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

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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* gene product NodD, which apparently interacts with the flavonoids. In the alfalfa symbiont *R. meliloti*, gene products from *nodABC* are involved in synthesis of a complex signal molecule that elicits several events associated with infection, including root hair curling (Lerouge *et al.* 1990). Flavonoids that induce *nod* genes have been identified in exudates from many legumes (Long 1989), but common bean (*Phaseolus vulgaris* L.) releases a particularly large number of active flavonoids from seeds (Hungria *et al.* 1991a), as well as a separate group of *nod*-inducing molecules in root exudates (Hungria *et al.* 1991b). Unlike the *Rhizobium*-alfalfa symbiosis, the bean inducers show little apparent specificity for different NodD proteins in *R. leguminosarum* bv. *phaseoli* (Hungria *et al.* 1992).

Flavonoids are important components of seed coat color in common bean, and five of the most active *nod*-gene inducers released from black bean seeds are anthocyanins (Hungria *et al.* 1991a). Bean mutants with dissimilar seed colors contain structurally different flavonoids in their seed coats (Feenstra 1960), and the patterns of compounds identified in that early study are consistent with flavonoid biosynthetic pathways now known (Stafford 1990). Other data show that seed color influences interactions with fungal pathogens in isogenic white- and colored-seeded beans (Deakin 1974; Dickson and Petzholdt 1988), but no phenolics were identified as active factors in those studies.

Common bean is an important crop in tropical and subtropical countries, where poor root nodulation often limits symbiotic N_2 fixation in this plant (Neves and Hungria 1987). Flavonoids can restrict root nodulation under certain conditions in alfalfa (Kapulnik *et al.* 1987; Jain *et al.* 1990), and thus it is reasonable to examine the extent of their contribution to that process in bean. Having identified nine flavonoid aglycone *nod*-gene inducers released from seeds and roots of a black-seeded bean (Hungria *et al.* 1991a, 1991b), we compared that bean line with its isogenic white-seeded mutant for root nodulation by two *Rhizobium* species. When differences in nodulation were observed, we made a more detailed comparison of *nod*-inducing flavonoids released by the two lines.

RESULTS

Growth and nodulation of bean lines.

After 14 days of growth, CS and WS beans, which had been planted with intact, unrinsed seed coats, did not differ

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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, unrinsed 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 (Hungria *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 (Hungria *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.

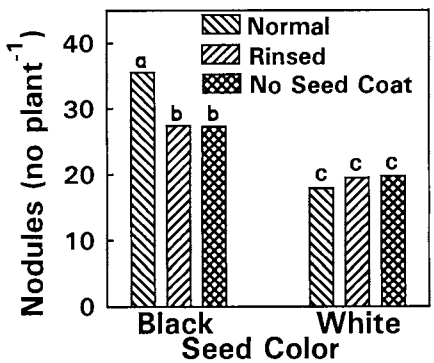

Fig. 1. Effects on root nodulation produced by altering the availability of seed flavonoids in isogenic black- and white-seeded beans. Primary root nodules were counted on 14-day-old seedlings grown from untreated normal seeds, seeds rinsed in water for 24 hr, and seeds from which the seed coats were 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.

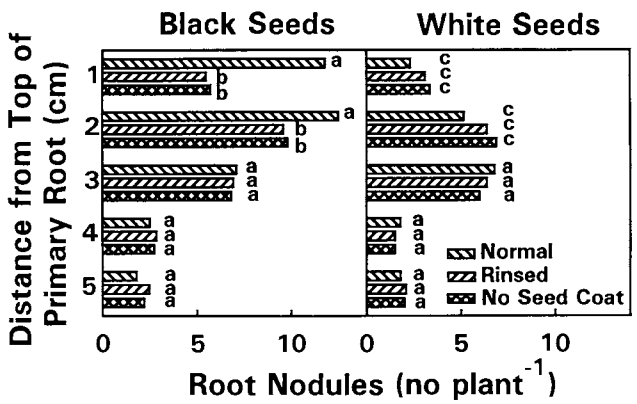

Fig. 2. Effects on root nodule distribution produced by altering the availability of seed flavonoids in isogenic black- and white-seeded beans. Mean numbers of nodules in each 1-cm primary root segment from eight replicates were determined for plants reported in Figure 1. Values within each root segment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

