Nodulation and nitrogen fixation in interspecies grafts of soybean and common bean is controlled by isoflavonoid signal molecules translocated from shoot

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ABSTRACT

Identification of common signals of nodulation control among legume species will facilitate progress in enhancing symbiotic nitrogen fixation of legumes in sustainable agriculture system. Grafting experiments between soybean and common bean were carried out to evaluate whether a common shoot signals control the expression of hypernodulation among the tow species. Grafting of a hypernodulating soybean mutant NOD1-3 shoots to three cultivars of normally nodulating common bean roots resulted in hypernodulation on roots of three tested cultivars of common bean. The shoot control of hypernodulation may be causally related to differential root isoflavonoid levels, which are also controlled by shoot factors. Isoflavonoid analysis from root extracts of grafted plants showed that NOD1-3 shoots had markedly higher root isoflavonoid concentrations in roots of both NOD1-3 and common bean cv. Adzuki compared with self-grafts of common bean Adzuki. Exogenous application of daidzein, genistein, coumestrol, glycitein and in combination at concentration of 10 μmol to the nutrient solution significantly increased the nodule numbers of common bean cv. Adzuki. Therefore, the control of hypernodulation expression by isoflavonoid signal molecules translocated from shoot is common among legume species.

Keywords: *Bradyrhizobium japonicum*; grafting; hypernodulation; isoflavonoids; N_2 fixation; *Rhizobium leguminosarum* bv. *phaseoli*

The common bean (*Phaseolus vulgaris* L.) is an agriculturally important legume crop which benefits from symbiosis with bacteria belonging to genus *Rhizobium* and is one of the most important food legume worldwide in both economic and nutritional aspects (Broughton et al. 2003).

The economic and environmental importance of legume crops is largely due to their ability to fix atmospheric dinitrogen in symbiosis with rhizobia. Although it is obvious that legumes help build soil fertility, their nitrogen fixation has other advantages in agro-ecosystem, including improved soil structure, deep rooting, erosion protection and contributing to greater biological activity and sustainability (Giller and Cadisch 1995).

Various reports indicate Egyptian soil do not contain indigenous *Bradyrhizobium* and *Rhizobium leguminosarium* biovar *phaseoli* (Hamdi et al. 1974, Abd-Alla 1999, Moawad et al. 2004). Consequently proper inoculation is required for enhancing legume nodulation and nitrogen fixation (Rengel 2002). Despite the widespread belief that beans are poor fixers of nitrogen a wide evolution of plant genotypes and *Rhizobium* strains showed that some accession are able to fix up to 100 kg N/ha (Hardarson and Atkins 2003).

Grafting techniques are used to evaluate whether the shoot or the root exercises control over a number of physiological process (Bezdicek et al. 1972). With respect to nodulation, it has been shown that supernodulation or hypernodulation of soybean is primarily controlled by shoot. Yet, there is indication that the shoot control may be modified by the root (Abd-Alla 1999). Hamaguchi et al. 1993, however, reported that the mechanism controlling nodulation or substances involved in autoregulation are different between soybean and common bean. Thus no firm conclusion can

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be drawn on whether shoot signal exists among legume species which impacts autoregulatory control of nodule number.

The current study was carried out to evaluate the impact of shoot and root on the nodulation process of self- and reciprocal-grafts between hypernodulating soybean and three cultivars of common bean inoculated with *Bradyrhizobium japonicum* and USDA 123 and *Rhizobium leguminosarum* biovar *phaseoli* RCR 3622 and TAL 1383, respectively. The objective was to determine if shoot control related to isoflavonoid levels, and to examine the effect of exogenous application of isoflavonoid compounds on nodulation of common bean cv. Adzuki inoculated with *Rhizobium leguminosarum* biovar *phaseoli* RCR 3622.

MATERIAL AND METHODS

Three normally nodulating common bean (*Phaseolus vulgaris* L.) cultivars (Widusa, Cannellini and Adzuki) and one hypernodulating soybean (*Glycine max* L. Merr.) mutant NOD1-3 were used. Two strains of *Rhizobium leguminosarum* biovar *phaseoli* strains (RCR 3622 or TAL 1383) and *Bradyrhizobium japonicum* strain USDA 123 were used to nodulate common bean cultivars and soybean, respectively. Germination and subsequent growth were carried out as described by Abd-Alla and Harper (1996). Four weeks after transplanting, the plant were detached from shoots and placed in 500 mL jar, injected with 50 mL acetylene through a rubber septum into the lid and incubation at 30°C for 30 min. Following incubation, 0.5 mL sample was analysed for ethylene (nitrogenase activity) by flam ionization gas chromatography Thermo Scientific TRACE GC Ultra (Rodano, Milan, Italy) equipped with FID detector and Capillary column CP-PoraBOND U fused silica plot 25 m × 0.32 mm, *df* = 7 μm. Afterwards nodules of each individual root were counted and nodule fresh mass were measured.

