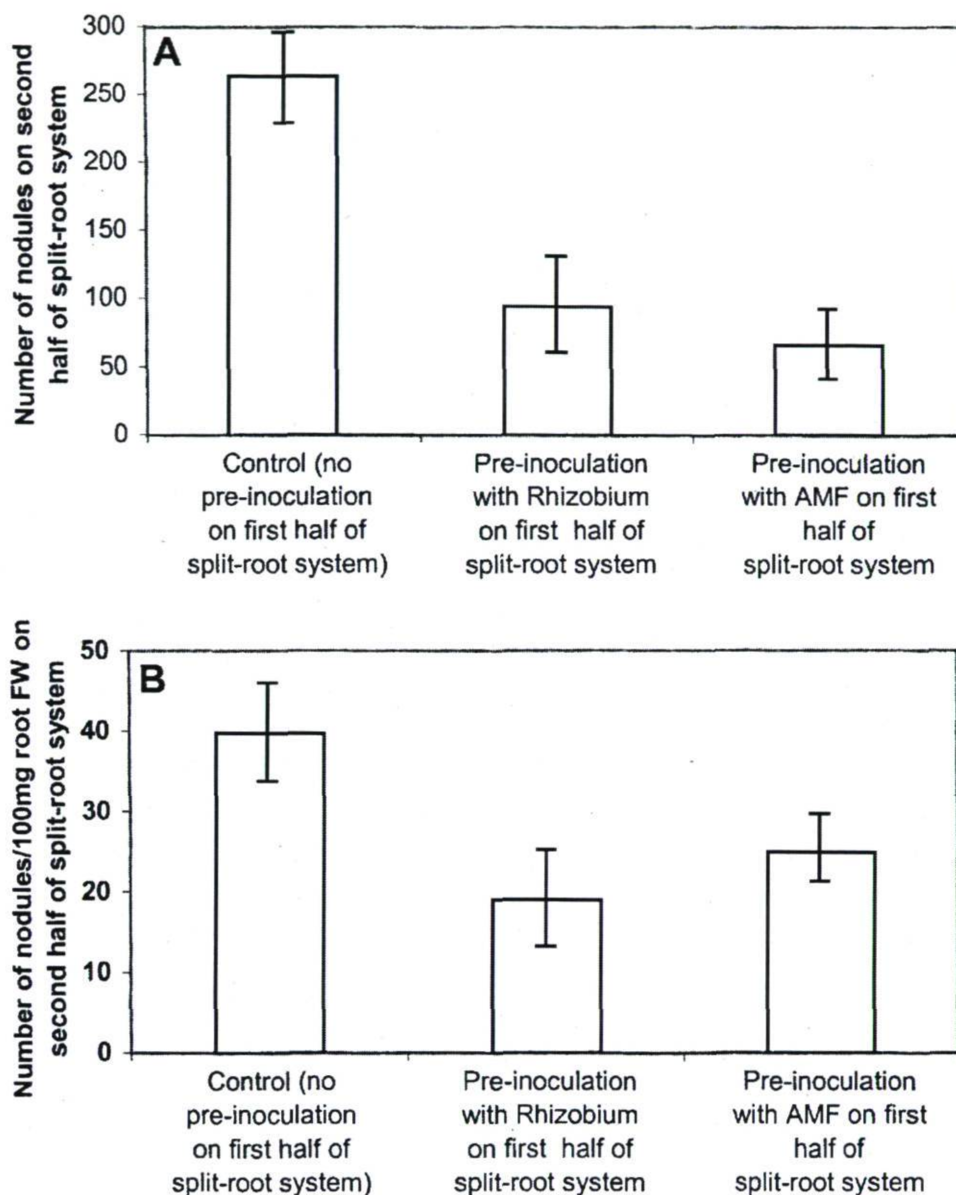

Figure 3: Effect of pre-inoculation on subsequent nodule formation. Data (means ±SE) indicate the number of nodules (A) and the number of nodules per 100 mg root FW (B) in the second compartment of the split-root system. The first half of the split-root system was pre-inoculated either with *G. mosseae* (root colonization at the time of harvest 63±3%) or with *S. meliloti* (228±46 nodules). Control plants were not pre-inoculated.

As seen in Fig. 4, the application of NodSm-IV(C16 :2, S) on one side of the split-root system inhibited nodule development on the other side, indicating an autoregulatory feedback response. Moreover, treatment of roots with NodSm-IV(C16 :2, S) also inhibited AMF colonization. Mycorrhizal colonization on the second part of the split-root system was approximately 2-fold lower compared with control plants which were not treated with Nod factors.

1.3.4 Carbon partitioning in split-root systems after application of Nod factors

To test the effect of Nod factors on carbon partitioning, NodSm-IV(C16 :2, S) was applied to one half of the split-root system and the leaves were treated with $^{14}CO_2$. Incorporation of ^{14}C was separately measured for the two compartments of the split-root system. Application of NodSm-IV(C16 :2, S) to one half of the split-root system did not affect carbon allocation. The carbon sink strength of the side treated with Nod factors (^{14}C=51 ± 6%) was similar to the non-treated side (^{14}C=49,±14%).

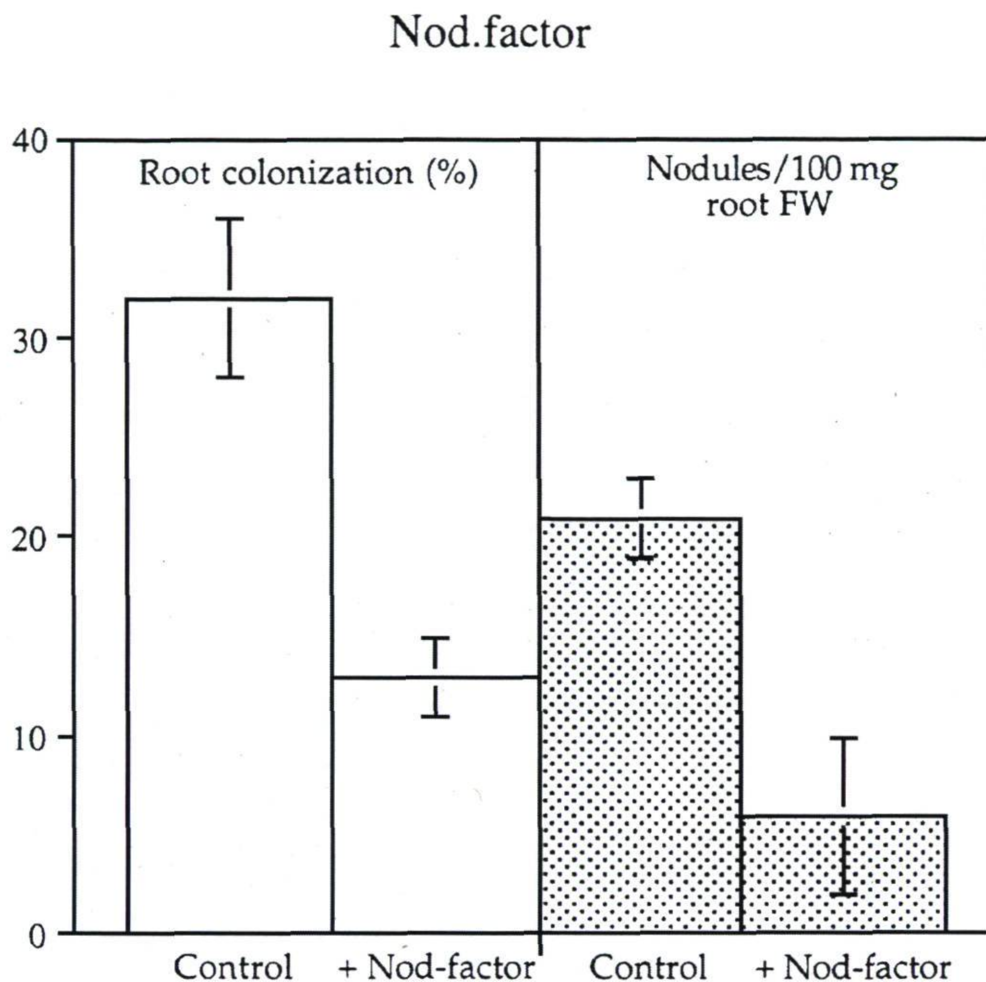

Figure 4: Application of Nod factors suppresses AMF colonization and nodule formation in split-root systems. The first compartment was daily treated with 4 ml of 10±8 M NodSm-IV(C16:2, S). Control plants were treated with 4 ml water. Eight days later, the second compartment was inoculated either with *G. mosseae* or with *S. meliloti.* Data (means ±SE) indicate the degree of AMF root colonization (left panel) and the number of formed nodules per 100 mg root FW (right panel) at the time of harvest.

