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Biological Characterization of Root Exudates and Extracts from Nonnodulating and Supernodulating Soybean Mutants

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The root exudates and extracts of soybean nonnodulation (nod$^-$) mutants nod49, nod139, and nod772 were compared with those of the wild-type parent cultivar Bragg and supernodulation (nitrate tolerant symbiotic) mutant nts382 to assess their effects on the early stages of nodulation by *Bradyrhizobium japonicum* strain USDA110. When nod$^-$ mutants were cocultured in proximity with wild-type or mutant nts382, nodulation was as expected for the individual genotypes. Coculturing nts382 with wild-type or nonnodulating plants decreased nodule number on the supernodulating mutant when compared to similar plant density of nts382 alone. Pretreatment of *B. japonicum* with collected root exudates of the soybean mutants did not affect nodulation efficiency or total nodule number per plant. Analysis of *in vivo* labeled proteins in root exudates of wild type, nts382, and nod49 showed similar patterns both on one- and two-dimensional gels. Radioactively (^{35}S) labeled root exudates were subjected to immunoprecipitation with the antibody to soybean lectin (SBL) and similar amounts were found in the nod49, nts382, and wild-type soybean. Seedling root extracts of nts382, nod49, and Bragg were tested for their ability to induce a *nodC::lacZ* fusion in *B. japonicum* strain USDA110. Similar levels of induction were found in all cases, indicating that uninoculated seedlings of cultivar Bragg, nod49, and nts382 contained similar stimulating compounds. Based on the observation that mutants nod49, nod139, and nod772 lack root hair curling, we suggest that the nonnodulation phenotype may be caused by an inability to respond efficiently to bacterial factors designed to stimulate plant cell divisions and concomitant root hair curling.

Additional keywords: symbiosis, rhizosphere, *Glycine max*, isoflavones.

Symbiotic nitrogen fixation involving *Rhizobium*/*Bradyrhizobium* and legumes depends on the genetic properties of both the bacteria and the host plant. The symbiotic association results in the formation of a specialized plant organ - the root nodule (Rolfe and Gresshoff 1988). Despite the advances made in the understanding of the molecular events taking place in the two symbionts (for example Nap *et al.* 1987; Fortin and Verma 1987; Hennecke *et al.* 1987), there is still relatively little known about how the organisms interact with each other during the process of nodule development.

There is evidence suggesting an exchange of signals between the plant and the bacteria when the bacterium first arrives in the root zone. Flavones or isoflavones, released by the roots of the plant, induce the common nodulation (*nodABC*) genes of the bacterium (Peters *et al.* 1986; Firmin *et al.* 1986; Redmond *et al.* 1986; Downie and Johnston 1986; Kosslak *et al.* 1987; Bassam *et al.* 1988) and also the bacterial host specificity genes (Horvath *et al.* 1986). In response to plant substance signaling, the bacteria appear to synthesize low molecular weight factors that induce root hair deformation and branching on clover (Bhuvaneswari and Solheim 1985) and in the case of small-seeded legumes, inhibit overall root growth (Van Brussel *et al.* 1986). Although these signals may not be specific for a legume-*Rhizobium* symbiosis, the early events induced by these signals permit the progression to later nodulation stages. Other plant signal substances are the lectins. These have been implied to be more specifically involved in legume-*Rhizobium* symbioses (Bohlool and Schmidt 1974; Sengupta-Gopalan *et al.* 1983; Su *et al.* 1980; Tsien *et al.* 1983; Halverson and Stacey 1985; Vodkin and Raikhel 1986). In the final analysis, all of these factors, and perhaps others, will be shown to play an interactive role in determining the specificity between host and infecting bacterium.

Carroll *et al.* (1986) described the isolation of several nonnodulation soybean mutants following ethylmethane sulfonate (EMS) mutagenesis and subsequent selection. These mutants, nod49, nod772, and nod139, are the only soybean nodulation mutants in addition to the naturally occurring variant rj_1 (Williams and Lynch 1954; Gresshoff *et al.* 1987). All three nod$^-$ mutants lacked root hair curling (Mathews *et al.* 1987), and grafting experiments showed that the nonnodulation phenotypes (as for rj_1) were controlled by the root (Delves *et al.* 1986; Mathews 1987). We have analyzed the root exudates and extracts of these nonnodulation mutants and compared them with the root exudates and extracts of their wild-type parent and a supernodulation mutant, nts382. Our data support the claim that Bragg, nod49, nts382, nod139, nod772, and rj_1 have similar root exudates and extracts with respect to proteins, biological activity, and factors that stimulate the induction of the *nodC::lacZ* fusion in strain USDA110 (pEA2-21). We thus conclude that the mutational block lies further along the ontogenetic pathway.

