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Rapid Colored-Nodule Assay for Assessing Root Exudate-Enhanced Competitiveness of *Bradyrhizobium japonicum*

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The effects of root exudate (RE) treatment on nodule occupancy by *Bradyrhizobium japonicum* were investigated by a rapid colored-nodule assay, which is based on the observation that *B. japonicum* L-110 and its antibiotically marked derivatives form dark-red nodules on certain soybean (*Glycine max*) cultivars, whereas other strains form beige nodules. The efficacy of the assay was confirmed by direct immunofluorescence and by antibiotic platings of nodule bacteria. Both logarithmic- and stationary-phase cultures of the reference strain, L-110Nal, were used in paired-competition studies with RE-treated or untreated cells of seven challenge strains. On the basis of field and greenhouse competition studies, these strains were placed into three competitiveness groups: high (AN-11, AN-16aStrRif, and AN-6), intermediate (AN-3 and 122SR), and low (I-110ARS and AN-18). Seedlings of *G. max* cv. Centennial were inoculated with two ratios of challenge to reference strain, 1:1 and 1:9, and nodule occupancy was determined after the V4 to V5 stage of ontogeny. Two of the strains showed increased occupancy in response to RE treatment at the 1:1 inoculation ratio. Logarithmic- and stationary-phase cultures of AN-6 showed increased occupancy, from 22 to 38% ($P < 0.10$) and from 23 to 39% ($P < 0.05$), respectively. While the maximum increase for stationary-phase cultures of AN-16aStrRif was from 34 to 47% ($P < 0.05$), logarithmic-phase cultures failed to respond to RE treatment. The results of these studies indicate that RE treatment increases the nodule occupancy of some, but not all, *B. japonicum* strains and that the colored-nodule assay could be rapidly and reliably used to determine the competitive ability of *B. japonicum*.

The soil bacterium *Bradyrhizobium japonicum* invades and forms a nitrogen-fixing symbiosis with soybeans (*Glycine max*). Of major concern in the introduction of improved *Bradyrhizobium* inoculant strains is their ability to compete successfully with indigenous soil populations of rhizobia (6, 14, 16, 19). Results of several studies have suggested that factors such as rhizosphere growth rates (27), temperature (30), rhizosphere numbers (23, 32, 36), bacterial antagonism (33, 34), and preferential host selection (31) may alter the competitiveness of a given rhizobial strain.

While the early periods of the *Bradyrhizobium*-soybean symbiosis are important for nodulation and competition (17), most of our knowledge of plant-bacterium interactions has been limited to postinfection events. Recently, substances found in legume root exudates (RE) have been shown to influence early events in the *Rhizobium*-legume symbiosis. Results from several studies indicate that RE may affect the chemotaxis of a particular strain to the host plant (7, 8), elicit a faster nodulation response (2), cause a phenotypic reversion in a slow-to-nodulate *B. japonicum* mutant (13), cause the development of lectin-binding receptors (3), and induce symbiosis-associated genes in *Rhizobium fredii* (28) and *R. meliloti* (26). Thus far, however, there have been no reports of RE-enhanced competitiveness of rhizobia.

In most *Rhizobium* competition studies, nodule occupants have been identified by platings on antibiotic-containing media (1, 22) or by immunological means (17, 21, 24, 32). While these methods are reliable in determining the identities of nodule bacteria, they tend to be time-consuming, costly, and labor intensive. Recently, Eaglesham et al. (10) demonstrated that dark-nodule-producing strains of cowpea rhizobia are useful in determining nodule occupancy in mixed-inoculum experiments. While a dark-nodule-producing strain of *B. japonicum* has been identified (18), its use in competition assays has not been reported.

In this study, we investigated the effects of RE treatment on the competitiveness of several *B. japonicum* strains. Nodule occupancy was determined by using a newly developed colored-nodule assay which allowed for the simple, rapid, and reliable determination of the competitive ability of *B. japonicum* strains.

