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Short Communication

Inoculum Density-Dependent Restriction of Nodulation in the Soybean-*Bradyrhizobium japonicum* Symbiosis

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Abstract

Bradyrhizobium japonicum USDA 110 is restricted for nodulation by *Glycine max* PI 417566 at relatively high inoculum levels (10^8–10^9 cells). Nodulation restriction conditioned by PI 417566 was suppressed at inoculation levels of 10^4–10^6 cells, although nodulation was delayed relative to that seen on a permissive soybean genotype. Over the range of concentrations tested (10^4–10^9 cells) on soybean cv. Kasota, there was no influence of inoculum concentration on nodulation by USDA 110 or by D4.2-5, a *Tn5*-induced, nodulation-competent mutant of USDA 110. The few nodules produced by USDA 110 on PI 417566 contained normal levels of nodulin-23, nodulin-26, cdc-2 gene, and LB23. Phenylalanine ammonia lyase mRNA levels were about 15% greater, 2 days post inoculation, when PI 417566 was

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inoculated with USDA 110 than was seen when strain D4.2-5 was the inoculum. There were no differences seen in the expression of chalcone synthase in nodulation-permissive or -restrictive plant genotypes inoculated with USDA 110 or D4.2-5. These results suggest that inoculum size and host defense-related responses may influence host-controlled restriction of nodulation in the *B. japonicum* soybean symbiosis.

Keywords: *Bradyrhizobium japonicum*, nodulation-restriction, inoculum density, phenylalanine ammonia lyase (PAL), chalcone synthase (CHS)

Bradyrhizobium japonicum forms nitrogen fixing nodules on the roots of soybean (*Glycine max*) plants. The early stages of nodulation involve bi-directional signal exchange between the plant and bacterium, with each member of the symbiosis playing an important role in the initiation and regulation of nodulation and nitrogen fixation (for review see Pueppke, 1996).

Soybean genotypes are differentially nodulated by strains of *B. japonicum*, and certain soybean genotypes restrict nodulation by specific strains or serogroups of Bradyrhizobium (Vest et al., 1973; Cregan and Keyser, 1986; Cregan et al., 1989a; Cregan et al., 1989b; Weiser, et al., 1990; Ferrey, et al., 1994). In 1989 we reported the identification of soybean genotype PI 417566, which restricts nodulation and reduces the competitiveness of strain USDA 110 (Cregan et al., 1989a; Cregan et al., 1989b) and several other serogroup 110 strains (Lohrke, et al., 1995). Reciprocal grafting studies showed that restricted nodulation conditioned by PI 417566 is due to soybean root factors and that plant growth temperatures influence host-controlled nodulation restriction (Sadowsky, et al., 1995). In addition, we determined that a single recessive host gene (tentatively called RJ 110) in PI 417566 conditions restriction of nodulation by *B. japonicum* USDA 110 (Lohrke et al., 1995). We also have reported that a *Tn5* mutant of *B. japonicum* USDA 110, strain D4.2-5, had the ability to overcome nodulation restriction conditioned by PI genotype 417566 (Lohrke et al., 1995).

The concentration of *B. japonicum* found in soil plays an important role in determining the extent of nodulation under natural conditions (Takats, 1986; Ferrey et al., 1994; Pazdernik et al., 1997). Moreover, inoculation with high levels of *B. japonicum*, similar to those not normally found in field soils, increases the length of time required to initiate nodulation, decreases total nodule number, and significantly alters the pattern of nodules found on the root (Pierce and Bauer, 1983; Takats, 1986).

To determine if inoculum concentration also influences the interaction of *B. japonicum* with the nodulation-restricting soybean genotypes, we conducted nodulation studies using PI417566 and the nodulation permissive soybean cv.

Kasota inoculated with different concentrations of USDA 110 and mutant D4.2-5. Soybean seeds were surface-sterilized as described (Vincent, 1970) and germinated, for 2–3 days, at 28°C in vermiculite. Individual seedlings of each genotype were aseptically transferred to each of a series of sterile growth pouches (Mega International, Minneapolis, MN) and watered with 10 ml of nitrogen-free nutrient solution (Keyser and Cregan, 1987). Mid-log phase, AG-grown (Sadowsky et al., 1987) cultures of wild-type USDA 110 and the mutant strain (at about 10^9 cells/ml) were diluted to 10^4 cells/ml in sterile AG medium and 1.0 ml of each dilution was used to inoculate individual soybean seedlings. Fifteen growth pouches were inoculated with each dilution. The influence of low inoculum density on the ability of USDA 110 to nodulate PI 417566 was repeated 3 times.

Plants were incubated at 20°C in a growth chamber as described (Sadowsky et al., 1995). Plants were watered every day and plant roots were examined daily for the appearance of nodules. Data were log-transformed and analyzed with the LIFETEST Procedure of SAS (Cary, NC) at $\alpha = 0.05$. At 21 days post-inoculation, plants were examined for nodule number and the location of nodules on the tap root, relative to the root tip mark, were determined using a dissecting microscope (Bhuvaneswari et al., 1980). The root tip mark is defined as the location of the root tip in the growth pouch at the time of inoculation.

