Effects of a Seed Color Mutation on Rhizobial
*nod*-Gene-Inducing Flavonoids and Nodulation
in Common Bean

Mariangela Hungria¹ and Donald A. Phillips²

¹EMBRAPA-CNPSoja, CEP-86001-970, Londrina, PR, Brazil; ²Department of Agronomy and Range Science, University of California, Davis 95616 U.S.A.
Received 25 January 1993. Accepted 26 April 1993.

A white-seeded mutant of common bean (*Phaseolus vulgaris* PI 165426WS) differed from its isogenic black-seeded parent, PI 165426CS, in the amounts and types of flavonoid *nod*-gene inducers released from the seed and in the pattern of root nodules formed by *Rhizobium tropici* CIAT 899 and by *R. leguminosarum* bv. *phaseoli* CNPAF 512. Comparisons of 14-day-old plants developing from black (CS) and white (WS) seeds showed that although there were no differences in any plant growth parameter measured, CS plants had at least 80% more nodules on the primary root. Rinsing seeds before planting decreased nodulation at the top of the primary root in CS, but not WS, seedlings. A direct role for seed compounds in the nodulation differences was supported by the fact that rinses from CS seeds induced 10-fold higher β-galactosidase activity from a *nodA*::lacZ fusion in *R. leguminosarum* bv. *phaseoli* than WS seed rinses. Analytical chemistry techniques showed that WS seeds lacked five *nod*-gene-inducing anthocyanins previously identified on CS seeds. WS seed rinses contained five *nod*-inducing flavonol glycosides released by CS seeds, but only 45% as much of those compounds was present. The *nod*-gene-inducing activity of WS root exudates, however, was much more similar to that from CS roots both quantitatively and qualitatively. Adding 20 μmoles of malvidin-3-O-glucoside or quercetin-3-O-glucoside to WS seeds inoculated either with CIAT 899 or with CNPAF 512 increased nodulation by at least 40%, but malvidin and quercetin aglycones had no effect on nodulation. No flavonoids tested altered nodulation on CS plants. These data indicate that initial root nodulation of WS, but not CS, beans was limited by availability of *nod*-gene inducers released from the seed coat.

Additional keywords: anthocyanins, anthocyanidins, flavonols.

Development of the *Rhizobium*-legume symbiosis requires that plant factors, such as particular flavonoids, induce transcription of rhizobial *nodABC* genes (Long 1989). The process is controlled by the regulatory *nodD*

Corresponding author D. A. Phillips.

MPMI Vol. 6, No. 4, 1993, pp. 418-422
This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1993.

RESULTS

**Growth and nodulation of bean lines.**

After 14 days of growth, CS and WS beans, which had been planted with intact, unrinse seed coats, did not differ
significantly in root dry weight (1.44 g), shoot dry weight (0.32 g), primary root length (28.3 cm), or number of lateral roots (54). Effective nodules distinguished by the presence of leghemoglobin were found only on the primary root. Untreated CS plants had at least 80% more nodules on the primary root than the comparable isogenic WS mutant (Fig. 1). Rinsing seeds before planting decreased nodulation at the top of the primary root in CS, but not WS, seedlings (Fig. 2). Removing the seed coat after rinsing caused no further decrease in nodulation of the primary root in CS beans and had no detectable effect on nodulation of WS plants relative to intact, unrinse controls. Comparable results were obtained with both R. tropici CIAT 899 and R. l. bv. phaseoli CNPAF 512 as an inoculum, and in all cases, treatments decreased nodulation of CS plants only at the top of the primary root.

**nod-gene inducers released by CS and WS beans.**

Quantitative measures of nod-gene-inducing activity in crude seed and root exudates showed that CS seed exudate had as much as 10-fold more nod-gene-inducing activity than WS seed exudate (Fig. 3). The nod-gene-inducing activity of WS root exudate also was initially lower than that of CS root exudates, but that difference narrowed greatly 240 hr after imbibition.

High-performance liquid chromatography (HPLC) analyses of seed rinses revealed several differences in flavonoids released by CS and WS beans (Fig. 4). Six previously observed nod-gene inducers (Hungría *et al.* 1991a) were identified in both CS and WS seed rinses by spectroscopic techniques, cochromatography, and β-galactosidase assays. Using peak identification numbers assigned previously (Hungría *et al.* 1991a), it is clear that WS seed rinses lacked the anthocyanins (peaks 2–6), but flavonols (peaks 7–11) were released from both bean lines. Three previously unobserved compounds (peaks 12–14) with minor nod-gene-inducing activities were present in WS seed rinse, and identities of those compounds are being sought.