1.4 Discussion

Establishment of symbiosis on one part of the root thwarts further microbial colonization on other parts of the root system. This systemic feedback-control, termed auto-regulation, has been described for nodule formation of legumes (Caetano-Anollés and Gresshoff, 1991a) and AMF colonization (Vierheilig *et al.*, 2000a, b). It has been suggested that plants evolved autoregulatory mechanisms to limit the costs for establishment of symbiosis (Caetano-Anollés and Gresshoff, 1991a; Vierheilig and Piché 2002). This study shows that precolonization of roots with AMF not only systemically inhibits further mycorrhization, but also inhibits nodule formation. Conversely, already existing nodules systemically suppress both further nodulation and subsequent AMF colonization. These findings indicate that autoregulatory mechanisms induced by the establishment of one type of symbiosis infuence another symbiotic association. This is reminiscent of certain supernodulating mutants of soybean and *Lotus japonicus*, which exhibited accelerated AMF colonization and increased formation of arbuscules compared to wildtype plants (Shrihari *et al.*, 2000; Solaiman *et al.*, 2000). It remains an open question to what extent the mechanisms involved in autoregulation are identical for both symbioses. Induction of a systemic signal, the signal itself, perception of this signal in the other root part, and blocking mechanisms could be specific for a given symbiotic interaction. Our experiments with alfalfa plants suggest a link between the two symbioses regarding the systemic signal itself and its perception on the other side of the split-root system. Recent results point towards a possible involvement of phytohormones in the rhizobial autoregulation. Apart from changes in the levels of other hormones, cytokinin levels are altered in roots of supernodulating mutants (Caba *et al.*, 2000). No data are available yet on the role of phytohormones in the regulation of mycorrhization, however, hormone level changes have been reported in roots of plants colonized by AMF (e.g. cytokinin Allen *et al.*, 1980; Shaul-Keinan *et al.*, 2002). van Brussel *et al.* (2002) reported recently that application of Nod factors from *Rhizobium leguminosarum bv.viciae* to one side of a split-root system inhibited nodule formation of *Vicia* plants on the other side. Their observations indicate that Nod factors elicit an autoregulatory

feedback-response. Our experiments show a similar effect of Nod factors in the interaction between alfalfa and *S. meliloti*. Moreover, it was found that application of Nod factors prevented AMF colonization on the other side of the split-root system. These findings indicate that Nod factors trigger a secondary signal involved in autoregulation of nodulation, which initiates a blockage of AMF colonization. In this context, it is worth mentioning that Nod factors may act as chito-oligosaccharide elicitors on legumes and non-legumes (Staehelin *et al*., 1994; Müller *et al*., 2000), thereby inducing plant defence reactions (Savoure *et al*., 1997; Xie *et al*., 1999). It is tempting to speculate that chito-oligosaccharides released from cell walls of AMF are stimuli triggering autoregulation of mycorrhization. The presence of AMF derived signals acting on the plant even before appressoria formation has been demonstrated recently (Larose *et al*., 2002).

The observed effect of Nod factors on mycorrhization is not contradictory to the results from Xie *et al*. (1995, 1998), who reported that application of Nod factors to legume roots promoted AMF colonization. In their experiments, treatment with Nod factors was performed simultaneously with the mycorrhizal inoculation, moreover, the same roots were treated with Nod factors and inoculated with AMF, thus a local, but not a systemic effect was studied. Hence, Nod factors locally promote symbiosis and desensitize other parts of the roots via an autoregulatory feedback mechanism in both the rhizobial and mycorrhizal interactions. Competition for carbohydrates within the root system is suggested to influence mycorrhizal colonization (Pearson *et al*., 1993). It is possible therefore that C-partitioning in alfalfa roots is involved in the autoregulation of symbiosis. Contrary to other AMF, however, *G. mosseae* strain BEG12 used in our experiment, does not induce any carbon sink strength in split-root systems (Lerat *et al*., 2003b). In this work, it is shown that symbiotically active Nod factors from *S. meliloti* did not affect the allocation of ^{14}C between the two parts of an alfafa split-root system, although other Nod factors locally changed the carbohydrate composition in *Lablab purpureus* roots (Xie *et al*.,1998). Taking these observations together, they suggested that autoregulation of symbiosis cannot be explained by competition for carbohydrates.

Future work is required to test whether autoregulation of symbiosis is related to salicylic acid (SA) accumulation and defence responses resulting in systemic acquired resistance (SAR) to pathogen attack (Martinez-Abarca *et al.*, 1998; Dong, 2001; Ramu *et al.*, 2002).

There are some indications for the involvement of SA in the regulation of nodulation and mycorrhization. In transgenic NahG *Lotus japonicus* plants with reduced SA levels, an increased number of nodules is formed (McAlvin *et al.*, 2001) and in NahG tobacco with reduced levels of SA and in transgenic CSA (constitutive SA biosynthesis) tobacco with enhanced SA levels it could be shown that the degree of root colonization by AMF is linked to the SA levels in the roots. Higher SA levels (in CSA plants) resulted in a reduced root colonization, whereas in roots with lower SA levels (in NahG plants), root colonization was increased (Herrera-Medina *et al.*, 2003).

Several studies indicate that mycorrhizal roots are more resistant to pathogens (reviewed by Azcon-Aguilar and Barea, 1996) and that legumes inoculated with rhizobia are less damaged by soil-borne pathogens (Chakraborty and Purkayastha, 1984; Chakraborty and Chakraborty, 1989; Tu, 1978). Indeed, it seems plausible that symbiotic roots develop systemic mechanisms to repulse colonization by pathogens, not discriminating between microsymbionts and soil-borne pathogens (Vierheilig and Piché, 2002). In his view, autoregulation would be a general strategy to resist pathogen attack, while reducing the costs for the already established symbiotic associations.

Chapter 2: Systemically suppressed isoflavonoids and their stimulating effects on nodulation and mycorrhization in alfalfa split-root systems

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Avant-propos

Le contenu de ce chapitre a fait l'objet d'une publication dans le journal scientifique : Plant Soil (2006) 285 : 257-266. La répartition des contributions des co-auteurs est la suivante : Christian Staehelin a fourni les facteurs Nod. Geneviève Larose a participé aux expériences de dosages des flavonoïdes par HPLC. Yves Piché et Horst Vierheilig ont dirigé le travail.

Ayant pour titre :

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

Résumé

En chambre bicompartimentée « Split-Roots », la séparation du système racinaire en deux parties distinctes permet de tester l'influence de la nodulation et de la mycorhization sur l'établissement d'une seconde symbiose, un phénomène qualifié d'autorégulation. Les racines de *Medicago sativa* soumises aux facteurs de signalisation rhizobiens, i.e. les facteurs Nod, affichent un comportement semblable de suppression systémique de leur propre symbiose. De plus, le rôle des flavonoïdes dans la régulation des deux symbioses a été investigué à la suite de l'inoculation de *Sinorhizobium meliloti* ou du *Glomus mosseae* de même qu'en présence de facteurs Nod. Les analyses par chromatographie en phase liquide à haute performance (HPLC) des deux parties du système racinaire montrent des patrons caractéristiques de flavonoïdes en réponse aux différents traitements. De

plus, deux flavonoïdes, la formononétine et l'ononine présentent une variation, une fluctuation semblable après l'inoculation microbienne ou fongique ou suite à l'application de facteurs Nod. L'application exogène de formononétine et d'ononine a restauré partiellement la suppression systémique de la nodulation et de la mycorhization. Ces données nous indiquent l'implication de ces deux métabolites secondaires au niveau de l'autorégulation de ces deux symbioses.

Abstract

In split-root systems of *Medicago sativa,* 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 similarily 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.

2.1. 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 Nod factors (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 1991b). 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 preinoculation 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 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.* 2005c, 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.

2.2. Materials and methods

2.2.1. 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 µmol/m^{-2} s^{-1}; relative humidity 50%]. All experiments were repeated twice with five replicate per treatment.

2.2.2. 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 impermeable 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 inoculated plants.

2.2.3. 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 (6000*g* x 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).

2.2.4. 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% acetonitrile/ 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.

2.2.5. 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 monofilament, 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 equipped with the Nylon screen. This donor compartment contained beans (*Phaseolus vulgaris* L. cv. Sun Gold) colonized by *G. mosseae.* Hyphae of the AMF penetrated the Nylon screen and colonized 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 flavonoid analysis. In all samples, the degree of AMF root colonization was determined at the time of harvest.

2.2.6. 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 formononetin 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.

2.2.7. 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-inoculated with *G. mosseae* as described above. After 20 days, when the AMF symbiosis was well established 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.

2.2.8. 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.

2.2.9. 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).

2.2.10. 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 (0.5-2.0 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 x 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), 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), daidzein (4',7-dihydroxyisoflavone) 17.20 min (Indofine), genistein (4',5,7-trihydroxyisoflavone) 23.11 min (Indofine), coumestrol 23.21 min (Fluka), formononetin (7-hydroxy-4'- methoxyisoflavone) 27.14 min (Indofine), medicarpin 31.44 min (Sequoia Research Products).

2.3. 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'-methoxychalcone 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-inoculated part of the split-root system exhibited levels comparable to those in non-inoculated control plants (Fig. 5). 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 downregulated 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. 6).

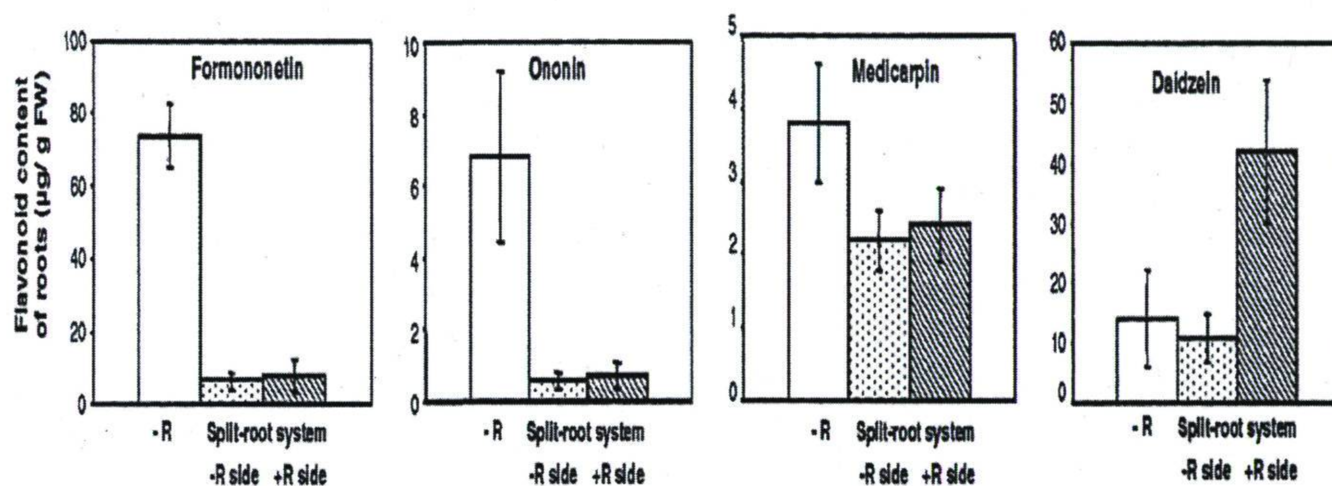


Figure 5: Flavonoid pattern in a split-root system of alfalfa with one side inoculated with *Sinorhizobium meliloti* (+R side) and the other side without inoculation (-R side) and in roots of non-inoculated control plants (-R). Data represent mean ± SE (n = 5).