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MATERIALS AND METHODS

Bacterial strains. *B. japonicum* Buchanan (Jordan) strain USDA110 was used for all of the plant nodulation studies. Plants were either inoculated with 5 ml (5×10^8 cells per milliliter) of a 3-day-old culture grown in YEMB medium (Vincent 1970) or with a peat inoculum (1–2 g per pot) containing 10^8 viable cells per gram. Pretreatment of strain USDA110 with exudate was as follows. Surface-sterilized seeds were germinated aseptically on YEMA medium (Vincent 1970) for 48 hr. Seedling roots were placed into half-strength plant nutrient solution (PNS; Jensen 1942), with the bulk of the seedling being supported by a petri dish lid into which holes had been punched. A sterilized plastic bag was placed around the whole assembly and the bottom part was wrapped in aluminum foil. Plants were grown hydroponically for 10 days in a Conviron growth chamber at 26° C, with 85% relative humidity, and for an 18-hr light period (approximately 400 $\mu E \cdot m^{-2} \cdot s^{-1}$). The conditioned plant medium was collected and passed through a 0.45-μm filter. Strain USDA110 was cultured to midlogarithmic phase in liquid YEMA medium (Vincent 1970), and 10 ml (at 2×10^9 cells per milliliter) was mixed with 50 ml of conditioned medium in 250-ml Erlenmeyer flasks and incubated without shaking at 30° C for 72 hr. Cells were centrifuged at $7{,}000 \times g$ for 10 min, and then washed once with half-strength PNS.

USDA110 (pEA2-21) containing a transcriptional *nodC*::*lacZ* fusion was used to monitor *nod* gene expression in response to mutant and wild-type extracts (Kosslak *et al.* 1987). *B. japonicum* strain USDA110 was originally obtained from J. Streeter (Ohio State University, Wooster).

Plant material. *Glycine max* (L.) Merr. cultivars Williams 79, Prize, and Bragg and mutants nod49, nod139, and nod772 (nonnodulating) as well as nts382 (supernodulating, nitrate tolerant nodulation) were used, and their nodulation parameters are previously described (Carroll *et al.* 1985a, 1985b; Carroll *et al.* 1986; Mathews *et al.* 1987). In short, nod49, nod139, and nod772 were isolated from EMS-mutated seed populations. Their Nod^- phenotype is controlled by two single Mendelian recessive genes (Mathews *et al.*, in press) acting through the root (Delves *et al.* 1986). No root hair curling is observed. They produced occasional nodules when grown in the laboratory. Such occasional nodules were normal in structure and fixed nitrogen effectively (Mathews *et al.* 1987). Cultivar Lee rj_1 (nonnodulating) was used as another nonnodulating cultivar.

Mutant nts382 was one of several supernodulation isolates derived from an induced mutagenesis (EMS) of cultivar Bragg (Carroll *et al.* 1985a, 1985b). The mutation resulted in a single Mendelian recessive gene, unlinked to nonnodulation mutant loci (Mathews, Carroll, and Gresshoff, unpublished data), and its phenotype is shoot-controlled (Delves *et al.* 1986; Gresshoff and Delves 1986). The supernodulation phenotype also made such mutants tolerant to the inhibitory action of nitrate on nodulation (Carroll *et al.* 1985a, 1985b; Gresshoff *et al.* 1988).

Plant growth conditions. Plants were raised either in glasshouses in Canberra (temperature range between 30° C and 16° C) with supplemented lighting to extend the photoperiod or in growth chambers (16-hr day, 28° C daytime and 20° C nighttime temperatures, 380 $\mu E \cdot m^{-2} s^{-1}$). Coculture experiments were conducted in Leonard jar assemblies (Vincent 1970; Carroll *et al.* 1985b). Six surface-sterilized seeds (Mathews *et al.* 1987) were placed per jar with differing combinations of wild type, nod49, and nts382 (three each). The seeds were inoculated with strain USDA110 at planting and harvested 4 wk later.

Plastic growth pouches were used for nodulation efficiency studies. Seeds were surface-sterilized before being pregerminated on YEMA plates (Vincent 1970) for 2.5 days. Three uniform-sized seedlings were placed into each premoistened (with half-strength PNS) pouch. Inoculation with pretreated (see previous description) *Bradyrhizobium* strain USDA110 occurred at that time. The positions of the root tip at the time of inoculation and the shortest emerging root hairs were marked on the plastic pouches. Pouches were watered with half-strength PNS as required and scored for nodulation in the zone between the root hair and root tip marks, and below the root tip mark. Such measurements gave an indication of the efficiency (speed) of nodulation, because rhizobia that were delayed in nodulation functions were expected to fail to nodulate in the the root hair-root tip region (Halverson and Stacey 1985).