MATERIALS AND METHODS

***Rhizobium* strains and growth conditions.** *B. japonicum* AN-3, AN-6, AN-11, AN-18, and AN-16aStrRif were obtained from the Allied-Nitragin culture collection. Strains L-110, I-110ARS, and 122SR were obtained from the U.S. Department of Agriculture Culture Collection, Beltsville, Md. On the basis of previous competition studies in the greenhouse and at field sites in the southern United States, these strains could be divided into three competitiveness groups: high (AN-6, AN-16aStrRif, and AN-11), intermediate (AN-3 and 122SR), and low (I-110ARS and AN-18). Cultures were grown aerobically at 28°C on yeast-extract-salts (YS) medium, which was essentially that of Bishop et al. (4), except that 0.1% yeast extract (Difco Laboratories,

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TABLE 1. Nodulation pattern of L-110 and its derivatives on *G. max* cv. Centennial, Braxton, and Corsoy

Cultivar and strain	No. of nodules[a]	% Nodules[a]	
		Red	Unknown
Centennial			
L-110 W.T.	40 ± 11	76 ± 12	24 ± 12
L-110 Str	42 ± 8	77 ± 11	23 ± 11
L-110 Kan	41 ± 10	87 ± 15	13 ± 15
L-110 Nal	50 ± 4	84 ± 12	16 ± 12
L-110 Rif	41 ± 11	76 ± 19	11 ± 15
Braxton			
L-110 W.T.	39 ± 11	87 ± 6	14 ± 6
L-110 Str	38 ± 10	89 ± 4	11 ± 4
L-110 Kan	39 ± 7	85 ± 13	15 ± 13
L-110 Nal	37 ± 5	89 ± 9	11 ± 9
L-110 Rif	40 ± 11	80 ± 18	19 ± 18
Corsoy			
L-110 W.T.	34 ± 9	89 ± 18	11 ± 18
L-110 Str	21 ± 5	83 ± 17	17 ± 17
L-110 Kan	24 ± 14	85 ± 11	15 ± 11
L-110 Nal	26 ± 9	86 ± 7	14 ± 7
L-110 Rif	27 ± 9	81 ± 13	19 ± 13

[a] Numbers are mean ± standard error of the mean for five replicates.

Detroit, Mich.) was substituted for sodium glutamate. Cultures were stored at −70°C as liquid suspensions containing 40% (vol/vol) glycerol. Mutants of strain L-110 which were spontaneously resistant to 500 μg each of streptomycin sulfate, kanamycin sulfate, or nalidixic acid per ml or to 150 μg of rifampin per ml were isolated by standard procedures. Antibiotics were obtained from Sigma Chemical Company, St. Louis, Mo. All antibiotic-resistant mutants were checked for phenotypic stability by repeated platings on YS medium and YS medium supplemented with the appropriate antibiotics and by in planta tests for nodulation.

Strain identification. *B. japonicum* strains in culture and in nodules were identified by platings on the appropriate antibiotic-containing media or by direct immunofluorescence with strain-specific fluorescent antibodies (32). In the latter case, gelatin-rhodamine isothiocyanate (5) was used to suppress nonspecific adsorption. Nodules were surface sterilized (35) prior to bacterial isolation.

Colored-nodule assay. Isolates of *B. japonicum* L-110 which were resistant to nalidix acid, streptomycin, kanamycin, and rifampin were used alone and in combination with strain AN-16aStrRif to inoculate soybean (*Glycine max* (L.) Merrill) cv. Braxton, Corsoy, or Centennial. Seeds were surface sterilized (35) and pregerminated prior to being planted. Rhizobia were grown in YS medium, and four seedlings in each of five replicate, modified Leonard jar assemblies (20) were inoculated with 1.0 ml of cell suspension to give a density of about 10^8 cells per seed. The Leonard jars contained a 1:1 (vol/vol) mixture of sand and vermiculite and received plant nutrient solution (PNS) (15) and 1 mM KNO_3 at planting. The pots were arranged in a randomized complete block design in a growth room at 28°C with a photoperiod of 14 h and illumination of 450 microeinsteins/m^2 per s. Seedlings were thinned to two per pot after 10 days and watered with nitrogen-free nutrient solution or deionized water as required. Plants were harvested after the V4 to V5 stage (12) of ontogeny (4 to 5 weeks). Nodules from the top 6.5 to 7.0 cm of the root system were picked and sorted into four color categories (red, beige, mottled, and unknown), and the percentage in each class was calculated. Small, immature, and nontypable nodules were placed in the unknown category, and nodules resulting from mixed infections were classified as mottled. Corroboration of the identity of bacteria in nodules of each class was obtained by the plating of homogenates of surface-sterilized nodules onto the appropriate antibiotic-containing medium or by immunofluorescence.