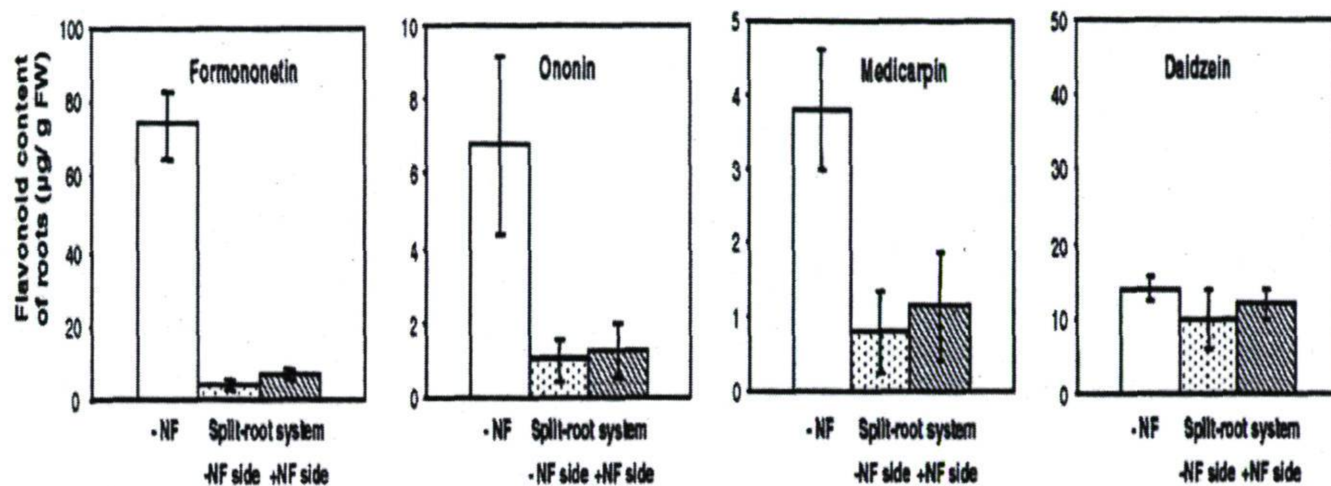


Figure 6: Flavonoid pattern in a split-root system of alfalfa with one side treated with nod factor (+NF side) and the other side without NF treatment (-NF side) and in roots of non-treated control plants (-NF). Data represent mean ± SE (n = 5).

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 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. 7).

Taken together these data 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 formononetin 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. 8, only few nodules were counted on the second half of the split-root system, whereas plants, which had been not pre-inoculated, formed about 10-fold more nodules. When formononetin was applied to the second side of the split-root system of preinoculated 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 formononetin, its glycoside ononin also promoted nodulation on the autoregulated side of split-roots, albeit to a lower extent than the aglycone (Fig. 8).

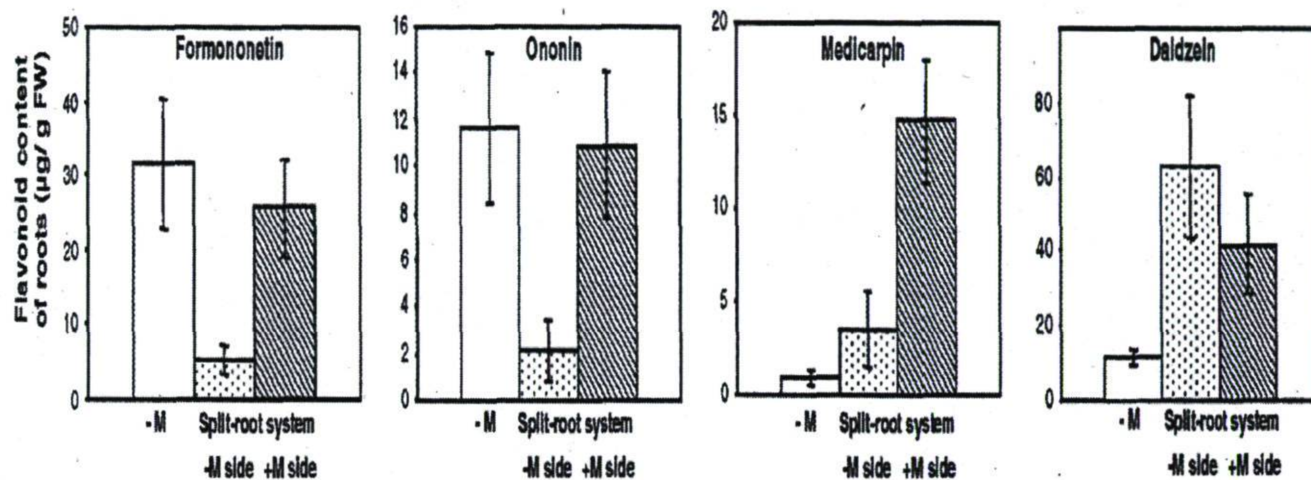


Figure 7: Flavonoid pattern in a split-root system of alfalfa with one side inoculated (+M side) with *Glomus mosseae* and the other side without inoculation (-M side) and in roots of non-inoculated control plants (-M). Data represent mean ± SE (n = 5).

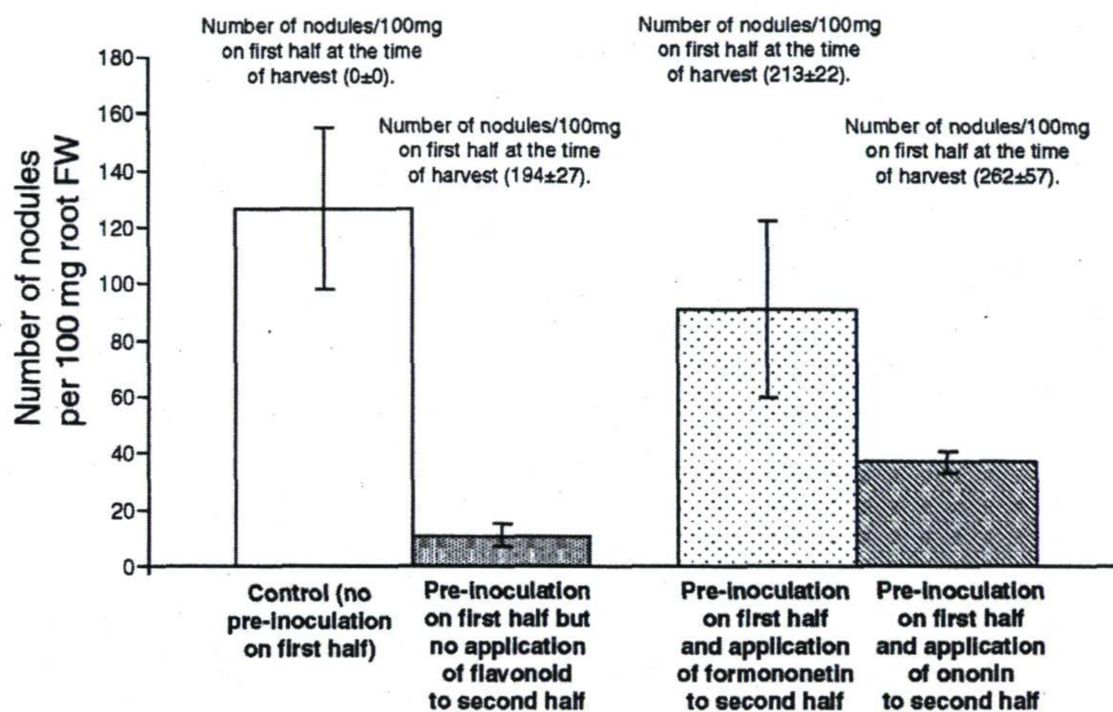


Figure 8: Effect of rhizobial pre-inoculation of one half of the split-root system of alfalfa on subsequent nodule formation on the second half of the split-root system with or without application of formononetin or ononin (10 ml of a 5 µM solution every second day) to the second half. Data represent 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. 9). 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. 9).