Grafting experiment. This experiment was carried out to determine whether the shoot of soybean NOD1-3 is involved in generation of signals that affects on nodulation of common bean cultivars. Surface-sterilized seeds were germinated as described above. Germination and subsequent growth were in growth chamber programmed for a 14-h photoperiod at 28°C, and a 10-h dark period at 20°C. Common bean seeds were planted one day later than soybean seed to synchronize germination for grafting purpose. Seedlings were grafted 6 days after planting soybean, when unifoliolate leaves were just emerging from between the cotyledons. Graft combinations include self-grafted soybean and common bean and soybean NOD1-3 shoot grafted to common bean cultivars root as described by Bezdicek et al. (1972). Vigorous seedlings with visible, unifoliate leaves were selected for grafting. Hypocotyls were severed with a razor blade at the mid-point between cotyledons and the sand surface. The upper hypocotyl section with cotyledons was then cut at an opposite side to form a V-shaped stem base. The lower hypocotyl section with roots was vertically split with a razor blade about 1 cm deep and V-shaped stem was inserted into the root slit. The graft was held in place by a 1-cm section of plastic drinking straw (2.5 mm), silt down one side to allow expansion as seedling grew. Grafted seedling were maintained in the germination sand tray for 5 days, covered by a transparent lid to maintain high humidity and increase survival rate of grafted seedlings as reported by Abd-Alla and Harper (1996). The seedlings were watered with 0.5 mmol $CaSO_4$ as needed. Five days after grafting, the graft union was sufficiently strong to allow transfer of plants. Seedlings were then transplanted to 2 L polyethylene plastic pots containing a modified, minus-nitrogen Hoagland nutrient solution (Gremaud and Harper 1989). Each pot contained six plants and was inoculated with 10 mL of *Rhizobium leguminosarum* biovar *phaseoli* strain RCR 3622 and TAL 1383 (10^7 cells/mL). Twenty-eight days after transplanting, plants were harvested and the plant roots were detached from shoots. Nitrogenase activity, nodule number and nodule mass were determined.

Determination of isoflavonoid compounds. Self and interspecies graft combination of soybean and common bean cv. Adzuki were analyzed for root isoflavonoid compounds after 12 days of grafting using UV-Visible detectors Perkin Elmer HPLC system (San Jose, California, USA). Isoflavonoid compounds were separated with mobile phase A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile) in the C18 column and the peaks were detected by UV detection at 260 nm. The flow rate was set at 0.8 mL/min. The peaks of separated isoflavones were compared with those in standard curve (daidzein, geneistein, coumestrol and glycitein).

Exogenous application of isoflavonoid compounds. This experiment was done to determine whether exogenous application of daidzein, genistein, coumestrol, glycitein, and combination of these compounds increased nodulation of common bean cv. Adzuki. Germination and subsequent growth were in growth chamber programmed as described by (Abd-Alla and Harper 1996). Each pot contained 4 plants and was inoculated at transplantation with 10 mL (10^7 cell/mL) of *Rhizobium leguminosarum* biovar *phaseoli* RCR 3622. Pots were grouped to four categories according to the type of isoflavonoid compounds applied. Daidzein, genistein, coumestrol, glycitein and mixture of these isoflavonoid compounds were applied at 10 μmol to the nutrient solution at transplantation and continued for 7-day growth period. Pots that did not receive any isoflavonoid compounds (0 μmol) were used as control. Twenty-eight days after transplanting and treatments, plants were harvested and nodule number was determined. Daidzein, genistein, coumestrol, glycitein, and mixture of daidzein, genistein, coumestrol and glycitein were applied at 10 μmol to yeast extract mannitol broth medium. Flasks containing 25 mL of medium were inoculated with 1 mL of *Rhizobium leguminosarum* biovar *phaseoli* RCR 3622 (10^7 cells/mL) and incubated on an orbital shaker at 120 rev/min at 28°C. The growth response of bacterial strains was assessed by following the optical density at 540 nm.

