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Rhizobial Nodulation Factors Stimulate Mycorrhizal Colonization of Nodulating and Nonnodulating Soybeans[1]

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Legumes form tripartite symbiotic associations with nodule-inducing rhizobia and vesicular-arbuscular mycorrhizal fungi. Co-inoculation of soybean (*Glycine max* [L.] Merr.) roots with *Bradyrhizobium japonicum* 61-A-101 considerably enhanced colonization by the mycorrhizal fungus *Glomus mosseae*. A similar stimulatory effect on mycorrhizal colonization was also observed in nonnodulating soybean mutants when inoculated with *Bradyrhizobium japonicum* and in wild-type soybean plants when inoculated with ineffective rhizobial strains, indicating that a functional rhizobial symbiosis is not necessary for enhanced mycorrhiza formation. Inoculation with the mutant *Rhizobium* sp. NGRΔ*nodABC*, unable to produce nodulation (Nod) factors, did not show any effect on mycorrhiza. Highly purified Nod factors also increased the degree of mycorrhizal colonization. Nod factors from *Rhizobium* sp. NGR234 differed in their potential to promote fungal colonization. The acetylated factor NodNGR-V (MeFuc, Ac), added at concentrations as low as 10^{-9} M, was active, whereas the sulfated factor, NodNGR-V (MeFuc, S), was inactive. Several soybean flavonoids known to accumulate in response to the acetylated Nod factor showed a similar promoting effect on mycorrhiza. These results suggest that plant flavonoids mediate the Nod factor-induced stimulation of mycorrhizal colonization in soybean roots.

Legumes form tripartite symbiotic associations with nodule-inducing soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium* (Caetano-Anollés and Gresshoff, 1991; Hirsch, 1992) and with VAM fungi (Bonfante-Fasolo, 1987; Koide and Schreiner, 1992). Both the rhizobial and fungal microsymbionts improve the mineral nutrition of the host plant in exchange for assimilates provided by the latter. The nitrogenase enzyme of rhizobia fixes atmospheric nitrogen in the nodules (Thorneley, 1992), and fungal hyphae facilitate the uptake of ions, mainly phosphate, in mycorrhizal roots (Smith and Gianinazzi-Pearson, 1988). In most cases investigated, especially when both nitrogen and phosphate are limiting factors, VAM fungi and rhizobia appear to act synergistically since combined inoculation with mycorrhiza and rhizobia enhances plant growth and reproduction more than inoculation with either microsymbiont alone and also leads to a higher degree of host colonization by the two symbionts (Daft and El-Giahmi, 1974; Cluett and Boucher, 1983; Kawai and Yamamoto, 1986; Pacovsky et al., 1986; Chaturvedi and Singh, 1989). Nevertheless, VAM fungi and rhizobia in established nodules may compete for photosynthate (Harris et al., 1985). Moreover, antagonistic effects in the establishment of the symbiosis between the mycorrhizal fungus *Glomus* and *Bradyrhizobium* were reported for soybeans when one of the microsymbionts had colonized the root system prior to the other (Bethlenfalvay et al., 1985).

There are many similarities between rhizobial and VAM symbioses, which suggest common properties in interactions with plants. Both microsymbionts are surrounded in the established stage of the symbiosis by plant-derived membranes: the peribacteroid membranes in the infected nodule cells and the perihaustorial membranes around arbuscules in the mycorrhizal roots, respectively. These interfaces are characterized by symbiosis-specific proteins (Wyss et al., 1990a; Verma, 1992; Perotto et al., 1994).

Soybean mutants that are unable to form nodules (Nod^-) may be colonized by VAM fungi (Kawai and Yamamoto, 1986; Wyss et al., 1990b), although Nod^- mutants of other legumes sometimes fail to establish a mycorrhizal symbiosis (Duc et al., 1989; Bradbury et al., 1991), indicating that common elements of the infection process may exist in both associations. Flavonoids and isoflavonoids, metabolites of the phenylpropanoid pathway, are exuded by the roots of the host plant and appear to play a role as early signals for both microsymbionts. They increase germination of VAM spores or hyphal growth in vitro (Gianinazzi-Pearson et al., 1989; Tsai and Phillips, 1991; Bécard et al., 1992; Kape et al., 1992; Morandi et al., 1992) and facilitate colonization by the mycorrhizal symbiont in vivo (Nair et al., 1991; Siqueira et al., 1991). Flavonoids and isoflavonoids of legumes act in the interaction with rhizobia as specific inducers of bacterial nodulation genes, the so-called *nod* genes (Firmin et al., 1986; Peters et al., 1986; Redmond et al., 1986). These *nod*

