Research Notes

Common Bean Root Exudates Contain Elevated Levels of Daidzein and Coumestrol in Response to *Rhizobium* Inoculation

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Received 1 March 1993. Accepted 11 June 1993.

Root exudate of common bean (*Phaseolus vulgaris*) inoculated with symbiotic *Rhizobium leguminosarum* bv. *phaseoli* bacteria contained more of the phytoalexin coumestrol and its isoflavonoid precursor daidzein than did exudates of uninoculated plants. Both compounds induced transcription of a *nodC::lacZ* fusion in *R. l.* bv. *phaseoli*. Because coumestrol is elicited in bean by pathogens, these results indicate that parallel biochemical changes occur when this legume responds to symbiotic and pathogenic microbes.

Pathogenic microbes often elicit synthesis of isolavonoid phytoalexins in legumes (Dixon and Lamb 1990). These compounds slow or stop pathogen growth, and they generally are viewed as one mechanism by which plants control undesirable bacteria and fungi. It is well established that soybean root nodules formed by *Bradyrhizobium japonicum* contain glyceollin, an isoflavonoid phytoalexin (Parniske *et al.* 1990), but little is known about the extent to which other symbiotic rhizobia elicit phytoalexin synthesis (Djordjevic *et al.* 1987). While it is recognized that legumes treated with rhizobia release increased amounts of *nod*-gene-inducing flavonoids (Rolfe *et al.* 1988; van Brussel *et al.* 1990; Recourt *et al.* 1991), it was only recently demonstrated that a legume (alfalfa) inoculated with *Rhizobium* can *exude* a phytoalexin (medicarpin) (Dakora *et al.* 1993). That observation directly differs with data from *Vicia,* which showed that symbiotic rhizobia stimulated the release of other flavonoids, but not phytoalexins (Recourt *et al.* 1991). The purpose of this study was to obtain an initial indication of which response may be more typical of *Rhizobium*–legume interactions. Having previously identified flavonoid *nod*-gene inducers released by *Phaseolus vulgaris* in the absence of *Rhizobium* (Hungria *et al.* 1991b), we therefore analyzed root exudates of bean grown with or without *R. leguminosarum* bv. *phaseoli.*

Bean (*Phaseolus vulgaris* L. 'Black Turtle Soup') seeds were surface-sterilized, germinated, and grown hydroponically under controlled conditions (Hungria *et al.* 1991a). *R. l.* bv. *phaseoli* strain 4292 was cultured in YMA media (Vincent 1970), and rinsed cells were inoculated into N-free solution (Maxwell *et al.* 1989) surrounding roots of 5-day-old seedlings to produce suspensions with $A_{600} \approx 0.020$. Uninoculated controls received sterile nutrient solution. The solution bathing roots, termed root exudate, was collected 5 days later (10 days after imbibition) and frozen at –80° C.

The *nod*-gene-inducing capacity of root exudate and specific compounds was assayed as β-galactosidase activity (Miller 1972) transcribed from a *nodC::lacZ* fusion under the control of *nodD1* gene in *R. l.* bv. *phaseoli* 4292pIJ1737pIJ1730 (Davis and Johnston 1990) using methods described previously (Hungria *et al.* 1991a).

Root exudate was centrifuged at 6,200 *g* for 15 min, and passed through 0.8- and 0.2-μm polycarbonate filters (Nuclepore Corp., Pleasanton, CA). Subsamples (50 ml) were adsorbed to 900-mg C_{18} Maxi-Clean cartridges (Alltech Associates, Inc., Deerfield, IL). Flavonoids from each cartridge were eluted with 50, 80, and 100% methanol (MeOH), combined, lyophilized, and solubilized in 50% MeOH for high-performance liquid chromatography (HPLC) analysis. HPLC separations were done on a 250 × 4.6 mm Lichrosorb 5RP18 column (Phenomenex, Rancho Palos Verdes, CA), which was rinsed at rate of 0.5 $ml \cdot min^{-1}$ from 0 to 75 min with a linear gradient (0:99:1 to 99:0:1, v:v:v, MeOH/water/acetic acid) and isocratically from 75 to 90 min with 99:1 MeOH/acetic acid. Larger-scale purifications were done on a 250 × 10 mm Lichrosorb 5RP18 semi-preparative column (Alltech Associates, Inc.) at a flow rate of 2 $ml \cdot min^{-1}$. All eluates were monitored initially for absorbance at 200–560 nm with a photodiode array detector. Closely eluting fractions were purified by additional HPLC separations using appropriate MeOH concentrations.