Statistical analysis. The experimental design was a randomized complete block with a factorial arrangement of treatments with three replications. Experimental data were subjected to analysis of variance using PC-state computer software version 1A (copyright 1985, University of Georgia). Means were compared using *LSD* test when significant *F*-test occurred.

RESULTS

Nodulation and nitrogenase activity of selected cultivars. The hypernodulating soybean mutant (NOD1-3) produced significantly more nodules and higher absolute nitrogenase activity when inoculated with USDA123 than the commercial cultivars of common bean when inoculated either with compatible strain USDA 123 or RCR 3409 (Table 1).

Grafting observations. Data of Tables 2 and 3 show the nodulation characteristic of reciprocal- and self-grafted plants between the soybean NOD1-3 hypernodulating mutant and commercial cultivars of common bean. NOD1-3 shoots induced hypernodulation when grafted onto roots of three tested cultivars. Grafting of soybean NOD1-3 shoot to common bean cultivar also enhanced nodule mass and acetylene reduction activities compared with the self-grafted common bean plants. *Rhizobium leguminosarum* biovar *phaseoli* RCR 3622 produced slightly more nodules and had a higher nitrogenase activity than strain RCR 3409 on all tested cultivars.

Isoflavonoid concentrations. When hypernodulating mutant NOD1-3 shoots were grafted onto roots of common bean cv. Adzuki, higher root isoflavonoids concentrations were observed 12 days after inoculation and grafting, compared with self-grafted Adzuki plants (Figure 1). Self-grafts of NOD1-3 had even higher isoflavonoid concentrations than did reciprocal grafts of hypernodulating mutants NOD1-3 shoots onto Adzuki roots.

Effects of isoflavonoid compounds on nodulation and growth of *Rhizobium leguminosarum* biovar *phaseoli* strain RCR 3622. Exogenous application of daidzein, genistein, coumestrol, glycitein, and in combination at concentration of 10 μmol to the nutrient solution significantly increased the nodule numbers of common bean cv. Adzuki (Figure 2). Combination of isoflavonoid compounds (daidzein, genistein, coumestrol, and glycitein) had more stimulatory effects on

Table 1. Nodulation and nitrogenase activity of soybean NOD1-3 inoculated with *Bradyrhizobium japonicum* USDA 123 and common bean cultivars inoculated with *Rhizobium leguminosarum* biovar *phaseoli* strain RCR 3622 and TAL 1383

Plant species	Bacterial strain	Nodule number/plant	μmol C_2H_4/plant/h
Soybean NOD1-3	USDA123	472[a]	3.61[a]
Common bean cv.			
Adzuki	RCR 3622	19[b]	0.92[b]
	TAL 1384	15[b]	0.71[b]
Cannellini	RCR 3622	19[b]	0.56[bc]
	TAL 1384	14[b]	0.49[c]
Widusa	RCR 3622	17[b]	0.59[c]
	TAL 1384	15[b]	0.54[c]

Means followed by the same letter in each column were not significantly different at the 5% level using *LSD* test

Table 2. Nodulation response of self- and interspecies-graft combinations of soybean NOD1-3 and common bean cultivars inoculated with *Rhizobium eguminosarum* biovar *phaseoli* strain RCR 3622

Graft* Shoot/root	Bacterial strain	Nodule number/plant	Nodule mass (mg/plant)	µmol C_2H_4/plant/h
NOD1-3/NOD1-3	USDA 123	446[a]	179[a]	3.49[a]
CBA/CBA	RCR 3622	16[d]	13[d]	1.63[b]
CBC/CBC	RCR 3622	12[d]	9[d]	1.37[b]
CBW/CBW	RCR 3622	11[d]	9[d]	1.14[b]
NOD1-3/CBA	RCR 3622	410[b]	163[b]	3.26[a]
NOD1-3/CBC	RCR 3622	388[c]	154[c]	3.28[a]
NOD1-3/CBW	RCR 3622	385[c]	153[c]	3.06[a]

*Combinations involved self grafts of hypernodulating mutant (NOD1-3) and common bean cvs. Adzuki (CBA), Cannellini (CBC) and Widusa (CBW), and Soybean NOD1-3 shoot grafted to common bean cultivars roots. Means followed by the same letter in each column were not significantly different at the 5% level using *LSD* test

nodulation than applied each alone. Addition of isoflavonoid compounds (daidzein, genistein, coumestrol, and glycitein) each alone or in combination at concentration of 10 µmol to YMB significantly increased the growth of *Rhizobium leguminosarum* biovar *phaseoli* strain RCR 3622 (Figure 3). All of isoflavonoid compounds tested were effective on nodulation and rhizobial growth. Combination of isoflavonoid compounds (daidzein, geneistein, coumestrol, and glycitein) had more stimulatory effects followed by daidzein, geneistein, coumestrol and glycitein in descending order as to inducer effectiveness.