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Abbreviations: Ac, acetate; ANOVA, analysis of variance; MeFuc, methylfucose; Nod factor, nodulation factor; VAM, vesicular-arbuscular mycorrhizal.

genes are involved in the synthesis of Nod factors, a family of lipo-oligosaccharide signal molecules, i.e. β-1,4-linked oligomers of *N*-acetylglucosamine with an *N*-linked fatty acid moiety on the nonreducing end. Nod factors stimulate various responses on host plants, such as root hair deformation, expression of early nodulin genes, mitosis in the root cortex, and the formation of nodule-like structures (Fisher and Long, 1992; Dénarié and Cullimore, 1993).

The intriguing similarities between the mycorrhizal and rhizobial symbiosis prompted us to investigate signaling between the plant and its symbionts in the tripartite interaction, focusing on the possible effect of the rhizobial Nod factors on colonization of soybean roots by the mycorrhizal fungus *Glomus mosseae*.

MATERIALS AND METHODS

Plant Material and Fungal Inoculum

Soybean seeds (*Glycine max* [L.] Merr.) cv Bragg and its nonnodulating mutants *nod*139 and *nod*49 (Mathews et al., 1987, 1989) were kindly provided by Dr. P.M. Gresshoff (Knoxville, TN). In experiments with rhizobia, inoculum of the mycorrhizal fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. Trappe from the host plant *Tagetes tenuifolia* was prepared as described previously (Vierheilig et al., 1993) and contained sporocarps, spores, hyphae, and small, infected root pieces. A similar inoculum of *G. mosseae* cultivated on *Plantago lanceolata* was used in experiments involving isolated Nod factors and flavonoids.

Plant Culture and Growth Conditions

Soybean seeds were surface sterilized by treatment with 30% (w/v) H_2O_2 (Fluka) for 20 min, followed by washing with sterilized tap water, and germinated in trays containing moistened, sterilized vermiculite. After 4 d, young seedlings were transferred to 150-mL pots (one seedling per pot) that contained mycorrhizal inoculum, sand, and loam in the ratio 1:1:1 (v/v/v). Sand and loam were autoclaved prior to use. Plants were cultivated in a growth chamber (14-h day at a photon flux of 300 μmol m^{-2} s^{-1} and 26°C, 10-h night at 20°C) and watered with sterilized tap water. Unless indicated otherwise, plants were inoculated with rhizobia after 1 week and harvested after 5 weeks of co-cultivation (in this work plant age is defined as days after transfer of the pregerminated seedlings to pots).

Inoculation with *Rhizobium* and *Bradyrhizobium* Strains

Bradyrhizobium japonicum strain 61-A-101 (Stripf and Werner, 1978), USDA110*spc*4 (Regensburger and Hennecke, 1983), USDA9 (Fischer et al., 1986), *Rhizobium* sp. NGR234(Rif^R) (Lewin et al., 1990), and *Rhizobium* sp. NGRΔ*nodABC* (Price et al., 1992) were used for the inoculation experiments. The bacterial cultures were grown to stationary phase in 20-E medium (Werner et al., 1975) at 27°C on a rotary shaker at 140 rpm. Where indicated, plants were inoculated with the *Rhizobium* and *Bradyrhizobium* cultures (5 mL/150-mL pot) or given a control treatment with 20-E medium. The ability of rhizobial strains to establish a functional symbiosis was tested as described previously, monitoring acetylene reduction as a measure of nitrogen fixation (Müller et al., 1994).

Application of Nod Factors and Flavonoids

Acetylated and sulfated Nod factors from *Rhizobium* sp. NGR234 were purified as described by Price et al. (1992). Stock solutions of the flavonoids apigenin (Fluka), coumestrol (Kodak), daidzein (Roth, Karlsruhe, Germany), and genistein (Sigma) were prepared in DMSO. Pots containing soybeans and mycorrhizal inoculum were watered daily with solutions of Nod factors and flavonoids (10 mL per pot) for 1 week. Controls in the experiments with flavonoids received DMSO at an appropriate dilution.