Analysis of proteins in root exudates. Roots of intact 4-day-old seedlings were incubated with gentle aeration in 10 mM potassium phosphate buffer containing [^{35}S]methionine (New England Nuclear, Boston, MA) and chloramphenicol, 50 $\mu g \cdot ml^{-1}$. After 8 hr, the incubation medium was concentrated by lyophilization and then dialyzed against 10 mM potassium phosphate buffer to remove small molecular weight contaminants.

Proteins were then fractionated by sodium dodecyl sulfate (SDS)-PAGE (Laemmli 1970) or two-dimensional gel electrophoresis (O'Farrell 1975) and visualized by fluorography (Laskey and Mills 1975). Immunoprecipitation of excreted proteins with soybean seed lectin antibody (obtained from Y. E. Laboratories, Sacramento, CA) was done by the double-precipitation method described by Lingappa *et al.* (1978).

Beta-galactosidase assays. Three-day-old seedling roots of Bragg, nod49, nts382, and Williams 79 were extracted as described previously (Kosslak *et al.* 1987). Assays for the induction of the *nodC*::*lacZ* fusion product were performed as described previously (Kosslak *et al.* 1987), except that cells were permeabilized with chloroform plus 0.1% SDS (Miller 1972) rather than toluene. Soybean root extracts were assayed for inducing ability by adding 1.5 ml of extract to 1.5 ml of cells. Three replicate cultures were assayed for each extract tested.

RESULTS

Coculture experiments to test broad biological effects. Table 1 shows the nodulation phenotype and plant dry weight of Bragg and its mutants tested in a single genotype culture. In uninoculated plants, growth was primarily or completely dependent on seed reserves, and variable plant dry weights were a reflection of variable seed reserves. When

seedlings were inoculated with *B. japonicum* strain USDA110, nodulation occurred in the appropriate genotypes. When inoculation levels were at approximately 1×10^8 cells per jar, nts382 showed supernodulation while nonnodulation mutants failed to nodulate. At increased *Bradyrhizobium* titers (6×10^8 cells per jar), nodulation of Bragg and nts382 was as before, but mutants nod49, rj_1, and nod772 produced some occasional nodules. In the same experiment, the effects of cocultivation on nodulation of Bragg, nts382, and the nonnodulation mutants were tested (Table 2).

Under these culture conditions, the root systems of plants were tightly intertwined, generating extensive possibilities for interactions and cross-feeding. Sterile growth conditions (except for the presence of *Bradyrhizobium*) were used to prevent the breakdown of exudate substances, thus allowing for maximum possible stimulation or suppression of nodulation. However, no effect on the nodulation phenotype of the nod^- mutants was observed when they were cocultured with either Bragg or nts382. The number of nodules that developed on Bragg (10–20 per plant) was also unaffected by the nod^- lines. Cocultivation of nts382 with either Bragg or the nod^- mutants altered the extent of the supernodulation phenotype. Nodule numbers per nts382 plant were about 100–150 when cultured with other genotypes, which was less than observed when nts382 was grown alone (289 ± 60).

The results from Table 2 indicated that despite the opportunities for cross-feeding between the root systems, neither a positive nor negative effect on the nodulation patterns of Bragg or the nod^- mutants was observed with cocultivation. The data confirmed a previous experiment in which the following nodule numbers per plant were observed: Bragg alone, 15.4 ± 4.0; nts382 alone, 359 ± 66; nts382 plus nod49, none on nod49 and 178 ± 8.6 on nts382; and Bragg plus nod49, none on nod49 and 20.6 ± 6.4 on Bragg. These plants (about six plants per Leonard jar filled with vermiculite) were grown in glasshouses, inoculated with strain USDA110, irrigated with nitrogen-free nutrient solution, and harvested after 35 days of culture. In coculture with Bragg or the nonnodulating mutants, the roots of nts382 plants developed fewer nodules than in single culture, possibly because of competition from the large root system of the Bragg plants. Alternatively, both Bragg and the nonnodulation mutants may produce exudates that antagonized nodulation on nts382. This requires further investigation.