---

**Fig. 1.** Effects on root nodulation produced by altering the availability of seed flavonoids in isogenic black- and white-seeded beans. Primary root nodules were counted on 14-day-old seedlings grown from untreated normal seeds, seeds rinsed in water for 24 hr, and seeds from which the seed coats were removed after rinsing. Means of eight replicates associated with different letters (a, b, c) show significant ($P < 0.05$) treatment or germplasm effects. All plants were inoculated with *Rhizobium tropici* strain CIAT 899.

**Fig. 2.** Effects on root nodule distribution produced by altering the availability of seed flavonoids in isogenic black- and white-seeded beans. Mean numbers of nodules in each 1-cm primary root segment from eight replicates were determined for plants reported in Figure 1. Values within each root segment followed by different letters indicate significant ($P < 0.05$) treatment or germplasm effects.

**Fig. 3.** Release of nod-gene-inducing activity from seeds and roots of isogenic black- and white-seeded beans. Values represent means of exudates from six replicates assayed for β-galactosidase activity induced from a nodA::lacZ fusion controlled by nodD from R. l. bv. *phaseoli* in rhizobial strain RBL1283. Activity of the black-seeded line was significantly ($P < 0.05$) higher at all points.

**Fig. 4.** High-performance liquid chromatography characteristics of seed exudate released during the first 6 hr of imbibition by isogenic A, black- and B, white-seeded beans. In each case, rinses from two seeds were fractionated on a reverse-phase C18 column with a methanolic gradient (0–99%) and monitored for maximum eluate absorbance ($A_{max}$, 200–360 nm) each second.
UV/visible spectral traits of peaks 12–14 clearly distinguished them as being different from peaks 2 to 6. Aglycone structures produced by hydrolysis of WS peaks 7–11 were identified as follows: 7, myricetin; 8 and 9, quercetin; 10 and 11, kaempferol. Glycosylation at the C-3 position in unhydrolyzed compounds was confirmed by UV/visible spectral shift experiments as described previously (Hungria et al. 1991a).

HPLC, spectroscopic analyses, and nod-gene-inducing activity of WS root exudate collected 216–240 hr after imbibition identified the same nod-gene inducers found previously in CS root exudate (Hungria et al. 1991b): genistein, eriodictyol, and naringenin (data not shown). Genistein in both CS and WS root exudates was glycosylated as a 7-O-linkage.

Quantification of flavonoids in WS seed and root exudates.

WS seed exudates collected during the first 6 hr of imbibition contained the following amounts of nod-gene inducers (nmol seed⁻¹): compound 7, 14; compound 8, 6; compound 9, 7; compound 10, 6 for a total of 33 nmol of flavonol glycosides seed⁻¹ h⁻¹. That value corresponds to 45% of the flavonol glycosides released by CS seeds (Hungria et al. 1991a). WS root exudates collected between 216 and 240 hr after imbibition contained the following amounts of nod-gene-inducing flavonoids (nmol plant day⁻¹): genistein, 38; eriodictyol, 217; naringenin, 190, or a total of 445 nmol flavonoids plant day⁻¹. That represents 67% of the amount measured for the same compounds over the same period in CS plants (Hungria et al. 1991b).

Effects of exogenous flavonoids on nodulation of CS and WS seedlings.

CS and WS plants showed no statistical difference in root or shoot dry weight, length of primary root, or number of lateral roots after 14 days of growth in the presence or absence of supplemental flavonoids (data not shown). However, adding 20 μmole of malvidin-3-O-glucoside increased nodule number on the WS primary root by 39% with R. tropici CIAT 899 as an inoculum and by more than 50% with R. l. bv. phaseoli CNPAF 512 (Table 1). Similar results were produced on WS plants with 20 μmole of quercetin-3-O-glucoside, but aglycone forms of these flavonoids did not alter the WS nodulation pattern. At harvest, nodules from WS plants treated with either malvidin or quercetin glycosides appeared more mature because approximately 60% more nodules contained visible leghemoglobin than control plants receiving no flavonoids. Nodulation of CS beans was not affected by addition of any flavonoids tested.

