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SHORT NOTE

A *Medicago truncatula* mutant hyper-responsive to mycorrhiza and defective for nodulation

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Abstract One key strategy for the identification of plant genes required for mycorrhizal development is the use of plant mutants affected in mycorrhizal colonisation. In this paper, we report a new *Medicago truncatula* mutant defective for nodulation but hypermycorrhizal for symbiosis development and response. This mutant, called B9, presents a poor shoot and, especially, root development with short laterals. Inoculation with *Glomus intraradices* results in significantly higher root colonisation of the mutant than the wild-type genotype A17 (+20% for total root length, +16% for arbuscule frequency in the colonised part of the root, +39% for arbuscule frequency in the total root system). Mycorrhizal effects on shoot and root biomass of B9 plants are about twofold greater than in the wild-type genotype. The B9 mutant of *M. truncatula* is characterised by considerably higher root concentrations of the phytoestrogen coumestrol and by the novel synthesis of the coumestrol conjugate malonyl glycoside, absent from roots of wild-type plants. In conclusion, this is the first time that a hypermycorrhizal plant mutant affected negatively for nodulation (Myc^{++}, Nod $^{-/+}$ phenotype) is reported. This mutant represents a new tool for the study of plant genes differentially regulating mycorrhiza and nodulation symbioses, in particular, those related to autoregulation mechanisms.

Keywords *Medicago truncatula* · Myc^{++}, Nod $^{-/+}$ mutant · Coumestrol · *Glomus intraradices*

Introduction

Currently, much of the research on arbuscular mycorrhizal (AM) interactions is focussed on plant genes required for mycorrhizal development. One key tool for these studies is the use of plants mutated for genes affecting mycorrhizal colonisation. Many AM plant mutants have been isolated by chemical or physical mutagenesis since the first mycorrhiza-deficient mutants (noted Myc^{-}) were reported for *Pisum sativum* and *Vicia faba* (Duc et al. 1989). Apart from Myc^{-} mutants identified in tomato, (Barker et al. 1998; David-Schwartz et al. 2001; David-Schwartz et al. 2003) and in petunia (Reddy et al. 2007), the majority of Myc^{-} mutants have been reported for legumes previously selected for nodulation deficiency, and common genes involved in both rhizobial and mycorrhizal symbioses have been identified (Marsh and Schultze 2001; Parniske 2004). In addition, hypermycorrhizal (Myc^{++}) phenotypes have been found among supernodulating and nitrate tolerant mutants of *P. sativum*, *M. truncatula*, *Glycine max*, and *Lotus japonicus* (Morandi et al. 2000; Shrihari et al. 2000; Solaiman et al. 2000).

Phenolic compounds are known to play a role in signalling between plants and their microsymbionts, (Siqueira et al. 1991a; Phillips and Tsai 1992; Morandi 1996; Vierheilig et al. 1998). In the case of AM interactions, flavonoid/isoflavonoids have been shown to influence in vitro spore germination, growth of germination hyphae (Tsai and Phillips 1991; Bécard et al. 1992; Morandi et al. 1992; Scervino et al. 2005a, b) and AM colonisation (Siqueira et al. 1991b; Morandi 1996; Vierheilig et al. 1998; Scervino et al.

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2005c; Scervino et al. 2007). In particular, the isoflavonoid coumestrol, a plant phytoestrogen quite common in legumes, has been shown to accumulate to significant levels in mycorrhizal roots and to stimulate growth of germination hyphae in *Gigaspora margarita* (Morandi et al. 1984; Morandi et al. 1992).

The model legume *Medicago truncatula* has received consistent attention for the selection of nodulation and AM mutants (Sagan et al. 1995; Catoira et al. 2000; Morandi et al. 2000; Penmetsa and Cook 2000; Morandi et al. 2005). In this paper, we report isolation and characterisation of an EMS generated *M. truncatula* mutant having a poorly developed root system and which presents a novel hyper-mycorrhizal, nodulation-defective phenotype. This mutant is characterised by an unusually high root content in coumestrol isoflavonoid, and by the presence of a root coumestrol conjugate not previously reported in *M. truncatula*.