The ability of strain L-110 to produce dark nodules on other soybean cultivars was also investigated. Seedlings of *G. max* cv. Evans, Forrest, Lee, Kent, and Williams were inoculated with approximately 10^8 cells per seed, and plants were grown as described above. Plants were harvested after 4 to 5 weeks of growth, and the color of the nodules formed was determined.

RE effects on interstrain competition. Logarithmic- and stationary-phase cultures of the reference strain, L-110Nal, were used in four paired-competition experiments with RE-treated and untreated cells of the seven challenge strains, AN-3, AN-6, AN-11, AN-18, 122SR, I-110ARS, and AN-16aStrRif, of similar age to the reference strain. To allow comparisons between experiments, An-16aStrRif was used as an internal standard in all four experiments. Reference and challenge strains were grown axenically in YS medium to the appropriate growth phase as determined spectrophotometrically with a Klett Summerson colorimeter equipped with a number 66 red filter. Cells were pelleted by centrifugation at 10,000 × *g* for 10 min and suspended to a concentration of 2×10^8 cells per ml in either PNS (13) or RE from 9-day-old plants. RE was prepared from *G. max* cv. Centennial seedlings essentially as described previously (13). Plants were aseptically grown in half-strength PNS at 28°C with a photoperiod of 14 h and illumination of 450 microeinsteins/m^2 per s. PNS containing the RE was pooled from several plants, centrifuged at 15,000 × *g* and filter sterilized by passage through a 0.45-μm-pore-size filter. Cells were incubated with RE for 21 h at 28°C, and suspensions containing about 2×10^7 cells per ml were prepared in PNS at two ratios of challenge to reference strain, 1:1 and 1:9. *G. max*. cv. Centennial seedlings were inoculated with 1-ml portions of the resulting suspensions. Actual cell ratios in inocula were confirmed by plating dilutions onto the appropriate selective media. Plants were grown, nodules were harvested, and the proportion of nodules occupied by each strain was determined as described above.

To test for significant effects of RE treatment on occupancy, eight comparisons (four strains and two inoculum ratios) were made for each of the four experiments by using standard analysis of variance techniques. An increase in occupancy was calculated such that, if RE had no effect within a given experiment, there would only be a 5% (or 10%) chance that this increase would be exceeded for one or more of the eight comparisons. This is known as a one-sided test (experiment level) at $\alpha = 0.05$ (0.10), adjusted for multiple comparisons.

RESULTS

Colored-nodule assay. Wild-type *B. japonicum* L-110 or its antibiotic-resistant derivatives formed an average of about 40 nodules per pot on soybean cv. Centennial and Braxton and about 26 on soybean cv. Corsoy (Table 1). While an average of 80% of the nodules formed by the wild type and the mutants were small and dark red, the remainder, ranging from 11 to 24% of the nodules per pot, were small and white. Nodules of this latter phenotype were placed in a category called unknown. They tended to be located predominantly

TABLE 2. Nodule occupany of L-110 and its derivatives in paired competition assays with AN-16aStrRif on *G. max* cv. Centennial, Braxton, and Corsoy