Staining of Roots and Estimation of Mycorrhizal Colonization

Roots were separated from harvested plants and boiled in 10% (w/v) KOH for 10 to 20 min, depending on the age of the roots. Then the roots were washed three times with distilled water and placed in 0.3 M HCl for 2 to 3 min. After the HCl was poured off, roots were stained for 10 to 12 min with trypan blue 0.1% (w/v) in 24% (v/v) glycerin, 31% (v/v) lactic acid, 30% (v/v) phenol, and 15% (v/v) H_2O. Mycorrhizal colonization was estimated according to the gridline intersection method (Giovannetti and Mosse, 1980). Data are given as percentages of root length infected.

Statistical Analysis

All data were analyzed using one- or two-way ANOVA (Zar, 1984) with five replicates for every treatment combination. Except for the time courses shown in Figure 1, experiments were repeated at least once, as detailed in the legends of tables and figures.

RESULTS

When wild-type soybean plants (cv Bragg) were inoculated with the mycorrhizal fungus *G. mosseae* alone, their root systems were progressively colonized. Colonization, as measured by the gridline intersection method, showed considerable variation from experiment to experiment, depending on the quality of the inoculum, but it reached values as high as 50% after 5 weeks. However, when soybean roots were co-inoculated with the mycorrhizal fungus *G. mosseae* and *B. japonicum* 61-A-101, the mycorrhizal colonization was further stimulated, reaching values as high as 80%. Three examples of such experiments are summarized in Table I. The degree of colonization by mycorrhiza was significantly higher ($P < 0.001$) in the presence than in the absence of bacteria in each experiment. There was no significant bacteria × experiment effect according to two-way ANOVA, indicating that the bacterial effect was consistent. At the time of harvest, plants inoculated with the mycorrhizal inoculum alone had no nodules, but all plants inoculated with *B. japonicum* had formed many effective nitrogen-fixing nodules, as assessed by their red coloration

Table I. *Mycorrhizal colonization of the soybean cv Bragg in the absence and presence of rhizobia*

Plants were inoculated with *G. mosseae* and 1 week later with or without *B. japonicum*, strain 61-A-101. Data represent means ± SE for five plants, harvested 5 weeks later. Different letters in the same row indicate significantly different values ($P < 0.001$).

Experiment	Degree of Mycorrhizal Colonization	
	Without *B. japonicum*	With *B. japonicum*
	% root length	
1	6 ± 1a	27 ± 5b
2	48 ± 6a	80 ± 4b
3	36 ± 4a	64 ± 4b

and by their ability to reduce acetylene to ethylene (data not shown).

To test whether nodules are required for the establishment of the fungal symbiosis, the nonnodulating soybean mutants *nod*49 and *nod*139 (Mathews et al., 1987, 1989) were examined. These mutants very rarely form nodules upon inoculation with *Bradyrhizobium* (at most one or two nodules in one out of five plants in the experiments described here), but they form VAM associations like their wild-type parent cv Bragg, as described previously (Wyss et al., 1990b). Interestingly, co-inoculation of these mutants with *G. mosseae* and *Bradyrhizobium* also led to a significant increase ($P < 0.001$) in mycorrhizal colonization (Table II). The degree of stimulation was similar in wild-type and mutant plants; there was no significant bacteria × cultivar effect according to two-way ANOVA. In a time-course experiment, mycorrhiza formation of the nonnodulating mutant *nod*139 was followed during a 5-week period in the presence or absence of rhizobia. In this experiment no nodules were formed in any of the experimental plants. Mycorrhizal colonization was stimulated in the presence of rhizobia (Fig. 1A). The time × rhizobium treatment interaction was significant [$P < 0.001$, two-way ANOVA, f ratio $(4,34) = 6.5$], demonstrating that the presence of rhizobia increased the rate of mycorrhizal colonization. These results show that rhizobia present in the rhizosphere stimulate VAM colonization independently of the host plant's ability to form nodules, and they indicate that the increase in mycorrhiza formation in the presence of rhizobia is due to either a higher infection rate or an accelerated growth of penetrated hyphae.