Materials and methods

Biological material

A mutant plant population was generated from *M. truncatula* (Gaertn.) line A17 of the variety Jemalong using 0.15% ethyl methane-sulphonate (EMS; Le Signor et al., submitted). Following EMS treatment, the M1 seeds were sown and M2 seeds were harvested from individual plants. One M2 plant was grown from each M1 plant (Single Seed Descent design) and the range of mutations generated was defined from seed and seedling phenotypes in the M2 generation (albino frequency, percentage of chlorotic phenotypes and percentage of M2 embryo abortion). Screening for a mycorrhizal phenotype was set up using M2 seed batches from 300 families where more than 100 seeds were obtained. Twenty seeds from each selected seed batch were scarified for 2 min in 98% sulphuric acid, rinsed five times in water and surface disinfected for 10 min in 3.5% calcium hypochlorite. After rinsing five times in water, seeds were transferred to moist filter paper in Petri dishes and left to germinate for 4 days in the dark at 4°C. Seedlings were then transferred to room temperature for 1 day before transplanting to 20 ml growth substrate composed of 35% of a commercial *Glomus intraradices* inoculum (Agrauxine, Biorize SA) and 65% clay loam soil. Plants were raised in a growth chamber (16-h photoperiod, 350 μmol m^2 s^{-1} irradiation, 70% day and 80% night relative humidity, 22°C day and 19°C night), watered daily with osmosed water and harvested 3 weeks later. Root systems were washed under tap water and stained with trypan blue (Phillips and Hayman 1970). For each plant, the entire root system was observed under a stereomicroscope to evaluate mycorrhizal phenotype and root morphology. No Myc^- phenotype was found amongst 3,000 plants observed (ten plants per mutant line), but plants from one mutant line presented a poorly developed root system and intense AM colonisation. This line was called "B9" and was selfed to obtain a M3 generation progeny for further investigations.

Growth of wild-type and B9 mutant genotypes of *M. truncatula*

The two genotypes, A17 and B9 (M3), were inoculated or not with *G. intraradices*. Seedlings germinated as described above were transferred to 100 ml of a substrate composed of a mixture of 3/4 (*v*/*v*) gamma irradiated clay loam soil and 1/4 (*v*/*v*) zeolite. In the *G. intraradices*-inoculated treatments, zeolite was replaced by the zeolite-based commercial inoculum. Each non-inoculated plant received 1 ml of a water filtrate of the mycorrhizal inoculum (100 g inoculum filtered with 100 ml distilled water) at the beginning of the experiment, in order to provide the same bacterial environment as inoculated ones. For the wild-type A17 genotype, ten plants were grown per treatment: five for isoflavonoid analysis and five for estimation of AM colonisation. For the mutant line B9, 20 plants were grown per treatment: five replicates, each consisting of roots pooled from three plants, were used for isoflavonoid analyses, and the remaining five plants were used for estimation of AM colonisation. Plants were grown as described above, and watered three times a week with 20 ml Long Ashton nutrient solution (Hewitt 1966), modified in order to have a one-tenth phosphate concentration in the form of NaH_2PO_4. Plants were harvested after 5 weeks' growth. Roots were washed under tap water, separated from the shoot, the two parts weighed (fresh material), and roots immersed in liquid nitrogen and stored at −80°C until analysis.

Evaluation of mycorrhizal and nodulation phenotypes

Root systems were stained with trypan blue, as described above, cut into 1-cm fragments and thirty randomly collected for estimation of mycorrhizal development. A stereomicroscope at ×40 magnification was used to evaluate mycorrhizal root length and percentage of cortical cells containing arbuscules in the colonised part and in the total root system (Morandi et al. 2005).

To assess the nodulation phenotype, B9 (M3) seedlings were planted into 200-ml pots containing sterile clay loam soil and inoculated with *Sinorhizobium meliloti* strain 2011. Plants were watered every 2 days with Long Ashton nutrient solution without nitrogen (Ca $(NO_3)_2$ and KNO_3 were replaced by $CaCl_2$ and KCl, respectively). Five replicate plants were harvested after 4, 6 and 8 weeks

growth, roots washed under tap water and observed visually for nodulation.