Table 3 presents data obtained after pretreatment of USDA110 with exudates from uninoculated seedlings of nod49, nod139, nod772, rj_1, and Bragg. The pouch method developed by Bhuvaneswari *et al.* (1980) and Pierce and Bauer (1983) was used to determine the zone of nodule formation. If an exudate contained inhibitory substances that would affect nodulation, then the nodulation sites should be delayed to below the root tip mark. Pretreatment of strain USDA110 with any of the exudates did not alter the pattern of nodule initiation or total nodule numbers on Bragg. Control treatments involved a 72-hr pretreatment with plant nutrient solution alone. In all instances, few nodules were formed above the root tip mark, a burst occurred within the first 2–3 cm below that mark, and total nodule number per plant was similar. Results showed that our isolate of USDA110 (as grown here) did not initiate a majority of nodules in the most susceptible zone at inoculation (that is between the root tip and the shortest emerging root hair marks), but rather nodulation was

Table 1. Coculture of soybean nodulation mutants, single genotype culture[a]

Treatment[b]	Nodule number per plant	Nodule dry weight per plant (mg)	Mean nod size (mg)	Root dry weight plus nodule dry weight per plant (mg)	Plant dry weight per plant (mg)
Inoculated (at 1×10^8 cells per jar; six plants per jar)					
Bragg	27 ± 5	28 ± 4	1.04 ± 0.01	135 ± 4	515 ± 25
nts382	285 ± 14	57 ± 5	0.20 ± 0.01	94 ± 5	300 ± 7
nod49	0	0	0	99 ± 7	431 ± 5
nod772	0	0	0	108 ± 13	521 ± 84
rj_1 (Lee)	0	0	0	121 ± 15	456 ± 45
nod139	0	0	0	92 ± 8	431 ± 37
Inoculated (at 6×10^8 cells per jar; six plants per jar)					
Bragg	15 ± 1	14 ± 0	0.94 ± 0.06	113 ± 10.2	457 ± 21
nts382	289 ± 60	55 ± 3	0.20 ± 0.04	100 ± 1.41	314 ± 10
nod49	1.7 ± 0.6	3.5 ± 0.4	2.56 ± 0.44	92 ± 0.5	373 ± 6
nod772	0.4 ± 0	0.3 ± 0	0.63 ± 0	107 ± 5	452 ± 9
rj_1 (Lee)	2.0 ± 0.5	2.5 ± 0.4	1.25 ± 0.05	106 ± 5	481 ± 27
nod139	0	0	0	99 ± 6	350 ± 17
Inoculated (1×10^8 cells per jar; one plant per jar)					
Bragg	21 ± 2	28 ± 4	1.33 ± 0.2	161 ± 15	560 ± 43
nts382	364 ± 26	83 ± 5	0.23 ± 0.1	141 ± 2	426 ± 11
nod49	0	0	0	96 ± 13	353 ± 35
nod772	0	0	0	160 ± 5	574 ± 13
rj_1 (Lee)	0	0	0	164 ± 17	548 ± 58
nod139	0	0	0	89 ± 7	368 ± 5

[a]Data are means (± standard error) of four replicates.
[b]Inoculation was with a liquid culture of strain USDA110 at 1×10^8 or 6×10^8 inoculant bacteria per jar. Inoculation at a titer of 1×10^8 bacteria per jar prevented occasional nodulation of the nonnodulation mutants.

Table 2. Coculture of soybean nodulation mutants, Bragg and nonnodulators[a]

Treatment[b]	Nodule number per plant	Nodule dry weight per plant (mg)	Mean nod size (mg)	Root dry weight plus nodule dry weight per plant (mg)	Plant dry weight per plant (mg)
Bragg plus nts 382					
Bragg	14 ± 1	27 ± 4	1.94 ± 0.02	92 ± 13	437 ± 11
nts382	127 ± 14	46 ± 7	0.38 ± 0.1	101 ± 3	275 ± 19
Bragg plus nod49					
Bragg	8 ± 1	10 ± 3	1.32 ± 0.09	76 ± 10	362 ± 31
nod49	0.08 ± 0	0.4 ± 0	5 ± 0.0	68 ± 6	290 ± 15
Bragg plus nod772					
Bragg	13 ± 2	16 ± 5	1.21 ± 0.15	81 ± 2	414 ± 13
nod772	0	0	0	75 ± 3	391 ± 13
Bragg plus rj_1 (Lee)					
Bragg	23 ± 5	16 ± 4	0.70 ± 0.0	87 ± 5	397 ± 31
rj_1 (Lee)	0	0	0	66 ± 5	374 ± 30
Bragg plus nod139					
Bragg	10 ± 1	16 ± 2	1.58 ± 0.2	92 ± 7	416 ± 32
nod139	0	0	0	65 ± 4	310 ± 7
nts382 plus rj_1 (Lee)					
nts382	94 ± 0.6	59 ± 14	0.63 ± 0.0	110 ± 7	463 ± 20
rj_1 (Lee)	0	0	0	85 ± 10	404 ± 22
nts382 plus nod49					
nts382	100 ± 12	42 ± 4	0.44 ± 0.11	68 ± 1	296 ± 31
nod49	0	0	0	64 ± 11	342 ± 2
nts382 plus nod772					
nts382	153 ± 17	67 ± 5	0.46 ± 0.06	166 ± 16	390 ± 11
nod772	0	0	0	99 ± 16	458 ± 44
nts382 plus nod139					
nts382	107 ± 7	49 ± 5	0.47 ± 0.04	86 ± 10	302 ± 2
nod139	0	0	0	88 ± 10	416 ± 75

