



Effect of mycorrhization on the isoflavone content and the phytoestrogen activity of red clover

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Received 24 April 2007; received in revised form 23 July 2007; accepted 24 August 2007

KEYWORDS

Arbuscular
mycorrhiza;
Isoflavones;
Phytoestrogen;
Red clover

Summary

Red clover, known for its estrogenic activity due to its isoflavones content (biochanin A, genistein, daidzein and formononetin), was inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae*. Once the symbiotic fungus was well established, plants were harvested and we determined the root and shoot dry weight as well as the P-content. In roots and leaves the levels of biochanin A, genistein, daidzein and formononetin were quantified by reversed-phase HPLC and the estrogenic activity of the leaves was measured by a transactivation assay using a yeast two-plasmid system.

Mycorrhization increased the levels of biochanin A in the root and the shoot and reduced the levels of genistein in the shoot of red clover. The levels of the other isoflavones were not affected. The shoot biomass of mycorrhizal plants more than doubled compared with non-mycorrhizal control plants, and this growth-stimulating effect of arbuscular mycorrhiza did not affect the estrogenic activity of red clover. In a control P treatment, the biomass of red clover was greatly enhanced. However, the estrogenic activity was reduced.

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These results suggest that, in contrast to an enhanced shoot biomass production after P application with a reduced estrogenic activity, with arbuscular mycorrhiza the shoot biomass of red clover can be enhanced without a negative effect on estrogenic activity.

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Introduction

Plant extracts containing polyphenolic compounds with estrogenic activity, the so-called phytoestrogens, have become increasingly popular for treatment of menopausal disorders (Beck et al., 2005). One source of phytoestrogens is red clover (*Trifolium pratense*) (Saloniemi et al., 1995). In red clover, the isoflavones biochanin A, genistein, daidzein and formononetin, which exert estrogenic activity in mammals (Miksicek, 1993; Breithofer et al., 1998), are present in high concentrations (Setchell et al., 2001). Thus, red clover has become popular as a food supplement for the amelioration of menopausal disorders (Dornstauder et al., 2001; Beck et al., 2003). This positive effect on menopausal disorders has been confirmed by several clinical trials (Hidalgo et al., 2005; Imhof et al., 2006).

Legumes such as red clover are hosts for rhizobia. Signals released by rhizobia alter the pattern of flavonoid (including isoflavone) accumulation in legumes (Phillips and Tsai, 1992), including red clover (Edwards et al., 1997) roots. Moreover, rhizobial nodulation of red clover not only affects isoflavonoid accumulation in the root but also systemically reduces the levels of certain isoflavones in the leaves (Edwards et al., 1997).

Red clover is a host not only for rhizobia, but also for symbiotic arbuscular mycorrhizal fungi (AMF). Arbuscular mycorrhiza (AM) is a symbiosis between root colonizing soil-borne fungi and most land plants. Root colonization by AMF results in a positive effect on plant growth, primarily through an improved nutritional status of the host plant by transferring phosphate (P) through the AM hyphae from the soil to the plant (Smith and Read, 1997). The formation of the AM association is a result of a complex exchange of signals between the plant and the AMF. Abundant data show an effect of flavonoids with phytoestrogenic activity on spore germination, hyphal growth and root colonization by AMF (reviewed by Morandi, 1996; Vierheilig et al., 1998a). Moreover, AMF not only respond to flavonoids with a known estrogenic activity, but also to estrogens and antiestrogens (Poulin et al., 1997). During the establishment of AM symbiosis, the

levels of isoflavones with estrogenic activity (e.g. of biochanin A, formononetin, genistein or daidzein) are altered in roots of mycorrhizal plants (Vierheilig et al., 1998a), such as alfalfa (*Medicago sativa*) (Volpin et al., 1994; Larose et al., 2002; Catford et al., 2006), *Medicago truncatula* (Harrison and Dixon, 1993) and soybean (*Glycine max*) (Morandi, 1996). Little is currently known, however, about systemic alterations of isoflavone content in the shoot of mycorrhizal plants.

The aim of the present work was to examine whether mycorrhization can alter the isoflavone pattern in the root and shoot of red clover, and whether these alterations can be linked to phytoestrogen activity. To exclude a simple P-mediated effect through mycorrhization, we also tested the effect of P application on the isoflavone accumulation and the phytoestrogen activity.