Isoflavonoid analyses

Frozen root samples were ground in liquid nitrogen and then extracted with 95% ethanol (10 ml g^{-1} fresh material) under sonication for 1 h at room temperature. The extract was filtered, evaporated to dryness under vacuum and the dry extract redissolved in methanol/water (8/2 *v*/*v*) to obtain a final concentration corresponding to 4 g fresh material/ml. The methanol extract was analysed by HPLC in a Beckman Gold system consisting of a 507e autosampler module, a 126 solvent delivery system module, a reverse-phase C18 column (ultrasphere IP 4.6×250 mm), a 168 diode array detector module and Gold version 8 data analysis software. The solvent system was a mixture of H_2O/1.5% orthophosphoric acid (Sigma Aldrich; solvent A) and acetonitrile (Sigma Aldrich; solvent B) delivered at a flow rate of 1 ml min^{-1} with the following gradient: t=0 min, B=0%; t=42 min, B=70%; t=47 min, B=100%; t=52 min, B=0%. A coumestrol standard (Sigma Aldrich; 10 µg ml^{-1}) was used as external injection under the same HPLC conditions for quantification purposes (given as microgram per gram of fresh material).

Coumestrol conjugate purification and characterisation

HPLC analyses revealed the presence, in roots of the B9 mutant, of a compound (RT=23.1 min) absent from A17 roots. Due to its similar UV spectra to coumestrol, it was presumed to be a coumestrol conjugate and its characterisation was undertaken. Samples of B9 extracts remaining after HPLC analysis were bulked together and concentrated to a unique sample in order to purify the coumestrol conjugate. To collect the corresponding peak by HPLC, 1.5% phosphoric acid in solvent A was replaced by 1% acetic acid. All collected fractions were bulked together, solvents evaporated to dryness under vacuum and the dry fraction redissolved in 200 µl 80% MeOH. Twenty microlitres were used for standard HPLC analysis, and the remaining fraction was heated 16 h at 80°C in a sealed vial to determine if the conjugate was malonylated. This procedure converts flavonoid glycoside malonates into their glycosides without the production of other derivatives (Lin et al. 2000). Twenty microlitres of the resulting solution was reanalysed by standard HPLC, and the rest was concentrated under vacuum to eliminate methanol. The solution was adjusted to 200 µl with water, and hydrolysis was performed in a sealed vial using ß-glucosidase (Sigma Aldrich) for 8 h at 37°C (Mabry et al. 1970). Water was then evaporated under vacuum and the sample redissolved in 80% MeOH for HPLC analysis as described above.

Statistical analysis

All data were analysed by ANOVA, after arcsin √ transformation for percentages and mean values were compared with the Tukey–Kramer test ($P \leq 0.05$).

Results

Growth and symbiotic phenotype of the B9 mutant

The B9 mutant presented clearly poorer growth than the wild-type A17 genotype (Fig. 1). The root system was particularly poorly developed, as shown by the higher shoot/root ratio (nearly three times) for B9 as compared to A17 (Table 1). The morphology of the root system was characterised by short lateral root development. Total weight (fresh material) of B9 mutant was about 13 and five times lower than A17 genotype for non *G. intraradices*-inoculated and *G. intraradices*-inoculated plants, respectively (Table 1).