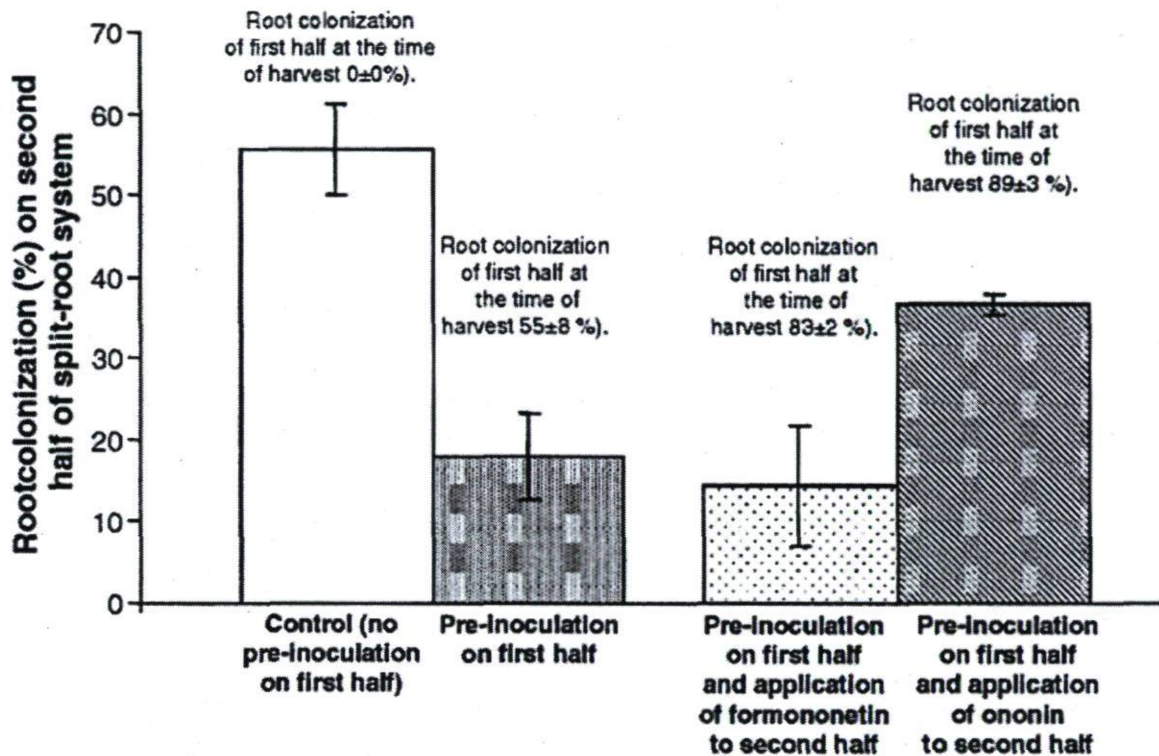


Figure 9: Effect of AM preinoculation of one half of the split-root system of alfalfa on subséquent AMF root colonization of the second half of the split-root system with orwithout application of formononetin or ononin (10 ml of a 5 µM solution every second day) to the second half. Data represent mean ± SE (n = 5).

2.4. 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 phytoalexine 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 altered isoflavonoids of the medicarpin synthesis pathway on the other side (Fig. 5). 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. 6). 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. 7). Thus, all known treatments eliciting autoregulation signalling – 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. 8) 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.* 2005a; Siqueira *et al.* 1991; Vierheilig *et al.* 1998a; Xie *et al.* 1995). Ononin promoted AMF root colonization in our experiments (Fig. 9), 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.

Chapter 3: The accumulation pattern of flavonoids in alfalfa roots is not strain specific when colonized by different strains of *Glomus mosseae*

Avant-propos

Ce chapitre est sous forme d'article en voie de parachèvement pour publication. Il sera soumis sous peu à un journal scientifique. La répartition des contributions des co-auteurs sera la suivante : Pierre Morin a assuré le support pour mon initiation au HPLC. Robert Chênevert m'a accueilli dans son laboratoire et il m'a permis d'avoir accès à des équipements essentiels pour ma recherche. Yves Piché et Horst Vierheilig ont dirigé le travail.

Résumé

Selon le genre et l'espèce de champignons mycorhiziens arbusculaires (MA), les flavonoïdes peuvent être favorables ou pas à la germination des spores MA, à la croissance de hyphes et aussi au degré de colonisation ultérieure des racines hôtes. Cet énoncé peut être aussi validé par les patrons d'accumulation de plusieurs flavonoïdes chez les racines colonisées par différentes souches de genre et d'espèce de champignons MA. Nous avons donc cherché à montrer qu'il y avait bien la présence d'un patron d'accumulation de flavonoïdes chez les racines de luzerne colonisées par trois différentes souches de *Glomus mosseae*. Pour toutes les souches confondues de *G. mosseae* les analyses chromatographiques ont bien révélées un patron similaire des flavonoïdes. Ces observations nous indiquent une absence de comportement spécifique des flavonoïdes pour les souches de *G. mosseae*.

Abstract

Depending on the arbuscular mycorrhizal (AM) fungal genus and species flavonoids have been shown to exhibit different effects on AM spore germination, hyphal growth and root colonization. This is also reflected in varying flavonoid accumulation patterns in roots colonized by AM fungi from distinct genera and species. We tested the flavonoid accumulation pattern in alfalfa roots colonized by three different strains of *Glomus mosseae*. The accumulation pattern of all flavonoids analyzed was similar with all *G. mosseae* strains, indicating the absence of a flavonoid specificity at the strain level within the species *G. mosseae*.

3.1 Introduction

The arbuscular mycorrhizal (AM) association between plants and Glomeromycota fungi (Schüssler *et al.* 2001) is playing a pivotal role in the evolution and functioning of vascular plants and terrestrial ecosystems. The AM symbiosis has arisen roughly 450 million years ago (Redecker *et al.* 2000) and this long co-evolution gave ample time to establish this root symbiosis in over 80% of all land plants (Remy *et al.* 1994; Heckman *et al.* 2001).

The establishment of the AM association involves a complex exchange of signals between the host plant and the AM fungus. Due to their effect on AM spore germination, hyphal growth and root colonization by AMF flavonoids have been suggested to be involved in AM signalling (reviewed by Morandi 1996; Vierheilig *et al.* 1998a; Steinkellner *et al.* 2007) and recently their implication in the autoregulation of nodulation and mycorrhization has been suggested (Catford *et al.* 2006).

AMF are found in several genera such as *Glomus, Gigaspora* and *Sclerocystis* (Walker and Trappe 1993). Comparing the data from several studies on the effect of flavonoids on AM fungi from different genera and species a certain AM genus- and even species-specific effect was suggested (Vierheilig *et al.* 1998a), however,

as the experimental conditions in all these studies always varied, data not always were comparable. Recently, in comparative studies with AM fungi from different genera and species a wide range of flavonoids was tested on their effect on spore germination and hyphal growth and AM root colonization. The data clearly confirmed an AM genus- and even species-specific effect of flavonoids (Scervino *et al.* 2005a, 2005b, 2005c; 2006; 2007).

Once an AM fungus has colonized the root, the flavonoid pattern in the root changes, indicating that during this stage of the interaction flavonoids play a signalling role (Harrison and Dixon 1993, Volpin *et al.* 1994, 1995 ; Larose *et al.* 2002; Catford *et al.* 2006; Schliemann *et al.* 2008; Carlsen *et al.* 2008). Varying requirements of AM fungi should lead to specific accumulation patterns of the signalling compounds once a fungus has colonized a plant root. To our knowledge there is only one study comparing the accumulation pattern of flavonoids after root colonization by AM fungi from different genera and species (Larose *et al.* 2002). In this study it could be shown that depending on the AM genus and even species the flavonoid accumulation pattern varied.

The purpose of the present study is to explore the flavonoid profile of alfalfa roots colonized by allopatric strains of *Glomus mosseae* in order to determine a possible AM fungal strain specificity in the accumulation pattern of flavonoid.

3.2 Material and Methods

3.2.1 Culture conditions

Alfalfa seeds (Sitel) were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water and germinated in pots in a steamsterilized (30 min, 121°C) mixture of silicate sand, expanded clay and soil (1:1:1 by vol). One week old seedlings were transplanted into the side compartments of a split-root system which had been joined (on the side equipped with a 30 µm mesh nylon screen) for 2 weeks with the inoculum compartment.

The inoculum compartment contained beans (*Phaseolus* vulgaris L.) colonized by one of the three *G. mosseae* and was equipped on both sides with a nylon screen (30 µm mesh). Thus, the AM hyphae could grow from the inoculum compartment through nylon screens to the two side-compartments, thereby rapidly colonizing the plants in the side-compartments (Wyss *et al.* 1991).

Starting two days before transplanting 2 ml of a N solution (KNO_3 0.808 g/l; $Ca(NO_3)_2.4H_2O$ 1.808 g/l) were added every second day to each plant compartment to prevent potential nodulation by contaminating rhizobial bacteria. Plants were harvested 4 weeks after inoculation with AMF.

Mycorrhizal root colonization was monitored using the technique described by Vierheilig *et al.* (1998b). Plants were inoculated with three different strains of *Glomus mosseae* from different provenances (BEG 12 isolated in England; BEG 54 isolated in Indonesia and BEG 55 isolated in the Philippines; International Bank of Glomeromycota; http://www.kent.ac.uk/bio/beg).

3.2.2 Plant extraction and high-performance liquid chromatography

At harvest roots were rinsed with tap water. Roots and shoots fresh weight (FW) were determined. Thereafter samples were frozen and lyophilized. The freeze-dried samples (0.5–2.0 g) were ground with liquid nitrogen using a mortar and pestle. Flavonoids were extracted in 100% methyl-alcohol overnight at 4°C. The debris were removed by centrifugation at 5,000g for 10 min. Contaminants were removed by partition against hexane two times. The extract was evaporated with a Rovaporator (BUCHI). The residue was dissolved in 0.5 ml of methanol and filtrated through 0,22 μm. 13 mm PVDF filtering unit. HPLC analysis was performed on a reverse-phase analytical column (Kingsorb C18, 150 · 4.6 mm id., 3 lm) 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, 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 corresponding standards. For dosimetry purpose standard curve of each flavonoid analysed was done.