DISCUSSION

Flavonoids responsible for seed color of black beans in this study had a markedly positive effect on initial root nodule formation by those plants. Rinsing seeds or removing the seed coat before planting decreased nodulation of CS beans, but these treatments did not alter nodulation of the isogenic WS mutant (Fig. 1). The effect of these treatments was evident only at the top of the primary root (Fig. 2) where compounds from the seed probably were present during early root development. A role for positive nodulation factors on the CS seeds was supported by the fact that much higher amounts of nod-gene-inducing activity eluted from CS than WS seeds (Fig. 3). The decline in nod-gene-inducing activity of WS seeds was associated with the specific absence of five nod-gene-inducing anthocyanins found on the CS seed coat (Fig. 4). When that loss of flavonoids on the WS seeds was offset with either malvidin-3-O-glucoside or quercetin-3-O-glucoside, nodulation was enhanced (Table 1). Thus, these results offer specific chemical data and a developmental effect consistent with previous suggestions that phenolics on dark-seeded beans contribute to plant growth and vigor (Deakin 1974; Dickson and Petzoldt 1988). Although plants in the current experiments were harvested before increased root nodulation associated with the presence of flavonoids enhanced N₂ fixation and growth, the benefits that follow early increases in nodule number are well established in alfalfa (Kapulnik et al. 1987; Jain et al. 1990). Other data for common bean indicate that early, prolific nodulation of the tap root also contributes significantly to a successful symbiosis in this species (Barradas and Hungria 1990). There is no evidence from this study that the mutation eliminating anthocyanins from WS seed coats had any marked effect on nod-gene-inducing flavonoids released from roots (Fig. 3). Thus, while WS seed rinses contained no detectable anthocyanins and only 45% of the flavonol glycosides reported for CS seeds (Hungria et al. 1991a), nod-gene-inducing flavonoids in WS root exudates were only slightly lower than the amount exuded after 10 days from CS roots. In related experiments, mutagenesis treatments eliminated anthocyanins from hypocotyls, flowers, and seeds of a black-seeded bean, but in all mutant lines, the flavonoids in root exudates remained qualitatively and quantitatively unchanged (D. A. Phillips, unpublished data). Taken with the data in Figure 3, these results strongly suggest that legume seedlings protect their capacity for signaling to symbiotic rhizobia by maintaining two sources of nod-gene-inducing flavonoids, seeds, and roots. Presumably genes responsible for flavonoid biosynthesis in those tissues are under the control of separate transcriptional promoters.

Mechanisms responsible for the differing effects of flavo-

---

**Table 1.** Effects of supplemental flavonoids on nodulation in isogenic black-seeded (CS) and white-seeded (WS) bean plants inoculated with either *Rhizobium tropici* CIAT 899 or *R. l. bv. phaseoli* CNPAF 512.*

<table>
<thead>
<tr>
<th>Flavonoid (20 μmole)</th>
<th>R. tropici</th>
<th>R. l. bv. phaseoli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>WS</td>
</tr>
<tr>
<td>Untreated control</td>
<td>51 a</td>
<td>23 c</td>
</tr>
<tr>
<td>Malvidin-3-O-glucoside</td>
<td>48 a</td>
<td>32 b</td>
</tr>
<tr>
<td>Malvidin</td>
<td>45 a</td>
<td>26 c</td>
</tr>
<tr>
<td>Quercetin-3-O-glucoside</td>
<td>48 a</td>
<td>33 b</td>
</tr>
<tr>
<td>Quercetin</td>
<td>45 a</td>
<td>24 c</td>
</tr>
</tbody>
</table>

*Mean numbers of nodules from eight replicates on day 14 followed by the same letter are not statistically different (P ≤ 0.05) within each *Rhizobium* species.
noid glycosides and aglycones (Table 1) are unclear. Natural glycosides released by CS seeds generally induce rhizobial nod genes at lower concentrations than the corresponding aglycones, but the aglycones certainly are biologically active (Hungria et al. 1991a). It is possible that promotive effects on rhizobial growth caused by the glycosides, but not the aglycones (M. Hungria, unpublished data), contributed to the enhancement of root nodule formation. Analytical data from R. meliloti show that in one case the intact malonylated isoflavanoid glycoside was required for regulating a bacterial gene (Dakora et al. 1993), so other, more complex factors may be involved.

Any agronomic benefits that may flow from this work remain to be defined. While adding flavonoid glucosides to WS seeds promoted nodulation, the total number of root nodules on the treated WS seedlings was not as great as CS control plants (Table 1). Perhaps larger amounts of flavonoids would have increased nodulation further, but the mutation in WS plants probably affected root nodulation through more than just differences in seed color. This possibility is supported by the fact that when CS and WS seed coats were completely removed, the WS seedlings still had significantly ($P < 0.05$) fewer nodules than CS plants (Fig. 1). One possible mechanism for that effect may involve interactions between flavonoids and plant hormones that are separate from the flavonoid induction of rhizobial nod genes (Hirsch 1992). The current results cannot be extrapolated to a soil environment where other microbes and physicochemical forces might decrease the biological effect of added flavonoids. These data, however, do indicate that, in the case of a bean mutant with altered flavonoid metabolism, the quantity of nod-gene-inducing flavonoids on the seed limited root nodulation.

MATERIALS AND METHODS

Nodulation tests.