Cultivar and strain	No. of nodules[a]	% Nodules[a]			
		Red	Beige	Mottled	Unknown
Centennial					
L-110 W.T.	36 ± 7	63 ± 8	17 ± 12	4 ± 4	10 ± 9
L-110 Str	37 ± 9	39 ± 8	48 ± 11	6 ± 6	7 ± 8
L-110 Kan	35 ± 7	22 ± 17	69 ± 27	0	8 ± 10
L-110 Nal	32 ± 5	71 ± 10	14 ± 2	6 ± 4	7 ± 6
L-110 Rif	39 ± 5	70 ± 13	19 ± 8	2 ± 2	9 ± 13
Braxton					
L-110 W.T.	30 ± 7	60 ± 17	35 ± 19	2 ± 3	4 ± 8
L-110 Str	38 ± 4	27 ± 11	59 ± 17	6 ± 12	8 ± 8
L-110 Kan	31 ± 5	11 ± 21	84 ± 21	0	6 ± 13
L-110 Nal	34 ± 9	67 ± 6	23 ± 6	1 ± 2	9 ± 9
L-110 Rif	26 ± 17	83 ± 8	15 ± 9	2 ± 2	3 ± 6
Corsoy					
L-110 W.T.	22 ± 8	67 ± 13	23 ± 18	7 ± 9	3 ± 4
L-110 Str	18 ± 3	22 ± 24	75 ± 29	3 ± 3	0
L-110 Kan	20 ± 4	21 ± 27	77 ± 31	0	2 ± 3
L-110 Nal	28 ± 9	64 ± 16	24 ± 17	1 ± 1	6 ± 5
L-110 Rif	22 ± 5	80 ± 12	9 ± 7	4 ± 8	7 ± 8

[a] Numbers are mean ± standard error of the mean of five replicates.

on the lower portions of tap roots or on laterals and were probably immature nodules resulting from later infections.

Dark-red nodules were also observed when wild-type strain L-110 was inoculated onto *G. max* cv. Kent and Lee. On cv. Evans, small brown nodules were formed, and on cv. Williams and Forrest, beige nodules were formed (data not shown). Thus, formation of the small red-nodule phenotype is dependent on the soybean cultivar used.

When plants were inoculated with 1:1 mixtures of wild-type strain L-110 or its mutants and *B. japonicum* AN-16aStrRif, the average nodule number per plant remained about the same as in the single-inoculum experiments with strain L-110. However, four nodule phenotypes were observed (Table 2). Small dark-red nodules produced by wild-type strain L-110 and its mutants were easily distinguished from the large beige nodules of AN-16aStrRif (Fig. 1). Mixed infections containing both inoculum strains were evident as mottled nodules and occurred at frequencies of 0 to 7% in all treatments. Nodules of unknown phenotype, representing 0 to 10%, were also observed.

We used immunofluorescence or antibiotic platings to corroborate the identities of the occupants of red and beige nodules. When 84 red nodules from an L-110Nal:AN-16aStrRif competition experiment were examined by immunofluorescence, 78 (92.9%) reacted positively. All 15 of the beige nodules examined reacted positively to antiserum raised against AN-16aStrRif. Also, all the beige nodules of 122SR, I-110ARS, and AN-11 examined in later experiments reacted positively to strain-specific antisera. Antibiotic resistance platings of 10 red and 10 beige nodules from an L-110Nal:AN-6 competition experiment resulted in 100% recovery in the appropriate media.

The L-110 mutants exhibited marked differences in competitive abilities that appeared to be cultivar independent (Table 2). While mutants resistant to streptomycin and