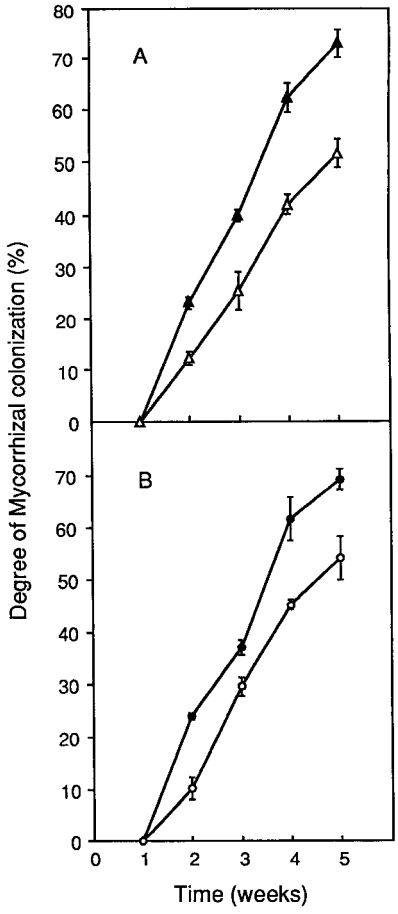

Figure 1. Time course of mycorrhizal root colonization by *G. mosseae* in the presence and absence of different rhizobia. A, One-week-old nonnodulating soybean *nod*139 were inoculated with *B. japonicum* 61-A-101 (▲) or mock treated with 20-E medium (△). B, *Rhizobium* sp. NGR234 (●) or its mutant deficient in Nod factor formation, strain NGRΔ*nodABC* (○). Rhizobial cultures were added to 1-week-old nonnodulating soybean mutant *nod*139. Data represent means ± SE, $n = 5$.

Table II. *Enhancement of mycorrhizal colonization induced by rhizobia in the soybean cv Bragg and in its nonnodulating mutants*

Plants were inoculated with *G. mosseae* and 1 week later with or without *B. japonicum*, strain 61-A-101. Data represent means ± SE for five plants, harvested 5 weeks later. Different letters in the same row indicate significantly different values ($P < 0.001$). This experiment was repeated once and yielded similar results.

Host Plant	Phenotype	Degree of Mycorrhizal Colonization	
		Without *B. japonicum*	With *B. japonicum*
		% root length	
cv Bragg	Nod^+, wild type	6 ± 1a	27 ± 5b
*nod*49	Nod^- mutant of cv Bragg	6 ± 3a	31 ± 4b
*nod*139	Nod^- mutant of cv Bragg	6 ± 4a	31 ± 5b

To find out whether the stimulatory effect is restricted to specific rhizobial strains able to enter into a functional symbiosis, we first tested *B. japonicum* strain USDA9, a strain lacking nitrogenase due to a deletion of the *nifA* gene. This strain induced nodule formation in soybean, but the nodules formed were much smaller, lacking red coloration and the ability to reduce acetylene, i.e. they had a Fix^- phenotype (data not shown). This strain caused the same promotion of mycorrhiza as our standard strain 61-A-101, yielding Fix^+ nodules (Table III, experiment 1). As expected, the parent strain of USDA9, *B. japonicum* USDA110*spc4*, induced Fix^+ nodules and increased mycorrhiza formation to a similar extent as the standard strain (Table III, experiment 2). Next, we tested the broad host-range strain *Rhizobium* sp. NGR234, which is able to form functional nodules on many different legumes but fails to undergo a functional symbiosis with soybean (Stanley and Cervantes, 1991). This strain indeed formed only small Fix^- nodules lacking acetylene reduction ability, in our experiments (data not shown). Nevertheless, *Rhizobium* sp. NGR234 was able to enhance mycorrhizal colonization to the same extent as the standard strain (Table III, experiments 1 and 2). In addition, we tested a nonnodulating (Nod^-) mutant derived from strain *Rhizobium* sp. NGR234, strain NGRΔ*nodABC*, which is deficient in Nod factor biosynthesis (Price et al., 1992). This strain did not induce nodule formation in our experiments, and it did not lead to any significant increase in mycorrhizal colonization as compared to controls without rhizobia (Table III, experiments 1 and 2). A time-course study showed that this difference was already apparent 1 week after inoculation of soybean roots with *Rhizobium* sp. NGR234 as compared to inoculation with the Nod factor-deficient mutant NGRΔ*nodABC* (Fig. 1B). The time × rhizobium strain interaction was significant [$P < 0.02$, two-way ANOVA, f ratio (4,34) = 3.5], showing that the rate of mycorrhizal colonization was higher in the presence of the Nod factor-forming wild-type strain.