Five weeks after inoculation with *G. intraradices,* B9 root colonisation was statistically higher than in wild-type A17 plants for all the three parameters evaluated (Table 1): +20% for mycorrhizal root length intensity, +16% for the arbuscule frequency in the colonised parts of the root and +39% for the arbuscule frequency in the total root system. Although normal pink nodules were visible on A17 roots

Fig. 1 Wild-type A17 and mutant B9 *Medicago truncatula* cultivated during 5 weeks in a substrate of 75% disinfected clay loam soil and 25% zeolite

Table 1 Growth, AM colonisation parameters (L=mycorrhizal root length intensity, AC=arbuscule frequency in colonised parts of the root system, AT=arbuscule frequency in the total root system) and root isoflavonoid [coumestrol and coumestol conjugate (CG)] content of wild-type A17 and B9 mutant *Medicago truncatula*, 5 weeks after inoculation (M) or not (NM) with *Glomus intraradices*

	A17NM	A17M	B9NM	B9M
Fresh mass				
roots	4.18 c	4.61 c	0.18 a	0.43 b
shoots	2.78 c	4.10 d	0.33 a	1.05 b
total	6.96 c	8.72 d	0.51 a	1.48 b
Shoot/root	0.68 a	0.89 b	1.83 c	2.44 d
M/NM biomass				
roots		1.10 a		2.39 b
shoots		1.48 a		3.18 b
total		1.25 a		2.90 b
AM colonisation parameters				
L (%)		69 a		83 b
AC (%)		74 a		86 b
AT (%)		51 a		71 b
Root isoflavonoid contents				
coumestrol (µg g^{-1} fresh mass)	0.87 a	1.65 b	19.1 c	23.4 c
CG (µg CE g^{-1} fresh mass)	nd	nd	1.2 b	0.86 a

Means in each line followed by different letters are significantly different at $P \leq 0.05$ (Tukey–Kramer statistical analysis)

nd not detected, *CE* coumestrol equivalent

4 weeks after *S. meliloti* inoculation, none of the root systems of B9 plants had visible nodules after 4 and 6 weeks. Only a few white nodules were observed on two out of five plants of B9 after 8 weeks' growth, characterising a late, poorly nodulating phenotype ($Nod^{-/+}$).

A much stronger mycorrhizal growth stimulating effect was observed on B9 than on the wild-type A17 genotype (Table 1): the M/NM ratio for shoots and roots was more than twofold higher for B9 than for A17.

This hypermycorrhizal, poorly nodulating phenotype of the mutant B9 was homogeneous between plants in a progeny and between two successive generations, which indicates that this trait is genetically determined and that the mutation could be fixed after one selfing generation.

Isoflavonoid occurrence in roots

Isoflavonoid profiles of mutant B9 and wild-type A17 genotypes were analogous (data not shown) in 5-week old *M. truncatula* roots. Exceptions were (a) coumestrol which had a considerably higher concentration in B9 than in A17 roots, and (b) a coumestrol conjugate (CG) which was detected only in mutant B9 roots. Coumestrol concentrations were about 14 and 22 times greater in B9 roots than in A17 roots, in mycorrhizal and non-mycorrhizal plants, respectively (Table 1). *G. intraradices* colonisation induced a significant increase (about twofold) in root coumestrol concentration in A17 roots, whilst in the mutant B9 the increase was not statistically significant. The amount of CG, given in coumestrol equivalent, was significantly lower (−20%) in mycorrhizal B9 roots than in non-mycorrhizal ones. After HPLC purification of CG, heating at 80°C for 16 h gave a compound with a retention time (RT) of 20.5 min, 2.6 min lower than CG (RT=23.1 min). This indicates that CG was malonylated (Lin et al. 2000). Hydrolysis of the demalonlylated CG using ß-glucosidase gave a compound with the same HPLC characteristics (RT=28.4 min) and UV spectra as pure standard coumestrol confirming that CG was a glycosylated conjugate of the aglycone coumestrol.