Materials and methods

Plant material and growth conditions

Seeds of red clover *T. pratense* L. were surface sterilized in 50% commercial bleach for 5 min, rinsed several times in sterile distilled water and germinated in autoclaved (20 min; 121 °C) perlite. After 2 weeks plantlets were transferred to 300 mL pots (1 plant/pot) into a mixture (autoclaved 20 min 121 °C; 1:1:1; by vol.) of sand:expanded clay:soil. Plants were planted into a hole in the substrate, where the inoculum (5 g per plant) had been previously added. The inoculum consisted of colonized root pieces of bean (*Phaseolus vulgaris* L. cv. Sun Gold), sporocarps, spores and hyphae of *Glomus mosseae* (Vierheilig et al., 1993) (BEG 12; La Banque Européenne des Glomales; International Institute of Biotechnology; Kent; GB; <http://www.kent.ac.uk/bio/beg/>).

Plants were randomly grown in the greenhouse (day/night cycle: 16h; 22 °C/8h; 19 °C; rel. humidity 50–70%; light intensity 400 $\mu\text{E}/\text{s m}^2$ by Radium HRI-T4W/DH lamps) for 16 weeks (8 plants per treatment) and watered with a nutrient solution with or without P. The nutrient solution without P consisted of $\text{Ca}(\text{NO}_3)_2$ 0.472 g/L, K_2SO_4 0.256 g/L; MgSO_4 0.136 g/L; MoO_3 0.07 g; NH_4NO_3 8 mg/L; $\text{Fe}_6\text{H}_5\text{O}_7 \cdot 3 \text{H}_2\text{O}$ 50 mg/L; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 4 \text{H}_2\text{O}$ 1.3 mg/L; $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ 1.5 mg/L; $\text{ZnSO}_4 \cdot 7 \cdot \text{H}_2\text{O}$ 0.6 mg/L; $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ 0.54 mg/L; $\text{Al}_2(\text{SO}_4)_3$ 0.028 mg/L; $\text{NiSO}_4 \cdot 7 \text{H}_2\text{O}$

0.028 mg/L; $\text{Co}(\text{NO}_3)_2 \cdot 6 \cdot \text{H}_2\text{O}$ 0.028 mg/L; TiO_2 0.028 mg/L; LiCl_2 0.014 mg/L; SnCl_2 0.014 mg/L; KJ 0.014 mg/L; KBr 0.014 mg/L. KH_2PO_4 (0.136 g/L) was also added to the nutrient solution with P.

The 'no M, no P' and the 'plus M, no P' treatment received the nutrient solution without P, whereas the 'no M, plus P' treatment received the nutrient solution with P. At the end of the experiment roots were carefully washed with tap water and root and shoot tissue samples were freeze dried. The root and leaf biomass (dry weight (DW)) was determined.

Eight plants per treatment were tested. The experiment was repeated three times.

P-content

The P content in freeze-dried leaves was determined with the ammonium–vanadat–molybdat method (Gericke and Kurmies, 1952).

Determination of root colonization

To visualize the AMF colonization, fresh roots were cleared by boiling 4 min in 10% KOH, rinsed three times with tap water and stained by boiling for 4 min in a 5% ink (Shaeffer; jet-black)/household vinegar (= 5% acetic acid) solution (Vierheilig et al., 1998b, 2005). After staining, the percentage of root colonization was determined according to the method of Newman (1966).

Extraction of samples for isoflavone analysis and determination of hormonal activity

Freeze-dried leaves were pulverized in a mortar. The powder was suspended in DMSO (0.1 g/mL DMSO) and stirred at 450 U/min for 23 h. The suspension was centrifuged at 13,200 rpm for 15 min. The supernatant was collected for further analysis and stored until use at 4 °C.

HPLC—analysis of isoflavones

Hundred milligrams of pulverized leaves or roots were mazerated for 24 h in 2 mL water for enzymatic cleavage of the glycosides. Four milliliters of methanol was then added. The mixture was refluxed for 1 h, filtered and the filtrate evaporated. The root extracts were dissolved in 800 μL DMSO and 40 μL of standard (5 mg 6-methoxyflavanon/mL) were added to 500 μL of the solution. The leaf extracts were dissolved in 1.0 mL DMSO. Forty microliters of standard (10 mg 6-methoxyflavanon/mL) was added to 500 μL of these solutions. Ten microliters of each solution was analyzed by HPLC according to Krenn et al. (2002) for the quantification of daidzein, genistein, formononetin and biochanin A.

Yeast cultivation

Media was sterilized at 121 °C and 1 bar overpressure for 15 min and stored at 4 °C. The GOLD medium

without tryptophan and without uracil (Gold–Trp–Ura) was used for overnight culture, yeast stock and induction.