[a]Data are means (± standard error).
[b]Six plants were grown in each Leonard jar; three of each cultivar. Four replicates of each jar were inoculated at the time of sowing with strain USDA110 at a titer of 6×10^8 cells per jar.

Table 3. Effect of exudate pretreatment on nodule initiation in cultivar Bragg[a]

Exudate source	Test plant	Nodule number per tap root[b] SERH-RT	Nodule number per tap root[b] Below RT	Total nodule number per plant[c]
None[d]	Bragg	1.3 ± 0.3	2.9 ± 0.6	18.9 ± 1.3
Bragg	Bragg	1.4 ± 0.3	2.1 ± 0.7	17.0 ± 1.9
nod49	Bragg	0.8 ± 0.1	4.7 ± 1.3	14.8 ± 1.5
nod772	Bragg	1.7 ± 0.3	3.8 ± 0.5	19.0 ± 1.0
rj_1	Bragg	1.0 ± 0.2	1.0 ± 0.5	16.3 ± 1.0
nod139	Bragg	1.0 ± 0.2	3.9 ± 0.4	18.0 ± 0.9

[a]A total of 22 to 47 replicate plants were used per test. *Bradyrhizobium japonicum* strain USDA110 was used and, where indicated, preincubated with root exudate (conditioned medium). Tests were conducted in plastic pouches inoculated as described in the text. Pouches were harvested at 28 days after germination. Data are means (± standard error).
[b]SERH = shortest emerging root hair; RT = root tip position at the time of inoculation with approximately 10^8 bacteria per plant.
[c]Total nodule number per plant includes nodules on the lateral roots.
[d]A "none" control treatment means that inoculant bacteria were pretreated with plant nutrient solution for 72 hr before inoculation.

displaced significantly to below the root tip mark. The majority of nodules developed on lateral roots. Neither pretreatment with exudate from nonnodulating and wild-type plants improved the nodulation efficiency of USDA110.

Analysis of the proteins exuded by the roots. Earlier suggestions in the literature pointed toward the involvement of exudate (especially through lectins) in the nodulation process (Dazzo and Gardiol 1984). These were further supported by Halverson and Stacey (1985), who demonstrated that a slow-to-nodulate mutant of *B. japonicum* strain USDA110 (called HS111) could be made to nodulate at the wild-type rate by the addition of soybean root exudate or soybean lectin. Figure 1 shows a fluorogram of [^{35}S]methionine-labeled excreted root proteins of Bragg, nod49, and nts382 after one- or two- dimensional gel electrophoresis. One-dimensional analysis revealed about six major protein bands, which were further resolved into about 20 spots when the proteins were fractionated by isoelectric focusing and SDS-PAGE. Comparisons of the protein profiles in one- and two-dimensional gels showed no significant differences.

For quantitative analysis of lectin proteins, which constitute a minor component of the total proteins exuded from soybean roots, [^{35}S]methionine-labeled root exudates were subjected to immunoprecipitation with anti-soybean lectin antibody. The immunoprecipitate was then analyzed by SDS-PAGE, followed by fluorography. As seen in Figure 2, similar amounts of root lectin were detected in Bragg, nod49, and nts382 exudates. The amount of root lectin exuded by the cultivar Prize (lane 1) was considerably

higher than that exuded by cultivar Bragg, although both cultivars have similar nodulation capacities.

Figure 3 shows the β-galactosidase activity from a *nodC*::*lacZ* fusion in USDA110 (pEA2-21) induced by various dilutions of root extracts of uninoculated 5-day-old seedlings of cultivar Williams 79, Bragg, nts382, and nod49. At 50% of the maximum β-galactosidase activity, the root extract from Williams 79 was three to six times more active than the root extracts obtained from Bragg, nts382, and nod49. Furthermore, the root extract from the Bragg parent was not significantly different from the root extracts obtained from either nts382 or nod49. Similar results were obtained using the same gene fusion in a USDA123 background (data not shown). Thus, the observed nodulation phenotype of nts382 and nod49 did not appear to be correlated with either the absence or enhancement of inducing compounds. Inhibition of growth of USDA110 (pEA2-21) occurred with all root extracts at concentrations greater than 300 μg per milliliter (data not shown).