Discussion

This paper reports a novel hypermycorrhizal (Myc^{++}) mutant of *M. truncatula* which is hyper-responsive to AM and defective for nodulation. In contrast to previously reported Myc^{++} mutants which all express a Nod^{++} phenotype (Morandi et al. 2000; Shrihari et al. 2000; Solaiman et al. 2000), the new B9 mutant has a late, poorly nodulating, ineffective phenotype ($Nod^{-/+}$). The existence of Myc^{++} Nod^{++} mutants has led to the suggestion that common mechanisms of autoregulation are shared for AM colonisation and nodulation of legume roots (Vierheilig 2004; Vierheilig et al. 2008). This hypothesis was reinforced by the observation of loss of mycorrhizal autoregulation in a hypernodulating mutant of soybean (Meixner et al. 2005). The Myc^{++} character in the *M. truncatula* mutant B9 appears to depend on a mechanism which is independent of nodulation. However, gene mutation is associated with a particular root morphogenesis since it results in a strongly reduced growth (compared to the wild type) and a limited lateral root ramification. This poor development of the root system of the B9 mutant, which may reflect an impaired C supply, will lower the plants' capacity to absorb mineral nutrients rendering it more dependent on the

mycorrhizal symbiosis, which is reflected in the greater mycorrhizal effect on the growth of B9 than of wild-type *M. truncatula*.

An interesting feature of B9 mutant roots is the high concentration of the isoflavonoid coumestrol which reaches up to 22-fold in roots of wild-type *M. truncatula*. Coumestrol is a phytoestrogen with strong estrogenic activities (Tinwell et al. 2000) which can be involved in the control of plant pathogens (Lyon and Wood 1975; Feet and Osman 1982; O'Neill 1996). In symbiotic interactions, it has been reported to be a Nod-gene-inducer for *Bradyrhizobium japonicum* (Kosslak et al. 1987) but a Nod-gene-inhibitor for *S. meliloti* (Zuanazzi et al. 1998), so that the low and late nodulation of the B9 mutant of *M. truncatula* could be due, at least in part, to the greater root concentration of coumestrol. Also, coumestrol levels increased in the mycorrhizal roots of *M. truncatula*, which is consistent with that previously reported for soybean (Morandi et al. 1984) or *Medicago* roots (Harrison and Dixon 1993). Coumestrol has been shown to stimulate growth of hyphae from germinating spores of the AM fungus *G. margarita* (Morandi et al. 1992). It can therefore be hypothesised that the high level of coumestrol in B9 roots could contribute to the increased mycorrhizal colonisation observed in this mutant.

The B9 mutant is also characterised by the presence of a coumestrol conjugate (CG) absent from the wild-type A17 genotype. After HPLC purification, heating at 80°C and hydrolysis with ß-glucosidase, we can conclude that CG is a coumestrol malonyl glycoside. This is the first report of such a coumestrol conjugate in *M. truncatula*, although it has previously been described in roots of *Medicago sativa* (Tiller et al. 1994). The advantage for plants to form flavonoid glycoside malonates is to sequester less soluble and more toxic flavonoid aglycons. In addition, they can provide a preformed pool of antimicrobial compounds which can be rapidly released following pathogen attack (Graham et al. 1990; Graham and Graham 1991; Harborne 1994). The coumestrol glycoside present in the B9 mutant is associated with a high concentration of root coumestrol, compared to the wild-type genotype of *M. truncatula*. This high level of coumestrol may induce, as part of a regulation mechanism, synthesis of the coumestrol malonyl glycoside in order to better solubilise or store a part of the coumestrol produced. The fact that AM colonisation reduces accumulation of the CG in roots could reflect hydrolysis of the coumestrol glycoside through activation of a ß-glucosidase, or inhibition of a glycosyltransferase and/or malonyltransferase involved in the biosynthesis of CG (Hsieh and Graham 2001; Modolo et al. 2007). Increased expression, rather than down-regulation of a glycosyltransferase and a malonyltransferase gene has been reported in mycorrhizal *M. truncatula* roots (Manthey et al. 2004; Lohse et al. 2005).

In conclusion, the newly described Myc^{++}, $Nod^{-/+}$ mutant of *M. truncatula* constitutes a novel tool for the study of AM-regulated symbiotic plant genes, independent of those implicated in nodulation. Further research on the carbohydrate availability to the root system, dynamics of root coumestrol accumulation and early stages of bacterial and fungal interactions will help to better understand the mechanisms involved in symbiotic alterations in the B9 mutant. In addition, the mechanisms modulating coumestrol and glycoside derivative synthesis in this plant mutant merit investigation in order to evaluate the biological role of these compounds in an autoregulation process promoting AM interactions.