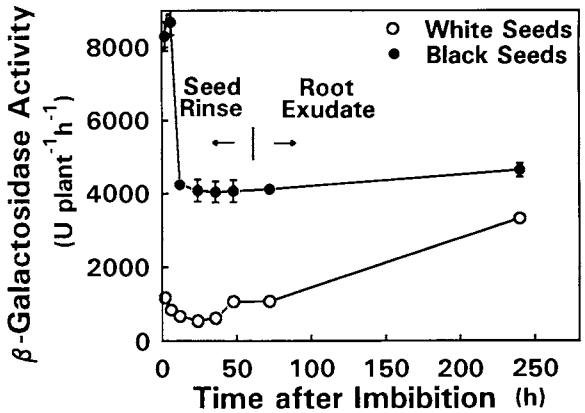

Fig. 3. Release of *nod*-gene-inducing activity from seeds and roots of isogenic black- and white-seeded beans. Values represent means of exudates from six replicates assayed for β-galactosidase activity induced from a *nodA::lacZ* fusion controlled by *nodD* from *R. l.* bv. *phaseoli* in rhizobial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

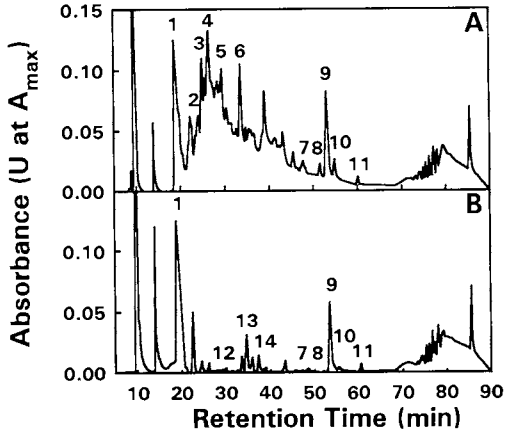

Fig. 4. High-performance liquid chromatograpy characteristics of seed exudate released during the first 6 hr of imbibition by isogenic **A,** black- and, **B,** white-seeded beans. In each case, rinses from two seeds were fractionated on a reverse-phase C18 column with a methanolic gradient (0–99%) and monitored for maximum eluate absorbance (A_{max}, 200–560 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 time 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 μmoles 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 μmoles 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.

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[a]

	Nodule number·plant^{-1}			
	R. tropici		*R.l.* bv. *phaseoli*	
Flavonoid (20 μmoles)	CS	WS	CS	WS
Untreated control	51 a	23 c	45 a	20 c
Malvidin-3-*O*-glucoside	48 a	32 b	42 a	31 b
Malvidin	45 a	26 c	47 a	23 c
Quercetin-3-*O*-glucoside	48 a	33 b	44 a	29 bc
Quercetin	45 a	24 c	42 a	25 c

[a] Mean numbers of nodules from eight replicates on day 14 followed by the same letter are not statistically different ($P \leq 0.05$) within each *Rhizobium* species.

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 N_2 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-

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 isoflavonoid 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 quanitity 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 10^8 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 μE 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 β-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 CE-3pA87(*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 μmoles of malvidin-3-*O*-glucoside $seed^{-1}$ at planting plus 10 μmoles 24 hr later (a total of 20 μmoles); 3, 20 μmoles of malvidin $seed^{-1}$ applied as in 2; 4, 20 μmoles of quercetin-3-*O*-glucoside $seed^{-1}$ applied as in 2; 5, 20 μmoles 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 μl 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.

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