DISCUSSION

The nonnodulation mutant nod49 possessed root exudates and root extracts from uninoculated seedlings similar to its parent, wild-type cultivar Bragg, on the basis of the biological (coculture and nodulation efficiency studies), biochemical (exudate proteins), and *nod* gene induction data presented. The other nonnodulation mutants, nod772 and nod139, had results similar to nod49 in coculture (Table 2) and nodulation efficiency studies (Table 3).

A naturally occurring nonnodulating variant (rj_1) was previously tested for its ability to affect nodulation when cocultured with wild-type soybean (Elkan 1961). The author concluded that rj_1 excreted an inhibitory compound. Eskew and Schrader (1977) tried to repeat the work and came up with the opposite conclusion, namely that high plant density, and no exudate, caused the lowered nodule number. We corroborate these findings with the newly induced nonnodulation mutants and have extended our analysis to the supernodulation mutant nts382. Not only did we find an absence of an inhibitory substance emanating from the roots of the nonnodulation mutants, but also the absence of "cross-feeding" of perhaps missing substances from either the wild-type or the nts mutant.

This was further tested by nodulation efficiency studies, which demonstrate that the phenotype of the nod^- mutants can now be extended to read Exu^+ (root exudate normal), Hac^- (no hair curling), and Nod^- (nodulation deficient). Table 3 showed that nodulation in the initially susceptible region of the roots was similar between differently pretreated inocula. The pouch inoculation data with Bragg and strain USDA110 showed that there was a shift of the position of maximum nodulation frequency from the shortest emerging root hair-root tip zone to below the root tip and the lateral roots. Interestingly, Pierce and Bauer (1983) and their associates used cultivar Williams in their experiments. Analysis of seedling extracts of Bragg and Williams showed that there was a 10-fold difference in the inducing activity of a *nodC*::*lacZ* fusion, perhaps explaining the difference between the Bragg inoculation data and those reported for cultivar Williams. Alternatively, Bhuvaneswari *et al.* (1980) used bacteria straight from the culture after only one centrifugation. To establish subtle differences between the nodulation mutants and wild type, inoculum dose-response curves need to be done. This was not done in this study because of the extensive work load involved and the apparent similarity of results.