References

Barker SJ, Stummer B, Gao L, Dispain I, O'Connor PJ, Smith SE (1998) A mutant in *Lycopersicon esculentum* Mill. with highly reduced VA mycorrhizal colonization: isolation and preliminary characterisation. Plant J 15:791–797. doi:10.1046/j.1365-313X.1998.00252.x

Bécard G, Douds DD, Pfeffer PE (1992) Extensive in vitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. Appl Environ Microbiol 58:821–825

Catoira R, Galera C, de Billy F, Penmetsa RV, Journet EP, Maillet F, Rosenberg C, Cook D, Gough C, Denarie J (2000) Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. Plant Cell 12:1647–1665

David-Schwartz R, Badani H, Smadar W, Levy A, Galili G, Kapulnik Y (2001) Identification of a novel genetically controlled step in mycorrhizal colonisation: plant resistance to infection by fungal spores but not extra-radical spores. Plant J 27:561–569. doi:10.1046/j.1365-313X.2001.01113.x

David-Schwartz R, Gadkar V, Smadar W, Bendov R, Galili G, Levy A, Kapulnik Y (2003) Isolation of a premycorrhizal infection (*pmi2*) mutant of tomato, resistant to arbuscular mycorrhizal fungal colonisation. Mol Plant Microbe Interact 16:382–388. doi:10.1094/MPMI.2003.16.5.382

Duc G, Trouvelot A, Gianinazzi-Pearson V, Gianinazzi S (1989) First report of non-mycorrhizal plant mutants (Myc^{-}) obtained in pea (*Pisum sativum* L.) and fababean (*Vicia faba* L.). Plant Sci 60:215–222. doi:10.1016/0168-9452(89)90169-6

Feet WF, Osman SF (1982) Inhibition of bacteria by the soybean isoflavonoids glyceollin and coumestrol. Phytopathology 72:775–760

Graham TL, Graham MY (1991) Glyceollin elicitors induce major but distinctly different shifts in Isoflavonoid metabolism in proximal and distal soybean cell populations. Mol Plant Microbe Interact 4:60–68

Graham TL, Kim JE, Graham MY (1990) Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. Mol Plant Microbe Interact 3:157–166

Harborne JB (1994) For the content of “Flavonoids”. In: Buckingham J (eds) Dictionary of Natural Products. Chapman and Hall, New York, pp 449-468 and 673-700

Harrison MJ, Dixon RA (1993) Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular-arbuscular mycorrhizal associations in roots of *Medicago truncatula*. Mol Plant Microbe Interact 6:643–654

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. In: (eds) Technical Commmunication No. 22. Commonwealth Agricultural Bureau, London, pp 430-434

Hsieh M-C, Graham TL (2001) Partial purification and characterization of a soybean ß-glucosidase with high specific activity towards isoflavone conjugates. Phytochemistry 58:995–1005

Kosslak RM, Bookland R, Barkei J, Paaren HE, Appelbaum ER (1987) Induction of *Bradyrhizobium japonicum* common nod genes by isoflavones isolated from *Glycine max*. Proc Natl Acad Sci USA 84:7428–7432. doi:10.1073/pnas.84.21.7428

Lin LZ, He XG, Lindenmaier M, Yang J, Cleary M, Qiu SX, Cordell GA (2000) LC–ESI–MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). J Agric Food Chem 48:354–365. doi:10.1021/jf991002+

Lohse S, Schliemann W, Ammer C, Kopka J, Strack D, Fester T (2005) Organization and metabolism of plastids and mitochondria in arbuscular mycorrhizal roots of *Medicago truncatula*. Plant Physiol 139:329–340. doi:10.1104/pp.105.061457

Lyon FM, Wood RKS (1975) Production of phaseollin, coumestrol and related compounds in bean leaves inoculated with *Pseudomonas* spp. Physiol Plant Pathol 6:117–124. doi:10.1016/0048-4059(75)90039-9