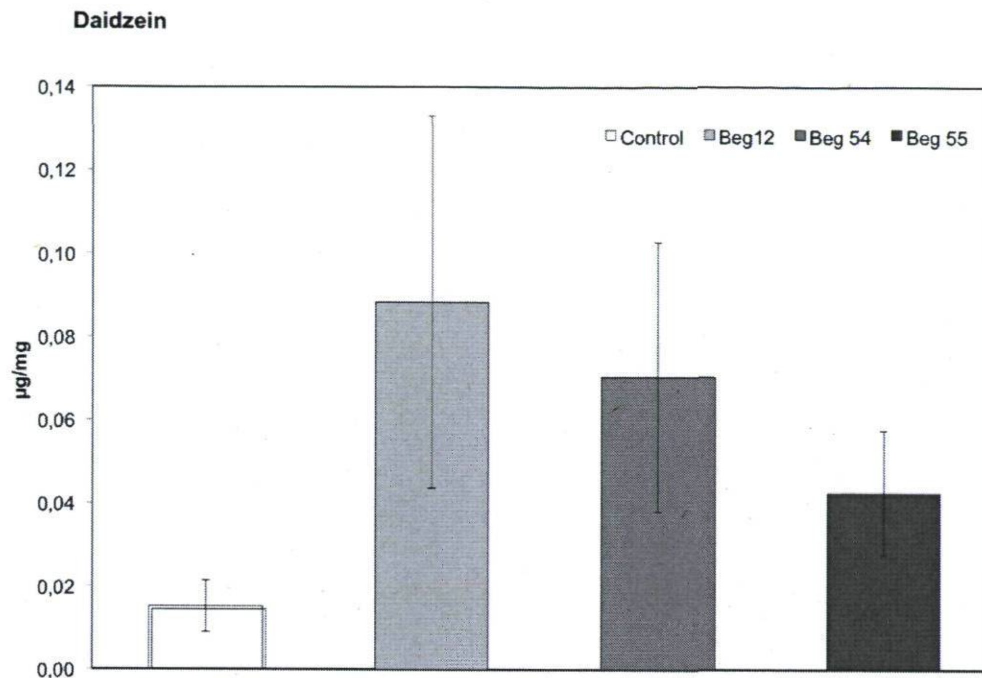
3.3 Results

At harvest time the three *G. mosseae* strains exhibited similarly high degrees of root colonization (%): BEG 12 = 82±8, BEG 54= 65±4, BEG 55= 71±5, indicating mycorrhization was well established and comparable between the three strains.

The following flavonoids were monitored: biochanin A, coumestrol, daidzein, formononetin, genistein, isoliquiritigenin, medicarpin, ononin and quercitin.

With all three *G. mosseae* strains a similarly enhanced accumulation of daidzein, formononetin, and medicarpin could be observed in mycorrhizal roots (Fig. 10).

The accumulation pattern was reversed with biochanin A, coumestrol, genistein, ononin and quercetin. Roots colonized by one of the three AM fungal strains showed similarly low levels of these compounds (Fig. 11), whereas isoliquiritigenin was not affected by mycorrhization (Fig.12).



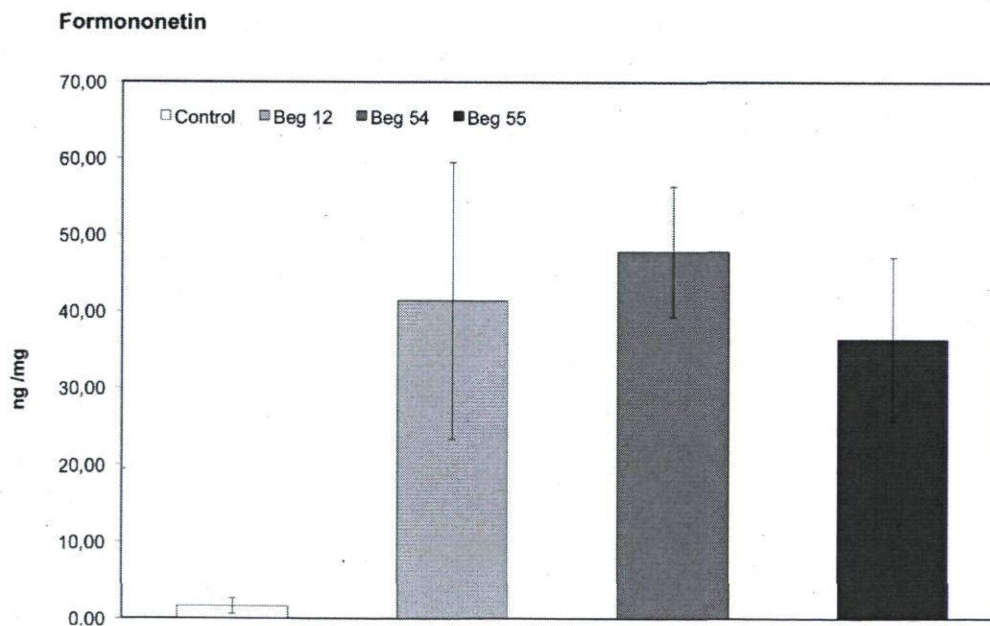


Figure 10: Flavonoid patterns of alfalfa roots for non-inoculated roots vs mycorrhized roots inoculated respectively with BEG12, BEG54, BEG55. Symbiosis stimulated flavonoid production. Data represent mean ± SE (n = 5).

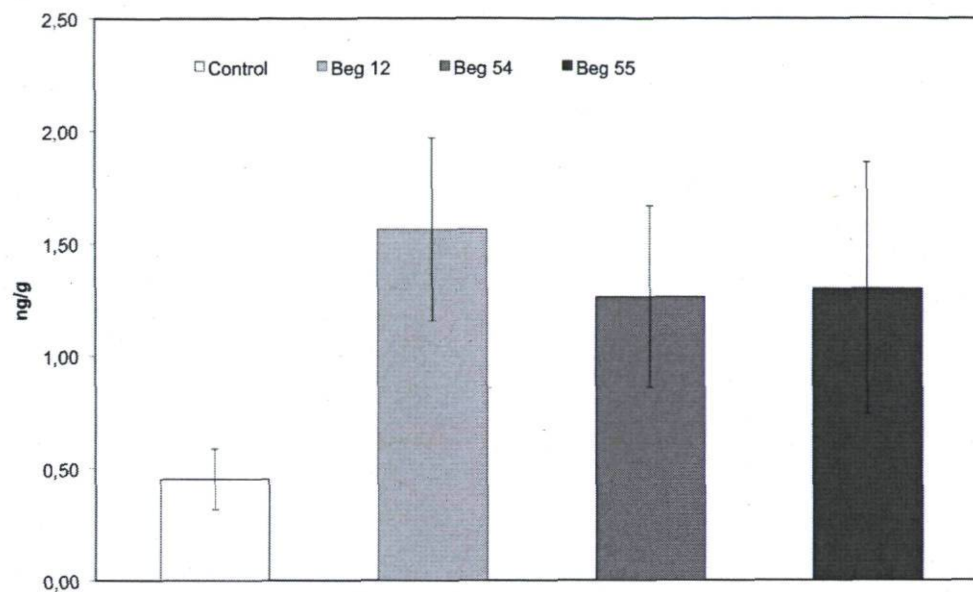


Figure 10 (bis): Flavonoid patterns of alfalfa roots for non-inoculated roots vs mycorrhized roots inoculated respectively with BEG12, BEG54, BEG55. Symbiosis stimulated flavonoid production. Data represent mean ± SE (n = 5)

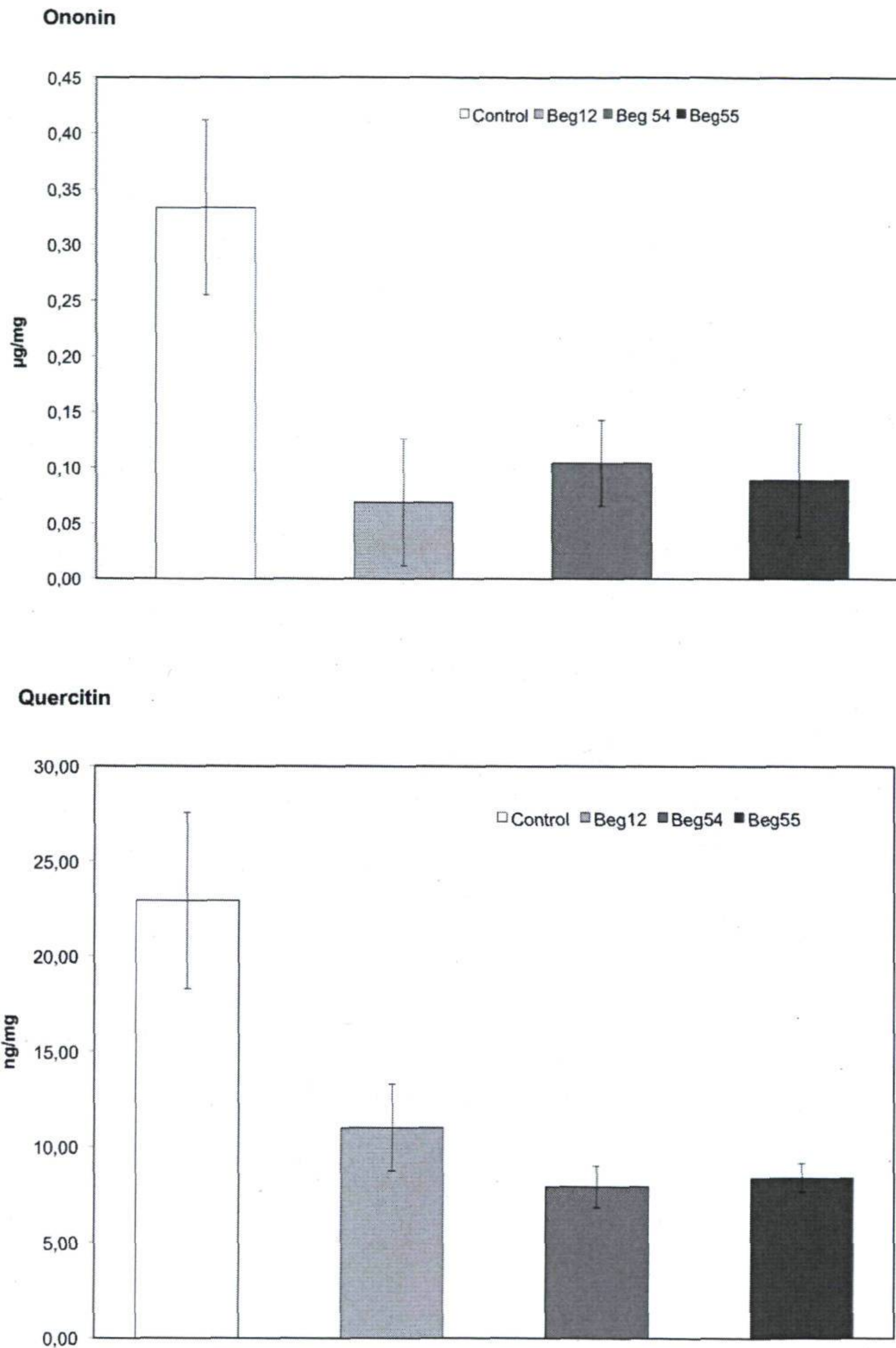
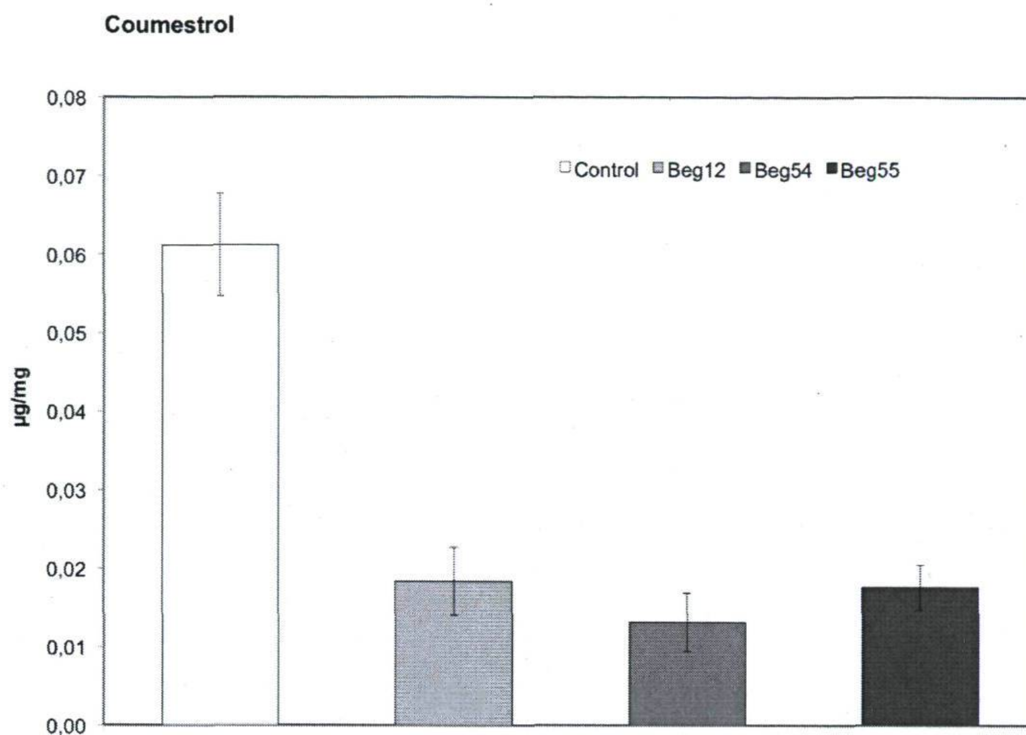