Yeast estrogen screen (YES)

The yeast estrogen screen is a bioassay based on yeast and is an established test system for detecting estrogenic activity. It is a two-plasmid system: the expression plasmid YE β E12 consists of a copper-inducible human estrogen receptor α gene, an ampicillin resistance gene and a tryptophan-auxotrophy marker, whereas the reporter plasmid carries the reporter lacZ, a uracil-auxotrophy marker, two copies of the estrogen response element (ERE) and the iso-1-cytochrome c promoter. Both plasmids are transformed into the *Saccharomyces cerevisiae* strain 188R1.

Upon induction with copper and activation of the estrogen receptor (ER) by an estrogenic active ligand, β -galactosidase is expressed. The amount of β -galactosidase is correlated to the estrogenic activity of the unknown compound and can be quantified by means of a logistic dose–response curve generated with the reference substance 17- β -estradiol, which is tested within each test run. The evaluation has been described previously by Jungbauer and Beck (2002).

For the assay, an overnight culture was prepared and diluted with medium to an OD_{600} of 0.4. Five microliters of 10 mM CuSO_4 , 45 μL of DMSO and 5 μL of test substance were added to 5 mL of the diluted yeast culture. For the blank value, only 50 μL DMSO and 5 μL CuSO_4 were added to the yeast culture. A calibration curve with 17- β -estradiol was performed within each assay.

After an incubation of 4 h at 30 °C, the cell suspension was centrifuged at 2500 rpm for 5 min. The pellet was washed with 1 mL lac-Z buffer (60 mM $\text{Na}_2\text{HPO}_4 \cdot 2 \cdot \text{H}_2\text{O}$, 40 mM $\text{NaH}_2\text{PO}_4 \cdot 2 \cdot \text{H}_2\text{O}$, 10 mM KCl, 1 mM $\text{MgSO}_4 \cdot 7 \cdot \text{H}_2\text{O}$ and 1 mM DTT, adjusted to pH 7.0 and filtrated (0.22 μm filter)) and after centrifugation at 10,000 rpm for 5 min the pellet was resuspended in 100 μL of lac-Z buffer. The cells were disintegrated mechanically with glass beads (vortexing three times for 30 s within 15 s of rest on ice between the vortexes). The disintegrated cells were centrifuged at 10,000 rpm for 10 min and the clear supernatant was used for the β -galactosidase and protein assays.

β -galactosidase assay

For the β -galactosidase assay, 5 μL of each test tube were transferred to a 96-well microtiter plate. 250 μL of an ONPG solution (*O*-nitrophenyl- β -galactopyranoside; 0.77 g dissolved in 1 L Lac-Z-buffer) were added to each well. The microtiter plate was incubated for 15 min at 37 °C until a yellow color had developed. The reaction was stopped by adding 100 μL of 1 M Na_2CO_3 . The absorption was measured at 405 nm with a reference wavelength at 620 nm. Each determination was performed in duplicate.

Protein assay

The protein content of each test tube was measured according to Bradford (1976) with a modified method. Five microliters of clear supernatant were transferred to a 96-well microtiter plate. Diluted Bio-Rad protein assay reagent (250 μ L) (1:10 dilution with water) was added. The absorption was measured at a wavelength of 570 nm with a reference wavelength of 690 nm. As reference substance BSA was used in each assay. Each determination was performed in duplicate.

Statistical analysis

Analysis of variance was performed after a variance check by Levene's test. Mean values were compared using Fisher's least significant difference. These analyses were performed using Statgraphics Plus 5.0 software.

Results

In inoculated plants (plus M, no P) a high level of root AM was reached (nearly 80%). Non-inoculated plants showed no signs of root colonization (Table 1). In plants supplied with P (no M, plus P) and in mycorrhizal plants (plus M, no P), a clear increase of the shoot DW and enhanced P levels in the leaves were observed (Table 1). The shoot DW and the P levels in the P treatment were always higher compared to the mycorrhizal treatment (plus M, no P). The root DW was enhanced only in the P treatment (no M, plus P).

Mycorrhization (plus M, no P) did not affect the levels of daidzein and formononetin in the root and the shoot compared to the control treatment (no M, no P). However, in mycorrhizal plants, biochanin A levels were increased in the root and the shoot and genistein levels were decreased in the shoot (Figures 1 and 2). Application of P (no M, plus P) never exhibited an effect on the isoflavone content in red clover plants, with the exception of daidzein, which was reduced in roots compared to levels in mycorrhizal plants, and genistein, which was reduced in the shoot compared to levels in mycorrhizal and control plants (Figures 1 and 2).

The total isoflavone content in the root was not affected in any of the treatments. However, in the shoot the total isoflavone content was increased in the mycorrhizal (plus M, no P) and the P treatment (no M, plus P) (Figure 3).