DISCUSSION

The current observations, that grafting of hypernodulated soybean shoots to root of normally nodulated common bean cultivars results in enhanced nodulation levels, support the hypothesis of nodule number in legumes via common translocatable signal (Harper et al. 1997). However, arguing against this conclusion is the study by Hamaguchi et al. (1993) in which it was concluded that grafted supernodulating soybean did not alter nodulation of common bean, and vice versa, using normally nodulating and supernodulating line of each species. Results of current study come in agreement with the observations of Harper et al. (1997) that expression of supernodulation in common bean, like that in soybean is known to be controlled by the shoot. Ito et al. (2008) reported that the autoregulation of nodule formation signaling might be related to the control system of leaf-cell proliferation. Isoflavonoid analysis from root extracts of grafted plants showed that NOD1-3 shoots had markedly higher root isoflavonoid concentrations in roots of both NOD1-3 and common bean cv. Adzuki compared with self-grafts of common bean Adzuki (Figure 1). These observations are consistent with the hypothesis that root isoflavonoid levels may be related to differential nodulation expression by a mechanism that is unrelated to the

Table 3. Nodulation response of self- and interspecies-graft combinations of soybean NOD1-3 inoculated with *Bradyrhizobium japonicum* USDA 123 and common bean cultivars inoculated with *Rhizobium leguminosarum* biovar *phaseoli* strain TAL 1384

Graft* Shoot/root	Bacterial strain	Nodule number/plant	Nodule mass (mg/plant)	µmol C_2H_4/plant/h
NOD1-3/NOD1-3	USDA 123	446[a]	179[a]	3.49[a]
CBA/CBA	TAL 1384	13[d]	10[d]	1.52[b]
CBC/CBC	TAL 1384	8[d]	8[d]	1.40[e]
CBW/CBW	TAL 1384	6[d]	9[d]	1.00[f]
NOD1-3/CBA	TAL 1384	390[b]	158[bc]	2.98[b]
NOD1-3/CBC	TAL 1384	350[c]	150[c]	2.78[c]
NOD1-3/CBW	TAL 1384	340[c]	148[c]	2.70[c]

*See Table 2

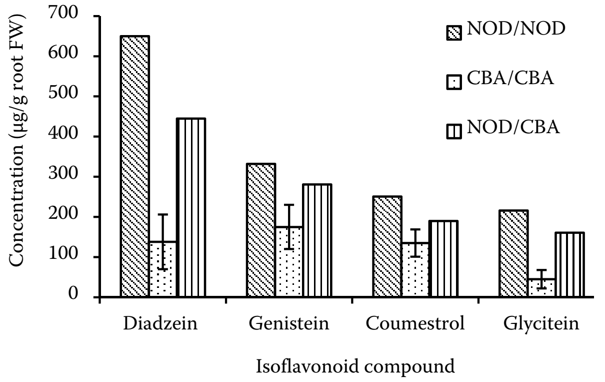

Figure 1. Isoflavonoid concentration in root extracts from self- and reciprocal-grafted between soybean NOD1-3 and common bean cv. Adzuki. Values represent means of three replicates for each grafting combination within an isoflavonoid compound and vertical bars are *LSD* at 0.05 level