It thus seems likely that rhizobial Nod factors may be responsible for the stimulatory effect on the fungus. To test this, purified Nod factors from *Rhizobium* sp. NGR234 were added to soybean roots inoculated with the VAM fungus *G. mosseae*. Two Nod factors differing in substitution of 2-*O*-methylfucose were used. The acetylated factor NodNGR-V (MeFuc, Ac) is characterized by an additional acetyl group, whereas NodNGR-V (MeFuc, S) possesses an additional sulfate group (Price et al., 1992). A stimulatory response on vesicular-arbuscular mycorrhiza was found after application of the acetylated factor NodNGR-V (MeFuc, Ac), even when added at concentrations as low as 10^{-9} M. Conversely, the sulfated Nod factor was ineffective in stimulating fungal colonization (Fig. 2). These results show that only specific Nod factors have the capacity to stimulate mycorrhiza formation.

It has been shown that the two NGR234 Nod factors, NodNGR-V (MeFuc, Ac) and NodNGR-V (MeFuc, S), differ in their ability to induce flavonoid secretion in soybean roots (Schmidt et al., 1994). Only the acetylated but not the sulfated Nod factor enhanced the secretion of daidzein, coumestrol, and genistein. To test the possibility that these flavonoids are involved in the stimulation of mycorrhiza formation, we examined directly the effect of these three flavonoids on fungal colonization. Apigenin was also included in the test, since it is known to be a good inducer of NGR234 Nod factors (Bassam et al., 1988; Price et al., 1992). Three of the flavonoids mentioned significantly stimulated fungal colonization (Table IV). The slight stimulation by genistein was not significant.

DISCUSSION

Here we show that mycorrhizal colonization of soybeans is not only enhanced in the presence of Nod factor-producing rhizobia but also by specific, exogenously applied Nod factors as well as by exogenously applied flavonoids. Stimulation of mycorrhiza formation by flavonoids and fungicides as well as by other microorganisms has been reported (Nair et al., 1991; Singh et al., 1991; Siqueira et al., 1991; von Alten et al., 1993). Synergistic effects of rhizobia on mycorrhiza were thought to be nutritional and restricted to the stage of nitrogen fixation. Our results demonstrate that rhizobia enhance mycorrhizal colonization before nodule formation and even on nonnodulating plants. Stimulation of mycorrhiza formation on the nonnodulating mutant

Table III. *Enhancement of mycorrhizal colonization induced by various rhizobia*

Soybean plants (cv Bragg) were inoculated with *G. mosseae* and 1 week later with culture medium only (control) or with various strains of rhizobia. Data represent means ± SE for five plants, harvested 5 weeks later. Different letters within an experiment indicate significantly different values [one-way ANOVA, $P < 0.002$, f ratio (4,20) = 12.0 in experiment 1, f ratio (4,19) = 22.7 in experiment 2].

Experiment	Bacterial Strain	Phenotype on Soybean	Degree of Colonization
			% root length
1	None (control)		36 ± 4a
	B. japonicum 61-A-101	Nod^+, Fix^+	64 ± 4b
	B. japonicum USDA9 (Δ*nifA*)	Nod^+, Fix^-	65 ± 4b
	Rhizobium sp. NGR234	Nod^+, Fix^-	77 ± 4b
	Rhizobium sp. NGRΔ*nodABC*	Nod^- mutant of NGR234	39 ± 3a
2	None (control)		48 ± 6a
	B. japonicum 61-A-101	Nod^+, Fix^+	80 ± 4b
	B. japonicum USDA110spc4	Nod^+, Fix^+	81 ± 2b
	Rhizobium sp. NGR234	Nod^+, Fix^-	73 ± 6b
	Rhizobium sp. NGRΔ*nodABC*	Nod^- mutant of NGR234	50 ± 7a