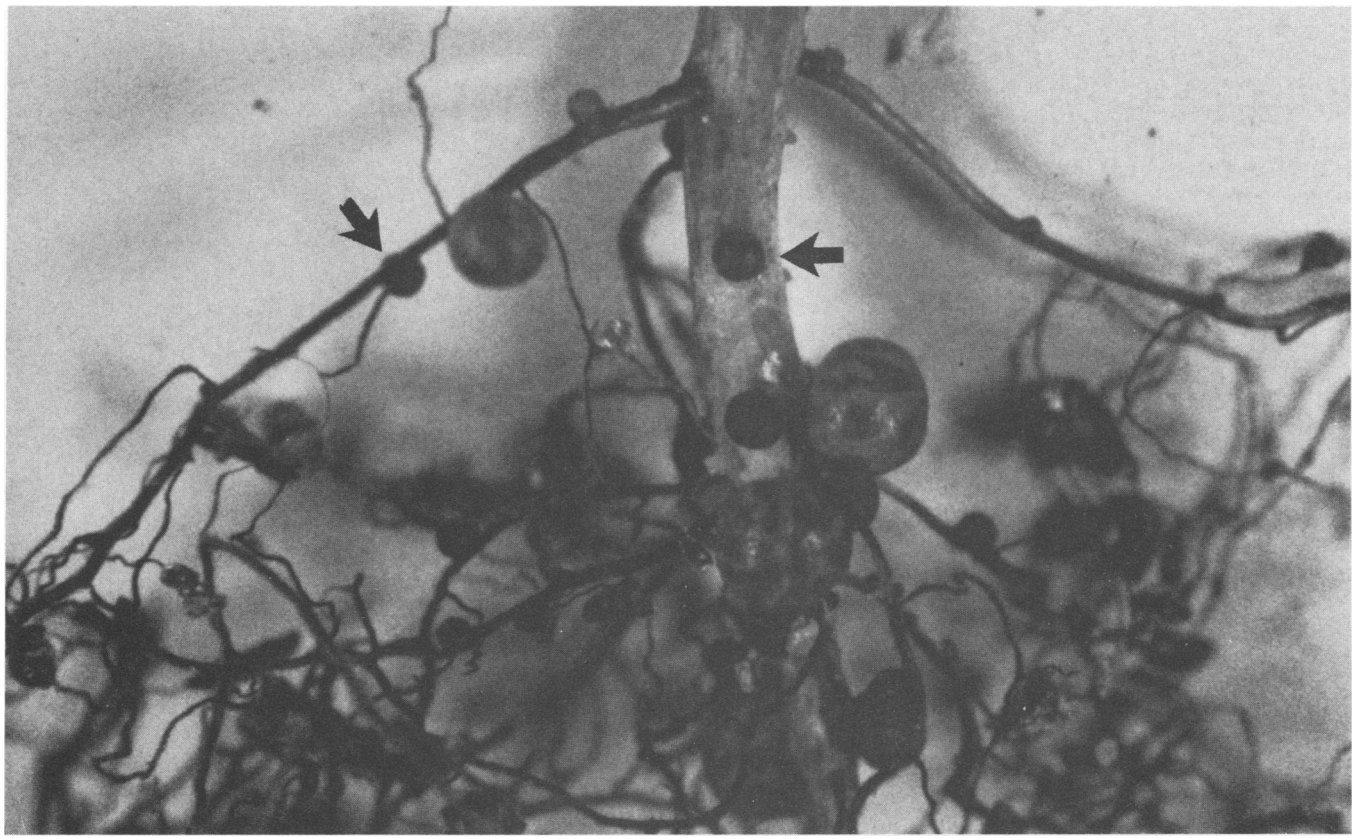

FIG. 1. Root system of *G. max* cv. Centennial showing the small dark-red nodules of L-110Nal (arrows) and the large beige nodules of AN-16aStrRif. The small white nodule near the crown is of the unknown phenotype described in the text.

TABLE 3. Effect of RE pretreatment on nodule occupancy by seven challenge strains in paired competition assays with reference strain L-110Nal.

Cell growth phase and challenge strain	Nodule occupancy by challenge strain (%)[a] at following ratio of challenge strain to L-110Nal			
	1:9		1:1	
	+RE	−RE	+RE	−RE
Logarithmic				
AN-18	4	5	4	5
I-110ARS	5	2	11	12
AN-11	9	5	12	9
AN-16aStrRif	11	10	30	31
Stationary				
AN-18	5	1	9	4
I-110ARS	6	4	24	25
AN-11	4	5	15	19
AN-16aStrRif	23	22	65	57
Logarithmic				
AN-6	9	15	38	22*
AN-3	10	19	21	20
122SR	25	22	36	35
AN-16aStrRif	32	28	73	63
Stationary				
AN-6	9	4	39	23**
AN-3	9	9	15	13
122SR	7	3	22	27
AN-16aStrRif	15	15	47	34*

[a] For each ratio of challenge to reference strain, the means of untreated samples were significantly increased by RE treatment at the $\alpha = 0.05$ (**) or 0.10 (*) levels.

kanamycin were inferior to the wild type in terms of the number of nodules formed and the competitive ability on all three cultivars, mutants resistant to nalidixic acid and rifampin were the most similar to the wild type. However, rifampin resistance was unstable, and for this reason, L-110Nal was selected for use in further experiments.