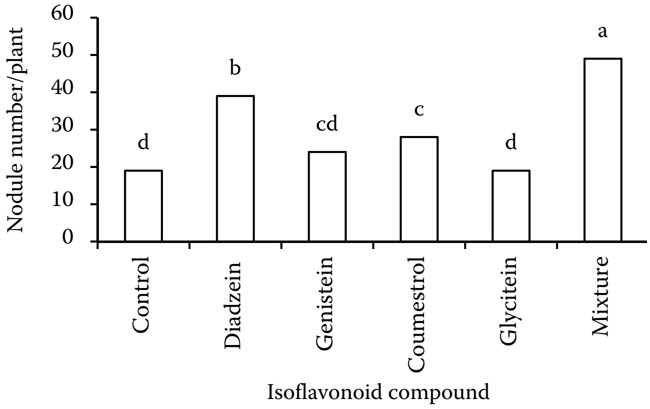

Figure 2. Effect of isoflavonoid compound application on nodule number of common bean cv. Adzuki inoculated with *Rhizobium leguminosarum* biovar *phaseoli* strain RCR 3622. Values represent means of three replicates for each isoflavonoid compound. Means with the same letter are not significantly different among isoflavonoid compound treatments at the 0.05 level using an *LSD* test

previously reported involvement of isoflavonoids on nod gene induction (Kosslak et al. 1987).

Isoflavonoids application to the nutrient solution significantly increased nodulation of common bean cv. Adzuki. Combination of isoflavonoid compounds (daidzein, genistein, coumestrol and glycitein) had more synergistic effects on nodulation than applied as single compound (Figure 2). These isoflavonoid compounds significantly increased the growth of *Rhizobium leguminosarum* biovar *phaseoli* strain RCR 3622. Addition of genistein to growth medium of *Bradyrhizobium japonicum* or soybean seeds significantly increased nodule formation and nitrogen fixation of soybean (Pan and Smith 2000). There are two possible explanations for the stimulatory effect of isoflavonoid compounds: (1) common bean cv. Adzuki plants are less able to synthesize signal molecules of isoflavonoid (Figure 1); (2) common bean plants are less able to excrete signal molecules.

A precise exchange of molecular signals between rhizobia and host plants is essential to the development of an effective legume root nodules (Novák et al. 2002). Flavonoids play an important role as signal molecules in the early stages of the legume-*Rhizobium* symbiosis. The isoflavones daidzein, coumestrol, naringenin, genistein, liquiritigenin, and isoliquiritigenin are the major components of *Phaseolus vulgaris* cv. Rab39 extract responsible for inducing expression of the nod genes of *Rhizobium tropici*, *R. etli*, and *R. leguminosarum* biovar *phaseoli* (Bolaños-Vásquez and Werner 1997). Long (2001) reported that each legume produces a distinct cocktail of flavonoids and that the quantity and spectrum of compounds may vary with the age and physiological state of the plant. In the early steps of symbiosis, a diverse array of compounds is exuded into the rhizosphere, including flavonoids and isoflavonoids. These compounds are chemoattractants for rhizobia (Caetano-Anolle's et al. 1992), influence bacterial growth, and induce the expression of nodulation genes (Peters et al. 1986, Hungria and Stacey 1997). This process is mediated by the regulatory gene, NOD, and it leads to synthesis of nodulation factors by the bacterial symbiont. These return signals elicit root hair deformation, cortical cell division, and sometimes nodule formation by the legume

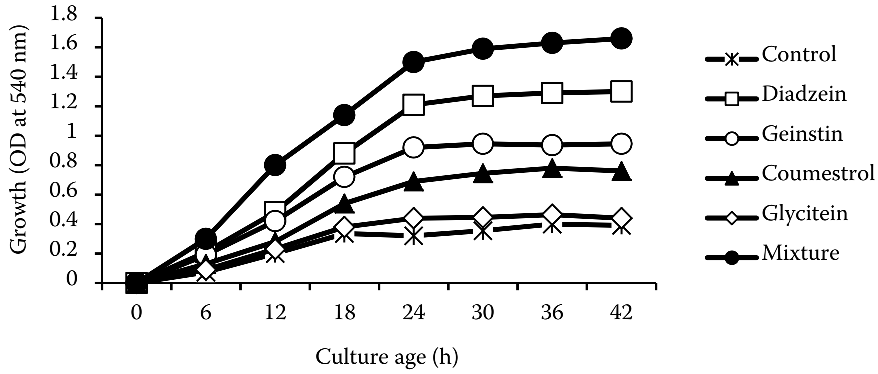

Figure 3. Effect of isoflavonoid compound addition on growth of *Rhizobium leguminosarum* biovar *phaseoli* strain RCR 3622

(Hirsch et al. 2001). The results have shown here support the previous conclusion (Gresshoff and Delves 1986) that the hypernodulation mutants lack some chemical signals which, when present, provide autoregulatory control over nodule number. It was concluded that hypernodulation expression by chemical signals of shoot is common among legumes species.

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