The estrogenic activity of the plant material was similar in the mycorrhizal (plus M, no P) and the

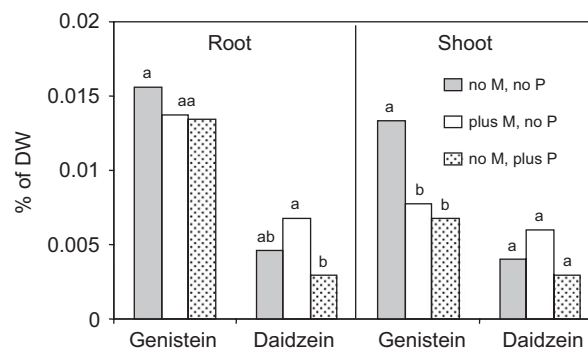


Figure 1. Genistein and daidzein content in roots and leaves of mycorrhizal (plus M) and non-mycorrhizal (no M) red clover plants supplied (plus P) or not supplied (no P) with a P solution. Within each group (e.g. root/genistein) columns with the same letter are not significantly different according to Fisher's LSD test ($P < 0.05$).

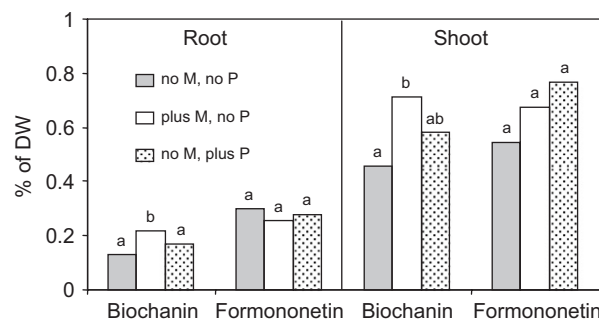


Figure 2. Biochanin A and formononetin content in roots and leaves of mycorrhizal (plus M) and non-mycorrhizal (no M) red clover plants supplied (plus P) or not supplied (no P) with a P solution. Within each group (e.g. root/biochanin A) columns with the same letter are not significantly different according to Fisher's LSD test ($P < 0.05$).

Table 1. Percentage AM root colonization, root and leaf biomass (dry weight (DW)) and P status of mycorrhizal (plus M, no P) and non-mycorrhizal (no M, no P) red clover plants and of non-mycorrhizal red clover plants supplied with a P solution (no M, plus P)

	% Root colonization	Root DW (g)	Shoot DW (g)	mg P/g DW shoot	mg P/shoot
No M, no P	0a	1.76a	0.67a	1.16a	0.58a
Plus M, no P	78b	2.19a	1.51b	1.36b	2.05b
No M, plus P	0a	4.02b	3.06c	3.23c	9.88c

Within each column values with the same letter are not significantly different.

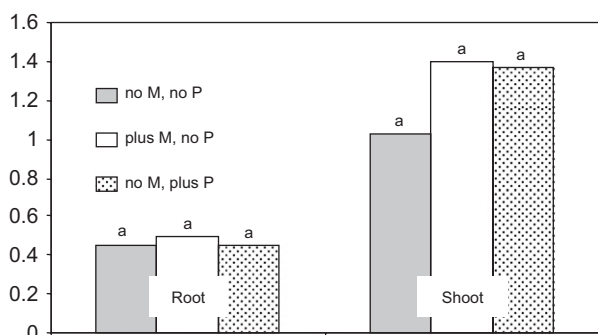


Figure 3. Total isoflavone (genistein, daidzein, Biochanin A and formononetin) content in roots and leaves of mycorrhizal (plus M) and non-mycorrhizal (no M) red clover plants supplied (plus P) or not supplied (no P) with a P solution. Within each group (roots or shoots) columns with the same letter are not significantly different according to Fisher's LSD test ($P < 0.05$).

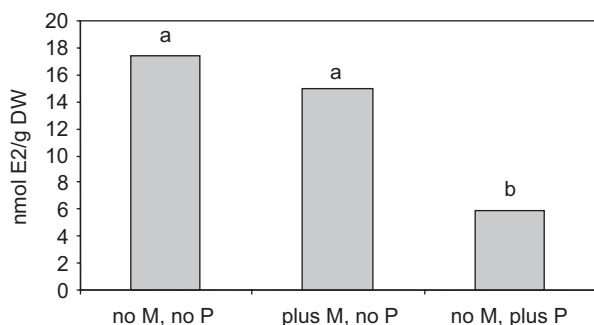


Figure 4. Estrogenic activity of leaves of mycorrhizal (plus M) and non-mycorrhizal (no M) red clover plants supplied (plus P) or not supplied (no P) with a P solution. Columns with the same letter are not significantly different according to Fisher's LSD test ($P < 0.05$).

control (no M, no P) treatment but was highly reduced in the P-treated plants (no M, plus P) (Figure 4).