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MPMI, Vol. 6., No. 5, 1993, pp. 665-668
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UV-visible spectral shift analyses (Mabry *et al.* 1970) were made with a Lambda 6 dual beam spectrophotometer (Perkin Elmer, Norwalk, CT). ^{1}H-NMR experiments were done in CD_3OD on a GN-300 Omega NMR spectrometer (General Electric Co., Fremont, CA), and spectra were referenced to tetramethylsilane. Fast atom bombardment (FAB) MS data were collected with a ZAB-HS-2F MS (VG Analytical, Wythenshawe, UK) using positive ionization (xenon, 8 keV, 1 mA). Samples were dissolved in MeOH and injected with continuous flow in 95:5 (v:v) water/glycerol at a rate of 5 $\mu l \cdot min^{-1}$.

Inoculating roots of 5-day-old bean seedlings with *R. l.* bv. *phaseoli* doubled total *nod*-gene-inducing activity of root exudates within 5 days. At that point, roots of the 10-day-old inoculated seedlings exuded 3,960 ± 387 β-galactosidase units per plant per day, while the comparable value for uninoculated controls was 1,520 ± 95.

HPLC chromatograms of the flavonoid fraction from root exudates of plants exposed to *R. l.* bv. *phaseoli* were generally similar to samples from uninoculated plants, except for a major enlargement of peak 5 and moderate increases in peaks 1–4 and 6 (Fig. 1). Assays containing 5% of peaks 1–5 induced *nod*-gene transcription in *R. l.* bv. *phaseoli* and gave values of 96, 63, 205, 361, and 389 β-galactosidase units, respectively. In tests with higher concentrations, 44 β-galactosidase units were detected with 20% of peak 6. Because only peaks 1, 4, 5, and 6 showed reproducible increases after inoculating with *Rhizobium* in other experiments, further studies were restricted to those compounds.

On the basis of UV-visible spectra, HPLC retention times, and *nod*-gene-inducing activities, peaks 4 and 1 were identified, respectively, as genistein aglycone (4′,-5,7-trihydroxyisoflavone) and the genistein glycoside reported previously in root exudates of uninoculated bean (Hungria *et al.* 1991b).

Absorbance measurements of peak 5 produced a UV-visible spectrum in MeOH with A_{max} at 202, 249, 301 nm, similar to daidzein (Mabry *et al.* 1970). The compound purified from peak 5 had proton resonances (δ_H ppm) in CD_3OD at 8.14 (1H, s, H-2), 8.05 (1H, *d*, J = 8.6 Hz, H-5), 7.37 (2H, *d*, J = 8.6 Hz, H-2′,6′), 6.92 (1H, *dd*, J = 2.4, 8.6 Hz, H-6), and 6.85 (3H, *m*, H-8,3′,5′). FAB-MS analyses produced a major ion at m/z = 255, which was identical to the MH^+ value observed for authentic daidzein. Retention time for peak 5 on the standard HPLC gradient also was identical to that of authentic daidzein.

Absorbance measurements of peak 6 produced a UV-visible spectrum in MeOH with A_{max} at 240, 265sh, 301, 342, 356sh nm, that closely matched authentic coumestrol: 243, 264sh, 303, 342, 356sh nm. The

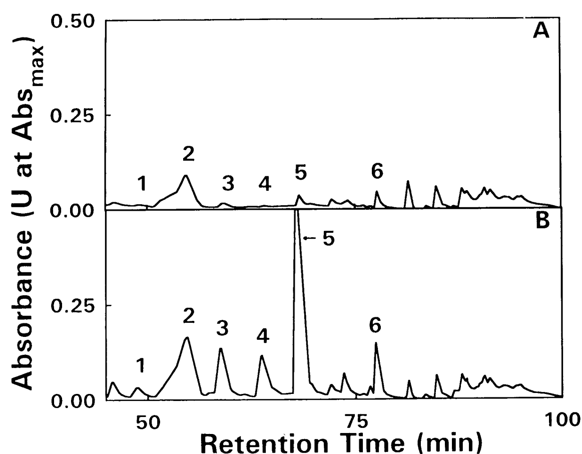

Fig. 1. HPLC characteristics of the flavonoid fraction from root exudate (50 ml) of 10-day-old bean seedlings collected from **A,** uninoculated plants or **B,** plants inoculated with *Rhizobium leguminorsarum* bv. *phaseoli*.