Figure 11: Flavonoid patterns of alfalfa roots for non-inoculated roots vs mycorrhized roots inoculated respectively with BEG12, BEG54, BEG55. Symbiosis repressed flavonoid production. Data represent mean ± SE (n = 5)



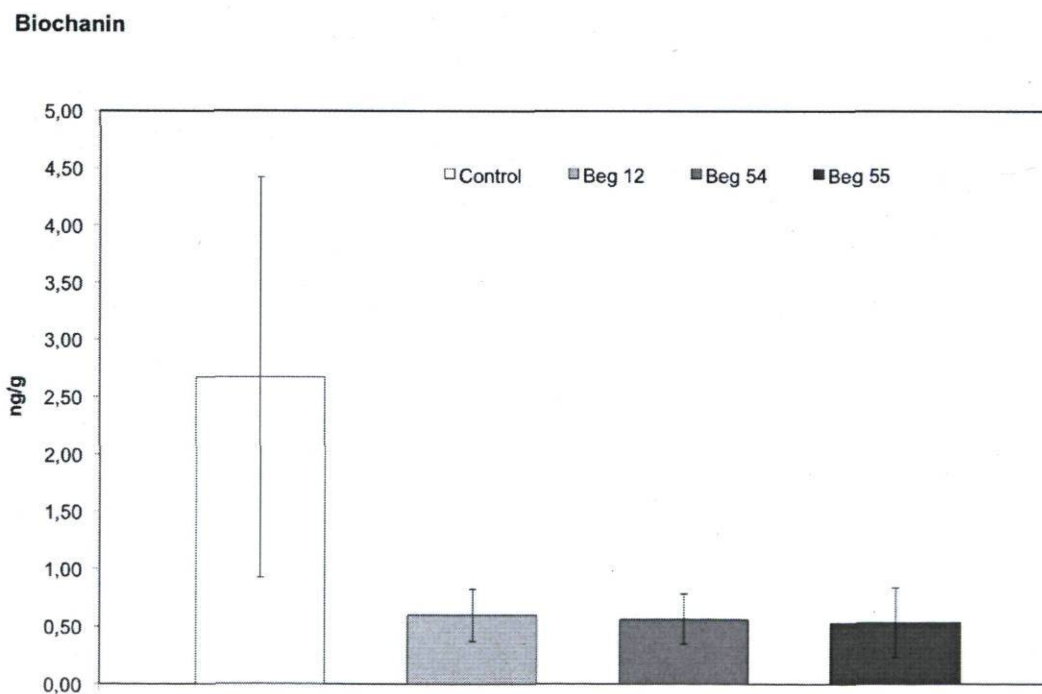


Figure 11 (bis): Flavonoid patterns of alfalfa roots for non-inoculated roots vs mycorrhized roots inoculated respectively with BEG12, BEG54, BEG55. Symbiosis repressed flavonoid production. Data represent mean ± SE (n = 5).

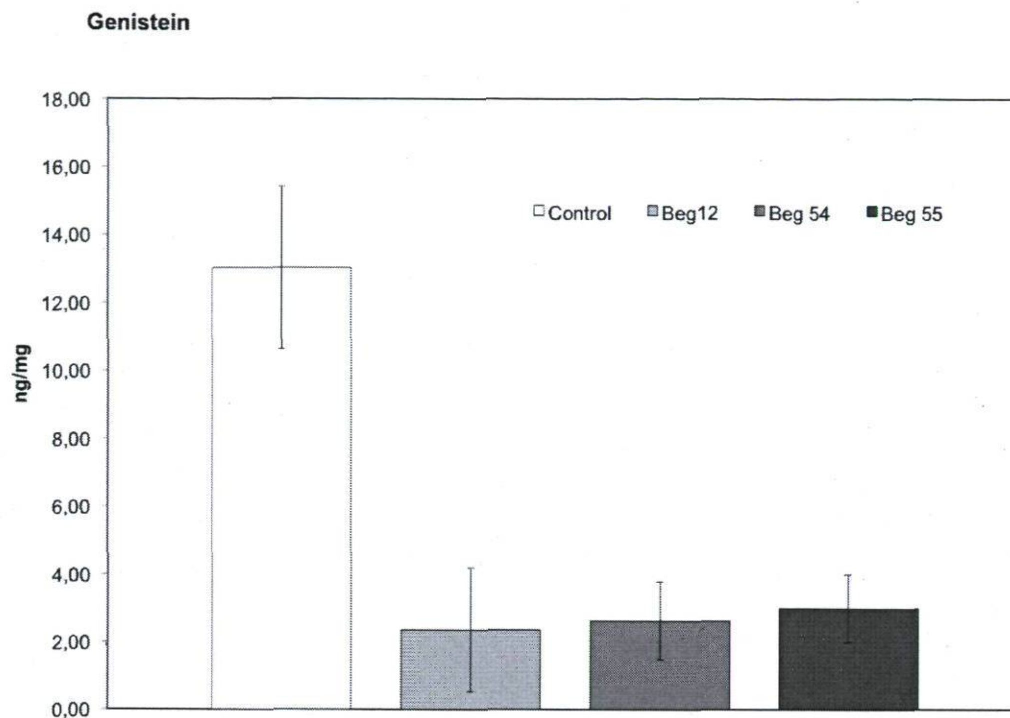


Figure 11 (bis): Flavonoid patterns of alfalfa roots for non-inoculated roots vs mycorrhized roots inoculated respectively with BEG12, BEG54, BEG55. Symbiosis repressed flavonoid production. Data represent mean ± SE (n = 5).