RE effects on interstrain competition. The influence of RE treatment on the proportion of nodules formed by the seven challenge strains in competition with L-110Nal are shown in Table 3. Two of the strains responded positively to RE treatment at the 1:1 ratio; there were no significant effects of RE at the 1:9 ratio. Logarithmic- and stationary-phase cultures of AN-6 showed increased occupancy from 22 to 38% ($P < 0.10$) and 23 to 39% ($P < 0.05$), respectively. Stationary-phase cultures of AN-16aStrRif showed an increase from 34 to 47% ($P < 0.05$) in one experiment and from 56 to 65% (almost significant; $P < 0.10 = 9.0\%$) in another. Logarithmic-phase cultures of this strain also approached a level of significance in one of two experiments.

The superior competitive ability of L-110Nal over the other strains was quite evident. At the 1:1 inoculum ratio in the absence of RE treatment, it outcompeted all but AN-16aStrRif. At the 1:9 ratio, only AN-16aStrRif was consistently as competitive as, or more competitive than, L-110Nal.

Statistical analysis of the data revealed that a minimum of 25 nodules per pot were required to detect statistically significant treatment effects. Therefore, while the mean number of nodules per pot in the four experiments varied between 40 and 80 (data not shown), this variation did not affect our ability to detect statistically significant treatment effects. In addition, since the nodules that were determined to be of unknown phenotype by visual criteria represented a relatively small fraction (mean of 4.99%) of the total number of nodules, they were not included in percentage occupancy values.

DISCUSSION

Rhizobium competition experiments can often be encumbered by the need to type large numbers of nodules. In such cases, the typing of nodules by visual means, as described in this report, offers advantages in both time and simplicity over more standard typing techniques such as antibiotic resistance or immunofluorescence staining (e.g., 25, 29). In addition, our visual typing results showed excellent agreement with the other methods and therefore did not appear to sacrifice accuracy.

A potential drawback of the visual typing assay stems from the occurrence of nodules which are untypable by this method. These small, unpigmented nodules, however, were always found in our experiments to represent a relatively small proportion of the total number and could be typed by other means or, alternatively, disregarded without affecting the conclusions drawn.

Our data on soybeans and those of Eaglesham et al. (10) on cowpeas show that expression of the dark-nodule phenotype is restricted to very few host-strain combinations. Therefore, a preliminary screening must be done to select the best combination for competitive assays, as was done here. An understanding of the nature of the colored compound and the genetic basis for its formation may make it possible in the future to increase, by genetic manipulation, the number of associations with this characteristic. In that regard, preliminary work has shown that the colored substance in cowpea nodules is not a heme or modified heme compound (9, 10) but possibly an anthro- or naphthoquinone (9).

Our initial application of the visual nodule-typing method was in an examination of RE pretreatment effects on the competitiveness of seven *B. japonicum* strains. Increased nodule occupancy relative to the L-110Nal reference strain resulted from such pretreatments in two (AN-6 and AN-16aStrRif) of the seven strains examined, although in the latter the response could not always be duplicated. These two strains, as well as strain AN-11, had been classified as highly competitive on the basis of data from several field studies in the southern United States. That AN-11, which belongs to serogroup 123, was not influenced by RE pretreatment could be due to properties peculiar to this group. Members of this serogroup are known to be poor competitors against members of serogroup 110 under gnotobiotic conditions (17; R. M. Zablotowicz, unpublished results) but excellent competitors in several field studies (11, 25).

We did not obtain a consistent response with AN-16aStrRif, nor did we observe a response with any strain at the 1:9 ratio. The possibility exists that modification of one or more of the parameters used in this first series of experiments, such as the number of cells in the inoculum or pretreatment with an increased concentration of RE, could result in even more dramatic and universal responses. Further investigation into the identity of the component(s) in RE which influences the infection process could also lead to a more conclusive demonstration of their effects on competitiveness.

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