Discussion

In general, root colonization by AMF results in a positive effect on plant growth, mainly through an improved P status of the host plant (Smith and Read, 1997). Recently, Stürmer (2004) reported an increased shoot DW of red clover when inoculated with different AMF; he did not, however, test *G. mosseae*. In our experiment with *G. mosseae*, we were able to confirm this growth-stimulating effect of mycorrhization on red clover in examination of the shoot DW, although the root DW was not affected. Moreover, similar to the report of Stürmer

(2004), in our experiment mycorrhization increased the P content.

It has been previously reported that isoflavone levels can be altered in roots of legumes when colonized by AMF (Harrison and Dixon, 1993; Volpin et al., 1994; Morandi, 1996; Larose et al., 2002; Catford et al., 2006). In our experiment the level of biochanin A was enhanced in roots of mycorrhizal red clover, but levels of genistein, daidzein and formononetin were not.

Mycorrhization can affect isoflavone levels in roots not only locally but also systemically, meaning that isoflavone levels are altered not only in mycorrhizal roots but also in non-mycorrhizal roots of mycorrhizal plants (Catford et al., 2006). A systemic effect of mycorrhization on flavonoids has been reported not only for the root system, but also for the shoot. In the shoot of white clover (*Trifolium repens*), the flavonoids levels were increased when roots were colonized by AMF (Ponce et al., 2004). In the shoot of red clover, we also found alterations of the flavonoid pattern when plants were mycorrhizal. The level of biochanin A was increased not only in the root but also in the shoot of red clover. Our data confirm that systemic alterations of the flavonoid pattern are a general pattern in mycorrhizal plants.

The main active compounds in red clover plant extracts exerting estrogenic activity are isoflavones such as biochanin A, genistein, daidzein and formononetin (Miksicek, 1993; Breithofer et al., 1998). We found that the total levels of these isoflavones were not affected in the different treatments, indicating the reduced estrogenic activity of the P treatment was not due to the total isoflavone content. Genistein is formed from biochanin A (Knight and Eden, 1996); thus, the enhanced levels of biochanin A in the mycorrhizal red clover plants compared to non-mycorrhizal plants could result in enhanced estrogenic activity. However, the estrogenic activity of mycorrhizal and non-mycorrhizal control plants was similar. This could be due to the relatively small increase of biochanin A in the mycorrhizal plant, which in its effect is possibly compensated by the decrease of genistein or a decrease of other compounds with estrogenic activity such as flavonols and coumestans. We did not determine these (Hänsel et al., 1994; Breithofer et al., 1998) by the HPLC analysis, but they should be detected by the bioactivity assay, if present.

There are some data available that a high P status can reduce the levels of flavonoids in roots. In soybean, P application resulted in reduced coumestrol levels (Morandi and Gianinazzi-Pearson, 1986) and in reduced daidzein levels in bean

(Malusa et al., 2006). To exclude a simple P-mediated effect through mycorrhization, we also tested the effect of P application on the isoflavone accumulation and the phytoestrogen activity. In contrast to other studies (Morandi and Gianinazzi-Pearson, 1986; Akiyama et al., 2002; Malusa et al., 2006), we did not find a reduction of flavonoid levels in the roots of P-treated plants, but genistein levels were reduced in the shoot.

The estrogenic activity in P-treated plants was highly reduced compared to the control and the mycorrhizal red clover plants. Application of P to red clover plants showed no effect on the shoot levels of daidzein, biochanin A and formononetin. However, genistein was reduced to a similar level as in the mycorrhizal plants. This reduction could be an explanation for the reduced estrogenic activity of P-treated plants. Moreover, other compounds with estrogenic activity such as flavonols and coumestans are possibly reduced in P-treated plants, thus explaining the lower activity.

In summary, we found that mycorrhization does alter the flavonoid pattern in the root and shoot of red clover. Moreover, the shoot biomass of mycorrhizal plants was doubled compared to non-mycorrhizal control plants without compromising estrogenic activity. P application resulted in drastic increase of biomass; the estrogenic activity, however, was highly reduced.

Acknowledgments

We thank Ms. E. Holub, Institut für Pflanzenbau und Pflanzenzüchtung, Universität für Bodenkultur Wien, for P analysis and the “Wirtschaftskammer Wien (WKO Wien)” (Chamber of Commerce Vienna) for funding.

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