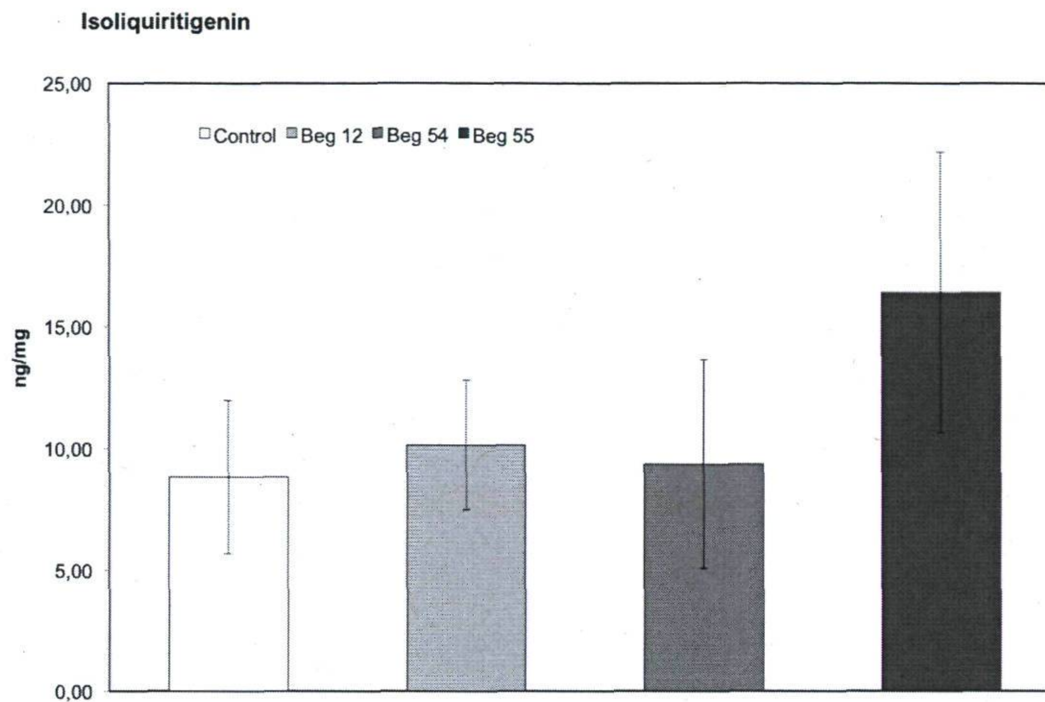


Figure 12: Flavonoid patterns of alfalfa roots for non-inoculated roots vs mycorrhized roots inoculated respectively with BEG12, BEG54, BEG55. Symbiosis had no direct effect on flavonoid production. Data represent mean ± SE (n = 5).

3.4 Discussion

To our knowledge, this is the first study comparing the accumulation pattern of flavonoids in roots colonized by different strains of the same AM species. It has been shown before that AM fungi from different genera and even species result in a different accumulation pattern of flavonoids (Larose *et al.* 2002). This has been attributed to varying flavonoid requirements by the AM fungi and could be explained by the intra-specific genetic variability reported for AM fungi and to different host specificity. (Lanfranco *et al.* 1995; Lloyd-Macgilp *et al.* 1996).

When looking at the same *G. mosseae* strains we used in the present study, Lerat *et al.* (2003a) demonstrated that the three strains exhibited a different carbon sink strength, indicating a some genetic variability.

This seems in contrast with the work of Lloyd-Macgilp *et al.* (1996), who reported that strains of *G. mosseae* are genetically similar. Our data also indicate a certain genetic similarity of the AM species *G. mosseae* as the accumulation pattern of all analysed flavonoids was similar independently of the root colonizing *G. mosseae* strain. Further studies are needed to elucidate whether a certain specificity of the reactions of plants to AM fungi is limited to the genus and the species level or in some cases also occur within a species, at the strain level.

Signalling molecules involed in AM symbiosis are still in the focus and non elucidated. It is well demonstrated that many flavonoids pattern changes with AM symbiosis evolution on a root system. Some are involved in symbiosis regulation and others not related, at least in this case, to a well established symbiosis.

To elucidate signalling in AM symbiosis it is important to rely on physiological basis to pinpoint molecules involved in succession of events leading to identify genes in controls. Flavonoids being good candidates for signalling (Peers *et al*, 2005) are involved with AM signals (Carlsen *et al*, 2008) or rhizobia signals (Werner, 2004). Furthermore, recently it was shown the existence of orthologs genes for AM and

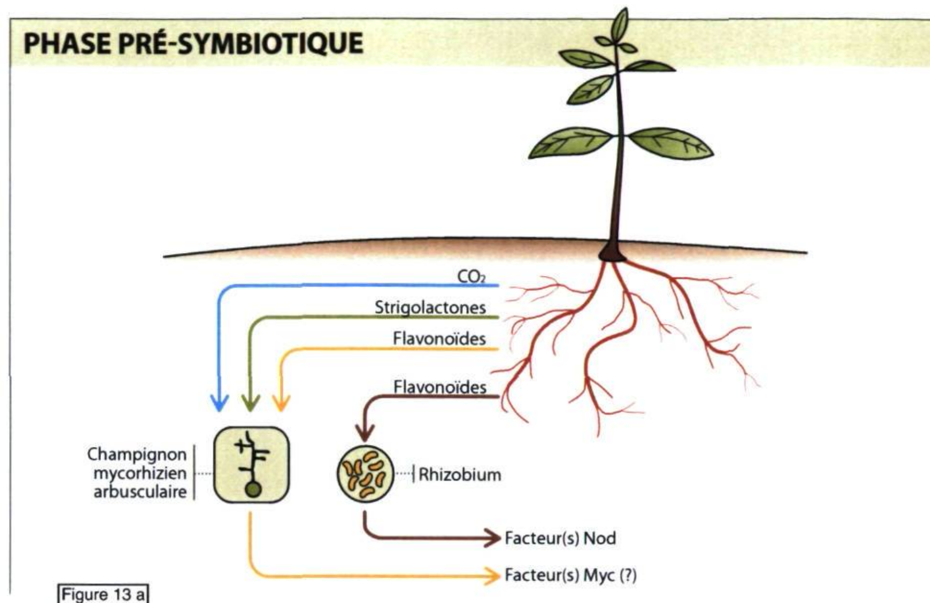
rhizobia symbiosis. These genes involved at regulating signals.level are related to flavonoids (Limpens *et al*, 2003) (Chen C. *et al*, 2008).

Signalling in AM symbiosis is definitely a more complicated process than previously stated. Many molecules, like flavonoids and strigolactones are part of these signals (Akiyama *et al*, 2005). A word on strigolactones, which appears to be part of signalling process are also well known, to be related to virulence in pathogenic fungi. Their exact role in AM symbiosis is still under debate.

The identical behaviour of flavoinoids, between strains of *Glomus mosseae*, lead for pursuing further in flavonoids studies with AM fungi and stimulate the search to elucidate their relation to Myc factors.

4. Conclusion générale

Jusqu'à ce jour, les champignons mycorhiziens arbusculaires (MA) ont résisté à tous les essais afin de réussir à les propager librement en milieux de culture pure. Pour arriver à comprendre cet état de dépendance, il faut être en mesure d'élucider les molécules "signal" qui contrôlent la croissance de ces champignons symbiotiques. De ce point de vue, le choix de la plante hôte *Medicago sativa* s'est avéré déterminant dans notre étude car elle permet d'examiner les voies communes de la signalisation qui sont responsables du développement de la symbiose endomycorhizienne et de la formation de nodules rhizobiens (Fig. 13a). Cette approche nous a rapidement dirigé vers l'étude des flavonoïdes puisque ces molécules "signal" sont capables de stimuler autant la croissance des champignons MA (Gianinazzi-Pearson *et al.* 1989; Chabot *et al.* 1992; Vierheilig *et al.* 1998a) que de contrôler l'expression des gènes *nod* (Nodulation) chez les *Rhizobia* spp. (Stacey 2007) (Fig. 13a). Les flavonoïdes agissent dans la signalisation et induisent l'activation de la transcription des gènes de la nodulation. Ils jouent un rôle prépondérant dans les mécanismes de reconnaissance impliqués dans une relation symbiotique plante/microorganisme. En effet, plusieurs études ont montré que les gènes *nod* codent pour la synthèse des facteurs Nod (lipochito-oligosaccharides) couramment identifiés comme étant les principaux inducteurs de la formation des nodules (Dénarié *et al.* 1996).



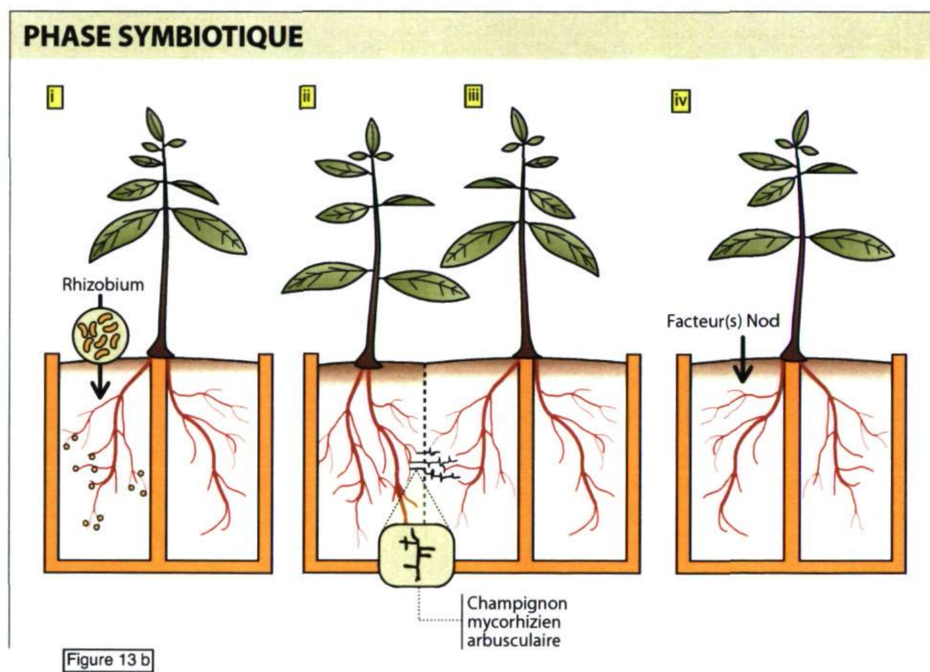


Figure 13 a) Analogie de signalisation entre la symbiose mycorhizienne et la symbiose rhizobienne. b) Système à bi-compartiments (Split-roots) avec les phases pré-symbiotiques et symbiotiques.

Dans la présente thèse, l'évaluation concomitante des deux symbioses nous a permis de nous interroger sur l'existence chez les champignons MA de molécules "signal" apparentées à ces facteurs Nod. En d'autres termes, l'augmentation de la croissance des hyphes des champignons MA en réponse au CO_2 (Poulin *et al.* 1993), aux flavonoïdes (Vierheilig *et al.* 1998a) ou aux strigolactones (Yoneyama *et al.* 2008) implique-t-elle la synthèse de molécules "signal" que l'on pourrait désigner par les facteurs Myc (Fig. 13a)? Toutes ces questions ont largement contribué à la conception et à la réalisation de la présente thèse dans les aspects les plus fondamentaux de la signalisation qui régularise la formation des symbioses MA.