Mabry TJ, Markham KR, Thomas MB (1970) The systematic identification of flavonoids. Springer, New York

Manthey K, Krajinski F, Hohnjec N, Firnhaber C, Pühler A, Perlick A, Küster H (2004) Transcriptome profiling in root nodules and arbuscular mycorrhiza identifies a collection of novel genes induced during *Medicago truncatula* root endosymbioses. Mol Plant Microbe Interact 17:1063–1077. doi:10.1094/MPMI.2004.17.10.1063

Marsh JF, Schultze M (2001) Analysis of arbuscular mycorrhizas using symbiosis-defective plant mutants. New Phytol 150:525–532. doi:10.1046/j.1469-8137.2001.00140.x

Meixner C, Ludwig-Muller J, Miersch O, Gresshoff P, Staehelin C, Vierheilig H (2005) Lack of mycorrhizal autoregulation and phytohormonal changes in the supernodulating soybean mutant nts1007. Planta 222:709–715. doi:10.1007/s00425-005-0003-4

Modolo LV, Blount JW, Achnine L, Naoumkina MA, Wang X, Dixon RA (2007) A functional genomics approach to (iso) flavonoid glycosylation in the model legume *Medicago truncatula*. Plant Mol Biol 64:499–518. doi:10.1007/s11103-007-9167-6

Morandi D (1996) Occurrence of phytoalexins and phenolic compounds in endomycorrhizal interactions, and their potential role in biological control. Plant Soil 185:241–251. doi:10.1007/BF02257529

Morandi D, Bailey JA, Gianinazzi-Pearson V (1984) Isoflavonoid accumulation in soybean roots infected with vesicular-arbuscular mycorrhizal fungi. Physiol Plant Pathol 24:357–364. doi:10.1016/0048-4059(84)90009-2

Morandi D, Branzanti B, Gianinazzi-Pearson V (1992) Effect of some plant flavonoids on in vitro behaviour of an arbuscular mycorrhizal fungus. Agronomie 12:811–816. doi:10.1051/agro:19921012

Morandi D, Sagan M, PradoVivant E, Duc G (2000) Influence of genes determining supernodulation on root colonization by the mycorrhizal fungus *Glomus mosseae* in *Pisum sativum* and *Medicago truncatula* mutants. Mycorrhiza 10:37–42. doi:10.1007/s005720050285

Morandi D, Prado E, Sagan M, Duc G (2005) Characterisation of new symbiotic *Medicago truncatula* (Gaertn.) mutants, and phenotypic or genotypic complementary information on previously described mutants. Mycorrhiza 15:283–289. doi:10.1007/s00572-004-0331-4

O'Neill NR (1996) Defense expression in protected tissues of *Medicago sativa* is enhanced during compatible interactions with *Colletotrichum trifolii*. Phytopathology 86:1045–1050. doi:10.1094/Phyto-86-1045

Parniske M (2004) Molecular genetics of the arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol 7:414–421. doi:10.1016/j.pbi.2004.05.011

Penmetsa RV, Cook DR (2000) Production and characterization of diverse developmental mutants of *Medicago truncatula*. Plant Physiol 123:1387–1397. doi:10.1104/pp.123.4.1387

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Phillips DA, Tsai SM (1992) Flavonoids as plant signals to rhizosphere microbes. Mycorrhiza 1:55–58. doi:10.1007/BF00206136

Reddy S, Schorderet M, Feller U, Reinhardt D (2007) A petunia mutant affected in intracellular accommodation and morphogenesis of arbuscular mycorrhizal fungi. Plant J 51:739–750. doi:10.1111/j.1365-313X.2007.03175.x

Sagan M, Morandi D, Tarenghi E, Duc G (1995) Selection of nodulation and mycorrhizal mutants in the model plant *Medicago truncatula* (Gaertn) after gamma-ray mutagenesis. Plant Sci 111:63–71. doi:10.1016/0168-9452(95)04229-N