Peters and Long (1988) reported that root exudates of

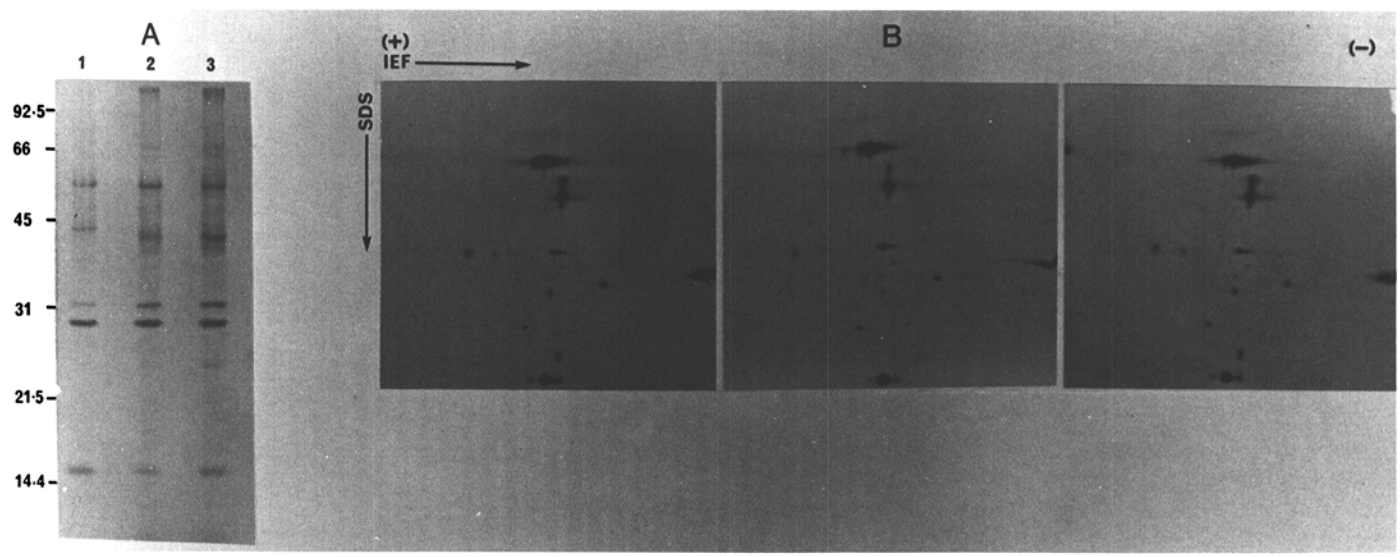

Fig. 1. Analysis of the ^{35}S-labeled excreted root proteins by $NaDodSO_4$-PAGE (**A**) and two-dimensional gel electrophoresis (**B**). The ^{35}S-labeled excreted root proteins were prepared, and 10,000 and 25,000 cpm of trichloroacetic acid (TCA) precipitable material were subjected to SDS-PAGE or two-dimensional gel electrophoresis, respectively, as described in the text. Lanes in panel **A** include: lane 1, excreted root proteins from the wild-type parent line, Bragg; lane 2, excreted root proteins from the nonnodulating mutant nod49; and lane 3, excreted root proteins from the supernodulating mutant line nts382. The molecular sizes (shown in kDa) were calculated from mobilities of known standard proteins (Bio-Rad) included in the same gel. IEF = isoelectric focusing; SDS = $NaDodSO_4$ = sodium dodecyl sulfate.

a nonnodulating alfalfa mutant were similar to wild-type plant exudates in their ability to induce *lacZ* gene fusions with bacterial nodulation genes. Furthermore, the presence of nitrate in the plant growth medium did not affect the amount of inducing activity, suggesting that nitrate tolerance of nodulation, as analyzed here with soybean mutants, is not regulated through the exudation of *nod* gene activating substances.

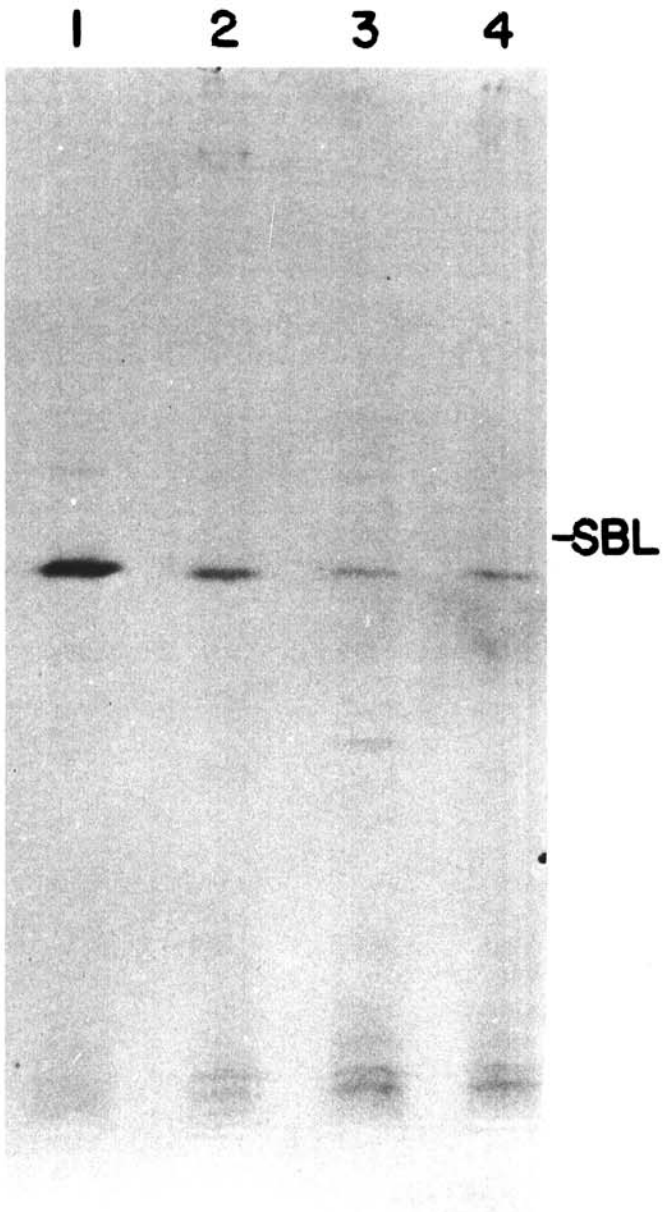

Fig. 2. Immunological detection of lectin proteins in the excreted root proteins. Ten thousand counts per minute of trichloroacetic acid (TCA) precipitable root exudate in each case was subjected to immunoprecipitation as described in the text. The immunoprecipitate was disassociated with sodium dodecyl sulfate and mercaptoethanol and subjected to $NaDodSO_4$-PAGE, followed by fluorography. Lane 1 contains cultivar Prize; lane 2, wild-type cultivar Bragg; lane 3, nonnodulating mutant nod49; and lane 4, supernodulating mutant nts382. The position of soybean lectin (SBL) was determined by including SBL in the same gel and staining with Coomassie blue.