Le système « Split-Roots » s'est avéré particulièrement bien adapté à l'étude de l'établissement des deux symbioses végétales (Fig.13 b). Pour faciliter la comprehension de cette figure, il faut condidérer la succession chronologique, si besoin est, des évènements comme suit. Il y a le premier compartiment que nous avons arbitrairement choisi comme étant le gauche. Le compartiment de droite a servi à mesurer l'effet à distance sur la symbiose. Nous avons utilisé ce système bicompartimenté en chambres de croissance afin de tester l'impact de l'infection rhizobienne sur l'établissement de la symbiose MA (Fig 13 b (i)), de la colonisation fongique MA sur la formation de nodosités (Fig 13 b (ii)(iii)) et finalement du rôle des facteurs Nod dans les interactions plante/champignon MA (Fig. 13 b (iv)).

4.1 Phénomène d'autorégulation chez les deux symbioses végétales

Dans le premier chapitre, nous avons mis en évidence un processus d'autorégulation bilatérale où la première symbiose établie réprime le développement de l'autre (Fig 13 b (i) et b (ii)). Nos résultats montrent assez clairement l'implication de molécules "signal". Leur action à distance est sans doute commune aux deux types de symbioses. Parmi les molécules médiatrices impliquées dans les relations plante/microorganisme, les facteurs Nod sont certainement les signaux chez la symbiose *Rhizobium*/légumineuse qui ont reçu le

plus d'attention. Nous avons observé, en conformité avec les travaux de van Brussel *et al.* (2002), que l'application dirigée de ces facteurs Nod sur le système racinaire de *M. sativa* aboutissait également à l'autorégulation de la symbiose rhizobienne (Fig 13b iv). Les expérimentations sur la même plante ont révélé un effet identique d'autorégulation par les facteurs Nod chez la symbiose MA. Nous pensons donc que les facteurs Nod affectent les deux symbioses de façon similaire par une rétroaction à distance.

Un autre aspect essentiel de ce premier chapitre a été de tester avec le système « Split-Roots » l'influence de la symbiose MA ou de l'application de facteurs Nod sur le bilan carboné du système racinaire de *M. sativa*. Nous avons cherché à montrer si les autorégulations décrites précédemment étaient causées ou non par une carence énergétique liée au manque d'hydrates de carbone. Nous avons donc vérifié par marquage radioactif si la symbiose MA et les facteurs Nod modifiaient l'exigence métabolique des racines de *M. sativa*. Selon les mesures de la radioactivité, nos résultats montrent que les besoins en hydrates de carbone sont identiques, peu importent les parties analysées du système racinaire de *M. sativa*. Nous concluons à une influence très limitée, voire minime, des besoins énergétiques sur le phénomène d'autorégulation pour chacune des symbioses à l'étude.

4.2 Dosage des flavonoïdes dans les racines nodulées, mycorhizées et soumises aux facteurs Nod

L'objectif du second chapitre était d'évaluer la variation des flavonoïdes en fonction de la colonisation des racines de *M. sativa* par les deux symbiotes microbiens et aussi, selon l'application des facteurs Nod. Nous avons principalement orienté nos analyses vers l'identification des isoflavonoïdes (Fig. 1). Ces molécules sont réputées d'être impliquées dans les mécanismes d'autorégulation des symbioses végétales (van Brussel *et al.* 2002).

Quatre isoflavonoïdes ont retenu notre attention au cours de nos travaux de recherche. Ce sont, par ordre alphabétique: la daidzéine, la formononétine, la médicarpine et l'ononine (Voir la Fig. 1 pour les structures moléculaires).

Pour interpréter l'implication de ces isoflavonoïdes dans les processus signalétiques des symbioses, il a fallu harmoniser tous nos chromatogrammes obtenus au HPLC en fonction des racines non nodulées et non mycorhizées. Ensuite, selon tous les cas de figures obtenus avec le système « Split-Roots » (Fig. 13 b), les racines ont été analysées relativement à la colonisation par le *Sinorhizobium meliloti*, le *Glomus mosseae* et sans oublier le traitement abiotique avec des facteurs Nod.

Les isoflavonoïdes affichent des teneurs totalement différentes selon les traitements: si les racines de *M. sativa* étaient colonisées ou pas avec S. *meliloti*, *G. mosseae* et les facteurs Nod. Dans toutes ces situations, les divergences entre les concentrations des quatre isoflavonoïdes s'expliquent par une rétroaction à distance. La formononétine et l'ononine affichent normalement une nette réduction lorsqu'une symbiose se développe sur la partie du système racinaire située dans l'autre compartiment. Dans nos conditions expérimentales, cette diminution de la formononétine et l'ononine est induite d'une façon similaire non seulement pour les deux symbioses mais aussi lors de l'application des facteurs Nod. Ces résultats nous amènent à proposer ces isoflavonoïdes comme d'excellents candidats pour être des molécules "signal" dans l'autorégulation à distance pour les deux symbioses végétales.

Les expériences subséquentes avaient comme corollaire d'essayer de rétablir les niveaux originaux de la formononétine et l'ononine et d'observer si l'addition de ces molécules "signal" vont s'interposer au processus d'autorégulation. Un ou l'autre de ces deux isoflavonoïdes ont été appliqués en concentration réduite sur des racines déjà autorégulées. Les résultats démontrent un effet réversible de l'autorégulation par l'augmentation de chacune des symbioses. Ces résultats sont

cohérents avec le rôle de molécules "signal" des isoflavonoïdes. Une exception observée est le cas de la formononétine. Elle ne réussit pas à contrer l'effet d'autorégulation causée par la symbiose endomycorhizienne. Pourtant cette molécule joue un rôle dans la restauration de la symbiose *Rhizobium*/légumineuse. En contrepartie, la molécule d'ononine contre efficacement l'autorégulation pour les deux symbioses. Ces deux résultats différents laissent supposer que les mécanismes d'autorégulation des deux symbioses sont similaires mais non identiques.

La suppression commune de production d'isoflavonoïdes par les symbioses endomycorhiziennes, rhizobiennes et par les facteurs Nod est le résultat le plus important de ce chapitre. Deux flavonoïdes, la formononétine et l'ononine, sont associés à l'autorégulation pour les deux symbioses et stimulent la nodulation. L'ononine semble être la seule molécule "signal" pouvant rétablir complètement la mycorhization.

4.3 Patron des flavonoïdes associé aux variations intraspécifiques de *Glomus mosseae*

Au troisième chapitre, nous avons analysé la variation des flavonoïdes dans les racines de *M. sativa* lorsqu'elles sont inoculées avec trois souches différentes de *G. mosseae*. Nous avons identifié au moins huit flavonoïdes que apparaissent être de bons candidats pour jouer un rôle dans la signalisation au niveau de l'autorégulation. On observe que l'accumulation de flavonoïdes est souvent typique pour un genre de champignon MA (Scervino *et al.* 2005a). Même si les souches de *G. mosseae* expriment des besoins énergétiques différents (Lerat *et al.* 2003a), nos résultats ont démontré une certaine homogénéité dans les patrons des flavonoïdes étudiés. Ces résultats confirment le rôle de signaux de certains flavonoïdes à comportement stable.

Grâce aux présents travaux, nous comprenons davantage l'implication des flavonoïdes dans la régulation de la symbiose endomycorhizienne. Nous avons également établi des liens avec une partie de la signalisation de la symbiose *Rhizobium*/légumineuse. Pour compléter ces résultats, il est intéressant de noter la récente découverte de gènes orthologues isolés chez *Medicago truncatula* (Zhu et al. 2006) et *Oriza sativa* (Chen *et al.* 2008) et codant pour l'établissement des deux symbioses que nous avons étudiées. Les séquences de DNA de même que les fonctions pour la formation des deux symbioses sont orthologues. Ces nouveaux faits viennent consolider le rôle probable des flavonoïdes dans la symbiose MA.

Les plantes colonisées par la symbiose mycorhizienne et celles colonisées par la symbiose rhizobienne présentent une susceptibilité réduite aux pathogènes (*Volpin et al.* 1994, *Deshwal et al.* 2003). Il est possible que les mécanismes d'autorégulation décrits précédemment jouent également un rôle dans cette protection.

L'étude de ces moyens de communication entre différents partenaires de mettent en perspective la kyrielle de mécanismes possibles impliqués dans la signalisation plante/microorganisme. Il est essentiel de considérer les multiples structures présentes dans la racine et la pluralité des échanges qui s'y déroulent. De nombreux mécanismes de régulation sont nécessaires et il semble que la signalisation ne soit pas la panacée d'un seul type de molécules. En effet, nous considérons que les strigolactones, un autre type de signal, seraient impliquées lors de la pré-colonisation de la symbiose MA (Fig. 13 a). La démarche à suivre pour comprendre la signalisation impliquée dans la symbiose endomycorhizienne serait de poursuivre le décryptage de la succession des évènements physiologiques présents. Nous devons aborder l'étude de la signalisation des symbioses par une approche globaliste. Pour ce faire il est important de rechercher les mécanismes signalétiques similaires voire meme même identiques, chez d'autres symbioses. Il est des résultats récents qui relient les deux symbioses qui ont captivé notre attention. En effet des récepteurs de kinases chez les plantes seraient derivés de récepteurs recrutés chez la symbiose mycorrhizienne

arbusculaire (Markmann *et al.* 2008). Il appert que des genes orthlogues communs pour les deux symbioses commencent à être identifié (Chen *et al.* 2008) ce qui vient conforter notre approche d'étude globale des symbioses. Il est aussi essentiel de ne pas oublier que la frontière, au niveau signalétique, qui les différencie des pathogènes est ténue.

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