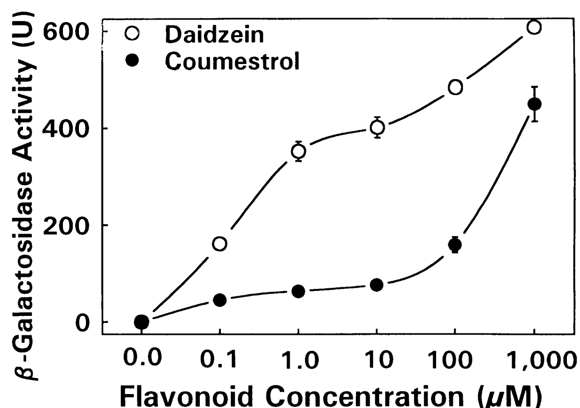

Fig. 2. Induction of *nod* genes in *Rhizobium leguminosarum* bv. *phaseoli* by daidzein and coumestrol. Authentic standards of compounds identified in peaks 5 and 6 (Fig. 1) were assayed in *R. l.* bv. *phaseoli* strain 4292pIJ1737pIJ1730. Mean values ± standard error from three replicates are reported for each concentration.

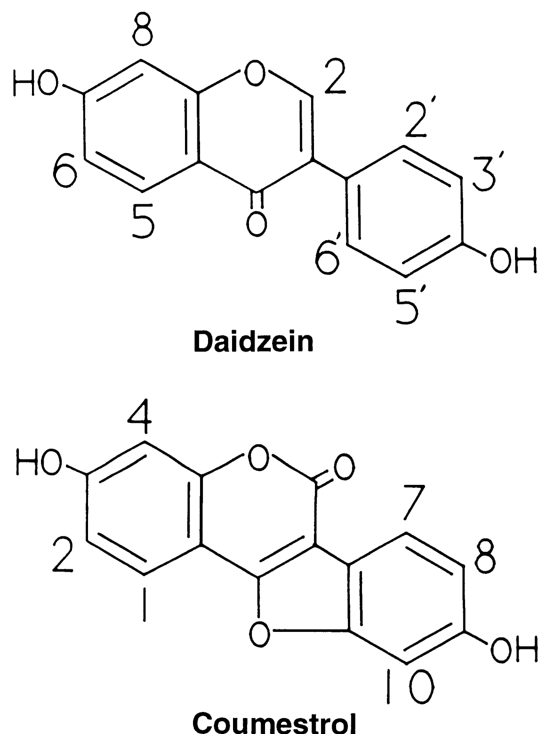

Fig. 3. Structure of daidzein and coumestrol identified in peaks 5 and 6 (Fig. 1).

compound purified from peak 6 had proton resonances (δ_H ppm) in CD_3OD at 7.83 (1H, *d*, J = 8.5 Hz, H-7), 7.75 (1H, *d*, J = 8.5 Hz, H-1), 7.10 (1H, *d*, J = 2.4 Hz, H-10), 7.02 (1H, *d*, J = 8.5 Hz, H-8), 6.94 (1H, *d*, J = 2.4 Hz, H-4), and 6.89 (1H, *dd*, J = 8.5, 2.4 Hz, H-2), which matched authentic coumestrol and were consistent with previously reported values (Zilg and Grisebach 1968; Le-Van 1984). Retention time for peak 6 on the standard HPLC gradient matched that of authentic coumestrol.

Tests with authentic daidzein and coumestrol showed that both compounds induced transcription of the *nodC::lacZ* fusion in *R. l.* bv. *phaseoli* strain 4292pIJ1737pIJ1730 (Fig. 2). On the basis of these biological and chemical data, it was concluded that *nod*-gene inducers in peaks 5 and 6 were daidzein (4′,7-dihydroxyisoflavone) and coumestrol, respectively (Fig. 3).