The analysis of labeled exudate proteins showed that any difference which did exist may be minimal or not detectable with the methodology used in this study. The precise analysis of the lectin concentration showed that root exudates of nts382 and nod49 have lectin levels similar to Bragg. The fact that cultivar Prize had much elevated levels of lectin protein in the exudate compared to Bragg may even suggest that lectin levels were not a limiting factor in the cultivars used here.

The analysis of plant root exudates has recently demonstrated that flavones (for *R. trifolii* Jordan, *R. meliloti* Dangeard, and *R. leguminosarum* Frank) and isoflavones (for *B. japonicum* [Kosslak *et al.* 1987]) are the compounds which, together with the *nodD* gene product, activate numerous bacterial *nod* genes, including *nodABC*. Our experiments showed that the level of inducing substances in the root extracts from the nts, nod^-, and wild-type plants is similar. The use of extracts for the induction studies appeared to be more sensitive for determining if a plant did have compounds capable of inducing *nod* genes. However, Yelton *et al.* (1987) recently pointed out that there were differences in some plants between the internal and external exudate contents which may be attributed to synthesis or the export of these compounds. Whether or not this was the case for the nts382 and nod49 lines remains to be examined, but based on the results presented here, it does not appear to be likely. Another yet unexplored facet involved the analysis of internal compounds after inoculation. The experimental system described here centered on the use of uninoculated seedlings and their root extract (or exudate for the pretreatment studies). It was conceivable that plants altered

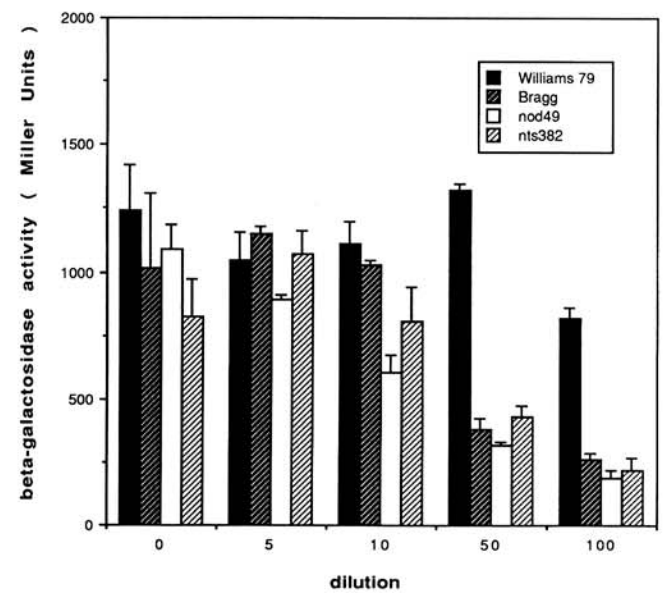

Fig. 3. Extract comparisons from uninoculated soybean roots. Extractions and assays using *lac*-fusion plasmid pEA2-21 were done as described in the text. The extracts had different initial concentrations and were as follows: Bragg, 1,710 μg/ml; Williams, 1,945 μg/ml; nod49, 1,650 μg/ml; and nts382, 1,638 μg/ml. Dilutions were conducted and plotted together because of the similarity of the initial extract concentrations. Data are means (± standard deviation).

the spectrum of inducing (and inhibitory) compounds following infection and that the mutants could differ from the wild type in this response.

Our data suggest that nts and nod$^-$ mutants of soybean were affected in other symbiotic functions which do not relate to the symbiotic signaling involving root exudate or extract from uninoculated roots. For nts mutants, we suggest the absence of an internally translocated shoot factor, which inhibits further nodulation in wild type following early nodulation events. Nonnodulation mutants like nod49 and nod139 are presumably unable to interact effectively with *Bradyrhizobium*, especially as increased cell inoculation titers increase the frequency of occasional nodules (Mathews *et al.* 1987). This presumes that the bacterium produces factors which initiate subepidermal cell divisions and pseudoinfection formation in response to the *nodD* cascade activation. The nonnodulation mutants thus would have a higher threshold to the activating principle of this substance, which at present is not isolated or defined.

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