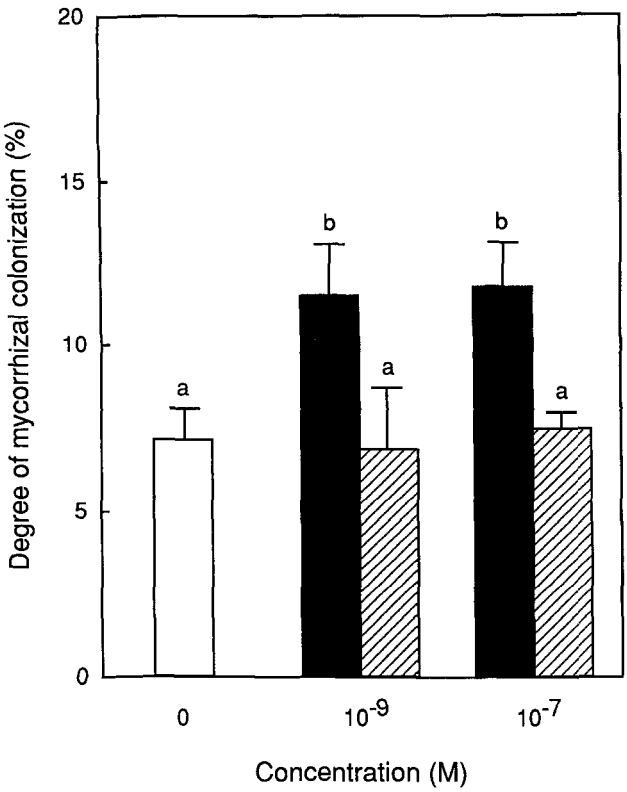

Figure 2. Effect of exogenously applied NodNGR factors on mycorrhizal colonization. Solutions containing 10^{-7} or 10^{-9} M NodNGR-V (MeFuc, Ac) (black bars), NodNGR-V (MeFuc, S) (hatched bars), or only water (open bar) were added to the nonnodulating soybean mutant *nod*139. The degree of mycorrhizal colonization by *G. mosseae* (mean ± SE, $n = 5$) was determined 7 d later. Different letters above bars denote significantly different values [$P < 0.03$, one-way ANOVA, f ratio (4,19) = 3.13]. This experiment was repeated once and yielded similar results.

nod49 by rhizobia is not unexpected, since this mutant shows some of the early responses to rhizobia, such as subepidermal cell divisions. More surprisingly, stimulation also occurs with the nonnodulating mutant *nod139*, which did not show any of the early responses to rhizobia (Caetano-Anollés and Gresshoff, 1990). Nod factors, the specific signal molecules of rhizobia, appear to be responsible for this stimulatory effect: all tested Nod factor-producing rhizobia, but not the mutant strain *Rhizobium* NGRΔ*nodABC*, enhanced the colonization of plants by VAM fungi. Moreover, exogenous application of specific Nod factors, such as NodNGR-V (MeFuc, Ac), clearly stimulated mycorrhizal colonization, whereas the structurally related sulfated Nod factor had no effect.

It remains an open question about how Nod factors increase fungal colonization. Although it cannot be excluded that Nod factors act directly as fungal growth regulators, it is more likely that active Nod factors trigger the colonization and development of VAM fungi via the so-called increased *nod* gene induction response (van Brussel et al., 1986). In this regulatory circuit, rhizobia as well as isolated rhizobial Nod factors induce the roots of host plants to secrete flavonoids that in turn further induce the rhizobial *nod* genes (Recourt et al., 1991, 1992; Dakora et al., 1993). This is also true for soybean roots that secrete compounds such as coumestrol, daidzein, and genistein upon incubation with Nod factors (Schmidt et al., 1994). Our observations show indeed that not only Nod factors but also flavonoids promote mycorrhiza formation. Stimulation begins during the very early stages of fungal root colonization. Based on these results, we postulate that VAM fungi and rhizobia may have evolved functionally similar recognition systems for plant flavonoids.

Our data suggest a general role of Nod factors or Nod factor-mediated plant responses in the establishment of symbiotic associations. It is tempting to speculate that Nod factors mimic related unknown signal molecules of VAM fungi in our system. In view of the highly sensitive perception systems present in plants for chitin oligomers as well as Nod factors (Staehelin et al., 1994), modified chitin oligomers released from the fungal cell wall might be good candidates for signals in the infection process of the VAM symbiosis.

Table IV. *Effect of various flavonoids on mycorrhizal colonization of soybean*

Each plant was watered daily with 10 mL of the indicated flavonoid and harvested after 1 week. Data represent means ± SE for five plants. Different letters indicate significantly different values [$P < 0.005$, one-way ANOVA, f ratio (4,19) = 6.1]. This experiment was repeated once and yielded similar results.

Flavonoid (10^{-7} M)	Degree of Mycorrhizal Colonization
	% root length
None	7.2 ± 0.9a
Apigenin	13.0 ± 0.9b
Coumestrol	14.0 ± 1.0b
Daidzein	15.4 ± 1.0b
Genistein	10.4 ± 1.7a,b

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