Inoculating bean roots with an infective strain of *R. l.* bv. *phaseoli* markedly increased total *nod*-gene-inducing activity of root exudate due primarily to increases in three isoflavonoids: a genistein glycoside, genistein, and daidzein (Fig. 1; peaks 1, 4, and 5). The *Rhizobium*-dependent increase in total *nod*-gene-inducing activity was consistent with earlier reports in clover (Rolfe *et al.* 1988), vetch (van Brussel *et al.* 1990), and alfalfa (Dakora *et al.* 1993). Bean typically has determinate root nodules while clover, vetch, and alfalfa all have indeterminate nodules. Thus the "Ini response" following rhizobial inoculation (van Brussel *et al.* 1990) occurs in legumes with both of these nodulation characteristics. Previous studies showed that bean plants treated with fungal or bacterial elicitors contained the isoflavonoid phytoalexin coumestrol (Rathmell and Bendall 1971; Lyon and Wood 1975). Therefore, the demonstration in these experiments that coumestrol also appeared in root exudate after rhizobial inoculation indicates a parallel in bean response to pathogens and symbionts. This finding is consistent with a recent report that showed the phytoalexin medicarpin appears in alfalfa root exudate after inoculation with *R. meliloti* (Dakora *et al.* 1993). These results contrast with the *Vicia* response to *Rhizobium* where additional chalcones and flavanones, but not isoflavonoids or phytoalexins, were exuded (Recourt *et al.* 1991).

HPLC analyses of *nod*-gene inducers in root exudate from uninoculated bean in this study were generally consistent with previous findings (Hungria *et al.* 1991b), and slight variations apparently reflected cultivar effects. Daidzein and coumestrol differ from genistein, a previously known isoflavonoid inducer of *nod*-genes in *R. l.* bv. *phaseoli* by the absence of a 5-hydroxyl group (Fig. 3). Because *R. l.* bv. *phaseoli* shows little capacity for distinguishing among flavonoid structures (Hungria *et al.* 1992), it was not surprising that daidzein and coumestrol too induced *nod* genes in this bacterium. Coumestrol is a more planar molecule than either daidzein or genistein, and that may account for its lower *nod*-gene-inducing activity (Fig. 2). The unexpected, secondary increase in *nod*-gene-inducing activity of coumestrol at higher concentrations (Fig. 2) may reflect new interactions at the same binding site, or it may be another manifestation of unexplained additive effects of eriodictyol and genistein on *nod*-gene induction in this organism (Hungria *et al.* 1992). Daidzein and coumestrol are present in sterile soybean root exudate (d'Arcy-Lameta 1986), and both compounds induce *nod* genes in *Bradyrhizobium japonicum* (Kosslak *et al.* 1990). The *nod*-gene-inducing activity of coumestrol contrasts with the fact that the phytoalexin medicarpin, which is exuded by alfalfa roots exposed to *R. meliloti*, does not induce *nod* genes in that rhizobial symbiont (Dakora *et al.* 1993).

No role for phytoalexins has yet been established in the *Rhizobium*–legume symbiosis. The recent demonstration that many *R. meliloti* infection threads abort in alfalfa roots during normal root nodule development (Vasse *et al.* 1992) suggests that phytoalexins may affect rhizobia. However, at least one *R. meliloti* strain grows in the presence of medicarpin (Pankhurst and Briggs 1980), and *B. japonicum* develops resistance to glyceollin, a soybean phytoalexin (Parniske *et al.* 1991). *R. l.* bv. *viciae* cells that nodulate *V. faba* also can grow in the presence of wyerone, a common phytoalexin in that plant (Görge and Werner 1991). Whether wyerone is released from *Vicia* roots in response to rhizobia has not been reported, but recent data indicate that the fungal symbiont *Glomus* elicits wyerone in that plant (Kape *et al.* 1992). No available data indicate how *R. l.* bv. *phaseoli* responds to coumestrol, but several levels of regulation involved in these responses may separate symbionts from pathogens and thereby influence root nodulation. Clearly daidzein, which can function as both a *nod*-gene inducer (Fig. 2) and as a precursor of the phytoalexin coumestrol (Dewick *et al.* 1970; Berlin *et al.* 1972), is a biochemically useful molecule even before the bean plant distinguishes symbiotic from pathogenic intruders.

ACKNOWLEDGMENT

This work was supported by U.S. Department of Agriculture National Research Initiative Competitive Grants Program award 91-37305-6513 and grant US-1884-90 from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD).

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