Studies were initiated with P. vulgaris seeds having similar weights (197 ± 8 mg) in both the black-seeded (CS) line PI 165426CS and its isogenic white-seeded (WS) mutant PI 165426WS (Dickson and Petzoldt 1988). After surface sterilization (Hungria et al. 1991a), the two lines were compared in three treatments: 1. Control—no additional handling; 2, rinsed seeds—bathed 24 hr in aerated sterile water; 3, no seed coats—seed coats were removed after a 24-hr rinse treatment. Seeds were planted in 15-cm-diameter pots containing sterile sand and vermiculite (1:2, v/v) before inoculating with $10^5$ cells of R. tropici strain CIAT 899 (CIAT, Cali, Colombia), which had been grown for 5 days at 28°C in YM medium (Vincent 1970). A completely randomized experimental design was used with eight replicates per treatment and two plants per replicate. Pots were maintained in a growth chamber under a 12/12 hr light/dark cycle, 25/22°C, 50% RH, and a photosynthetic photon flux density (400–700 nm) of 320 $\mu$E m$^{-2}$ s$^{-1}$. Every other day plants were given 150 ml of N-free nutrient solution (Maxwell et al. 1989), and after 14 days they were harvested and dried to a constant weight at 70°C. A second experiment followed the same procedure using R. l. bv. phaseoli strain CNPAF 512 (EMBRAPA-CNPAF, Goiânia, Brazil) as an inoculant.

Preparation of exudates and assays for nod-gene induction.

Seed exudates were collected in sterile, aerated distilled water (Hungria et al. 1991a) following 2, 4, 6, 12, 24, 36, and 48 hr of imbibition with complete replacement of sterile water at each time point. After 48 hr, seeds were transferred to containers that allowed roots to develop into sterile, aerated, N-free nutrient solution, and root exudate was subsequently collected (Hungria et al. 1991b). Samples were assayed for their capacity to induce $\beta$-galactosidase activity from a nod::lacZ fusions controlled by nodD from R. l. bv. phaseoli with strain RBL1283(nodA::lacZ) (generously supplied by R. J. H. Okker and B. J. J. Lugtenberg, Leiden University) or R. l. bv. phaseoli strain CE3pA87(nodC::lacZ) (a gift from F. Sanchez, UNAM, Cuernavaca) (Hungria et al. 1991a).

Flavonoid standards and purified compounds were prepared for assays as described previously (Hungria et al. 1991a). Initial assays for nod-gene-inducing activity of crude seed and root exudate or of HPLC eluant fractions were done with 1, 2, 5, 7.5, or 12.5% of the collected sample. Flavonoid concentrations were calculated spectrophotometrically using known extinction coefficients of authentic standards (Hungria et al. 1991a, 1991b).

Purification and identification of nod inducers.

The nod-gene inducers released from WS seeds and roots were purified by standard HPLC methods and identified by spectroscopic analyses (ultraviolet/visible absorbance, proton NMR, and MS) as described previously (Hungria et al. 1991a, 1991b). Identities were further verified by cochromatography with authentic standards and assays for nod-gene induction. Flavonoids were quantified by HPLC integration (Hungria et al. 1991a, 1991b), and amounts recovered during the normal purification process were corrected by adding known amounts of quercetin-3-O-galactoside to seed exudates and eriodictyol to root exudates (Hungria et al. 1991a, 1991b).

Effects on nodulation of CS and WS lines produced by the addition of flavonoids.

Surface-sterilized CS and WS seeds were inoculated with $10^8$ cells of R. tropici CIAT 899. Nodulation was compared in five treatments: 1, Control—no supplemental flavonoid; 2, 10 $\mu$moles of malvidin-3-O-glucoside seed$^{-1}$ at planting plus 10 $\mu$moles 24 hr later (a total of $20 \mu$moles); 3, 20 $\mu$moles of malvidin seed$^{-1}$ applied as in 2; 4, 20 $\mu$moles of quercetin-3-O-glucoside seed$^{-1}$ applied as in 2; 5, 20 $\mu$moles of quercetin seed$^{-1}$ applied as in 2. Flavonoid amounts were based on results showing that CS seeds released 423 nmole of anthocyanins seed$^{-1}$ h$^{-1}$ during the first 6 hr of imbibition (Hungria et al. 1991a). Aglycones were dissolved in methanol and glycosides in 50% methanol; a drop of concentrated HCl was added to improve the stability of malvidin and malvidin glucoside. No more than 30 $\mu$ of methanol was added to each seed. Malvidin and quercetin were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA). Malvidin-3-O-glucoside and quercetin-3-O-glucoside were purified from CS seed rinse as peaks 6 and 9, respectively, (Hungria et al. 1991a) and verified by NMR, MS, and GC analyses. A completely randomized experimental design was used with
eight replicates per treatment and two plants per replicate. Plants were grown and harvested 14 days after emergence, as described above for nodulation tests. The experiment was repeated with *R. l.* bv. *phaseoli* CNPAF 512 as an inoculum.

ACKNOWLEDGMENTS

This work was supported by U.S. Department of Agriculture National Research Initiative Competitive Grants Program award 91-37305-6513 and by EMBRAPA-CNPSO.

LITERATURE CITED