Scervino J, Ponce M, Erra-Bassels R, Vierheilig H, Ocampo J, Godeas A (2005a) Flavonoids exclusively present in mycorrhizal roots of white clover exhibit different effects on arbuscular mycorrhizal fungi than flavonoids exclusively present in non-mycorrhizal roots of white clover. J Plant Interact 15:22–30

Scervino J, Ponce M, Erra-Bassels R, Vierheilig H, Ocampo J, Godeas A (2005b) Flavonoids exhibit fungal species and genus specific effects on the presymbiotic growth of Gigaspora and Glomus. Mycol Res 109:789–794. doi:10.1017/S0953756205002881

Scervino J, Ponce M, Erra-Bassels R, Vierheilig H, Ocampo J, Godeas A (2005c) Arbuscular mycorrhizal colonization of tomato by *Gigaspora* and *Glomus* species in presence of roots flavonoids. J Plant Physiol 162:625–633. doi:10.1016/j.jplph.2004.08.010

Scervino J, Ponce M, Erra-Bassels R, Vierheilig H, Ocampo J, Godeas A (2007) The effect of flavones and flavonols on colonization of tomato plants by arbuscular mycorrhizal fungi of the genera *Gigaspora* and *Glomus*. Can J Microbiol 53:702–709. doi:10.1139/W07-036

Shrihari PC, Sakamoto K, Inubushi K, Akao S (2000) Interaction between supernodulating or non-nodulating mutants of soybean and two arbuscular mycorrhizal fungi. Mycorrhiza 10:101–106. doi:10.1007/s005720000064

Siqueira JO, Nair MG, Hammerschmidt R, Safir GR (1991a) Significance of phenolic compounds in plant-soil-microbial systems. Crit Rev Plant Sci 10:63–121. doi:10.1080/07352689109382307

Siqueira JO, Safir GR, Nair MG (1991b) Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. New Phytol 118:87–93. doi:10.1111/j.1469-8137.1991.tb00568.x

Solaiman MZ, Senoo K, Kawaguchi M, ImaizumiAnraku H, Akao S, Tanaka A, Obata H (2000) Characterization of mycorrhizas formed by *Glomus* sp on roots of hypernodulating mutants of *Lotus japonicus*. J Plant Res 113:443–448. doi:10.1007/PL00013953

Tiller SA, Parry AD, Edwards R (1994) Changes in the accumulation of flavonoid and isoflavonoid conjugates associated with plant-age and nodulation in alfalfa (*Medicago sativa*). Physiol Plant 91:27–36. doi:10.1111/j.1399-3054.1994.tb00655.x

Tinwell H, Soames AR, Foster JR, Ashby J (2000) Estradiol-type activity of coumestrol in mature and immature ovariectomized rat uterotrophic assays. Environ Health Perspect 108:631–634. doi:10.2307/3434883

Tsai SM, Phillips DA (1991) Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl Environ Microbiol 57:1485–1488

Vierheilig H (2004) Regulatory mechanisms during the plant-arbuscular mycorrhizal fungus interaction. Can J Bot 82:1166–1176. doi:10.1139/b04-015

Vierheilig H, Bago B, Albrecht C, Poulin MJ, Piche Y (1998) Flavonoids and arbuscular-mycorrhizal fungi. In: Manthey J, Buslig B (eds) Flavonoids in the living system. Plenum, New York, pp 9–33

Vierheilig H, Steinkellner S, Khaosaad T, Garcia-Garrido J (2008) The biocontrol effect of mycorrhization on soil-borne fungal pathogens and the autoregulation of the AM symbiosis: one mechanism, two effects? In: Varma A (ed) Mycorrhiza: genetics and molecular biology, eco-function, biotechnology, eco-physiology, structure and systematics. Springer, Heidelberg, Germany, pp 307–320

Zuanazzi JAS, Clergeot PH, Quirion JC, Husson HP, Kondorosi A, Ratet P (1998) Production of *Sinorhizobium meliloti* nod gene activator and repressor flavonoids from *Medicago sativa* roots. Mol Plant Microbe Interact 11:784–794. doi:10.1094/MPMI.1998.11.8.784