Results in Fig. 1A show that while *B. japonicum* USDA 110 has been characterized as being restricted for nodulation by PI 417566, this is clearly a function of inoculation and plant growth conditions. Diluted inocula (10^4–10^6 cells) of USDA 110 overcame nodulation restriction conditioned by this plant introduction genotype. When USDA 110 was inoculated onto PI 417566 at 10^4 cells, nodules first appeared 13 days after inoculation and 100% of the plants were nodulated after 18 days (Fig. 1A). In contrast, at 10^9 cells of USDA 110, only 13% of PI 417566 plants had nodules, even after 20 days of incubation. Over the range of USDA 110 inoculum concentrations examined, there is an apparent inverse relationship between inoculum size and the ability of strain USDA 110 to overcome nodulation restriction conditioned by PI 417566. On PI 417566, strain USDA 110 took significantly longer to produce nodules when inoculated with 10^6 cells than with 10^4 cells ($\chi^2 = 1.02$). Generally speaking, the lower the inoculum dosage of USDA 110 applied to PI 417566, the greater the percentage of plants that was nodulated, with the cut-off concentration for nodulation being about 10^7 cells.

Nodulation restriction conditioned by PI 417566 was not completely overcome by diluting the inoculum. Whereas 10^4 cells of USDA 110 produced an average of 6.5 nodules per plant on PI 417566, 10^4 cells of mutant D4.2-5 produced an average of 20.7 nodules per plant on the same genotype. Moreover, at 10^4 cells, it took significantly longer for USDA 110 to produce nodules on PI 417566 than did strain D4.2-5 ($\chi^2 = 1.61$).

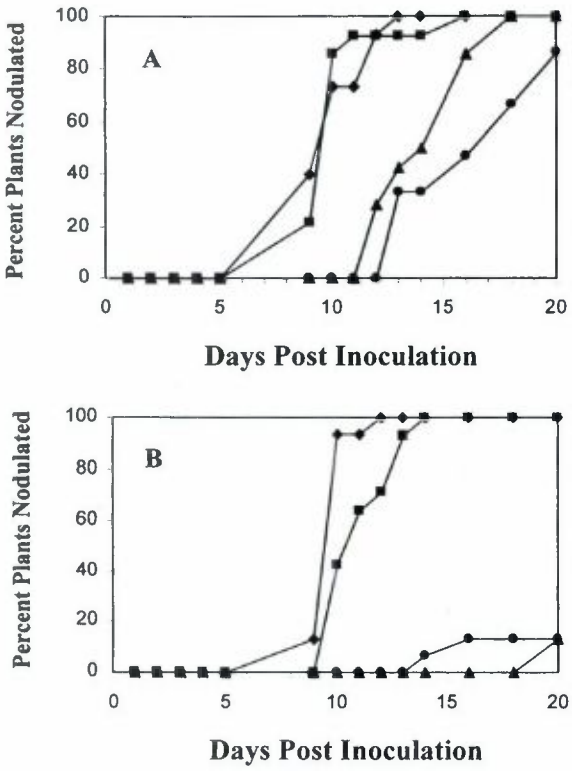

Figure 1. Timing and extent of nodulation by several concentrations of *B. japonicum* strains USDA 110 or D4.2-5 on Glycine max PI 417566. (A) 10^4 cells/ml USDA 110 (▲), 10^6 cells/ml USDA 110 (●), 10^4 ells/ml D4.2-5 (♦), and 10^6 cells/ml D4.2-5 (■); (B) 10^7 cells/ml USDA 110 (▲), 10^9 cells/ml USDA 110 (●), 10^7 cells/ml D4.2-5 (♦), and 10^9 cells/ml D4.2-5 (■). Fifteen plants of each treatment were analyzed at each time point. Data was analyzed for statistical significance using the LifeTest procedure of SAS at the $\alpha = 0.05$.

When 10^4 or 10^9 cells of D4.2-5 was inoculated on PI 417566, visible nodules were first formed 9 days after inoculation, and 100% of plants had nodules after 13–14 days (Fig. 1A). All PI 417566 plants inoculated with intermediate amounts of mutant D4.2-5, 10^5–10^8 cells, produced nodules (mean = 16.3 nodules/plant) about 14 days after inoculation. When PI 417566 was inoculated with either 10^4 or 10^9 cells of strain D4.2-5, there was no statistical difference in the timing of nodulation ($\chi^2 = 0.162$).

B. japonicum strain USDA 110 nodulated the permissive soybean cv. Kasota approximately equally at the 10^4 and 10^9 cell inoculation levels, with 100% of

the plants showing nodulation 11–12 days post inoculation (Fig. 1B) and plants producing an average of 7.5 and 9.6 nodules/plant, respectively. Relative to strain USDA 110, mutant D4.2-5 had a slight delay, 1–3 days, in the nodulation of cv. Kasota. Nevertheless, all plants receiving the 10^9 cells inoculum rate were nodulated 13 days post inoculation, and 100% of plant receiving 10^4 cells were nodulated 18 days post inoculation. Similar trends were obtained when cv. Kasota was inoculated with strain USDA 110 at the intermediate levels of 10^5–10^8 cells and initial nodulation occurred about 9 days after inoculation and all plants were nodulated after 12–14 days (data not shown). Likewise, soybean cv. Kasota plants inoculated with intermediate levels (10^5–10^8 cells) of strain D4.2-5 had initial nodule appearance after 10–11 days and all plants were nodulated 13–17 days after inoculation.

When USDA 110 and the compatible mutant strain D4.2-5 were individually inoculated at 10^4 cells, there was a dramatic difference in the nodule distribution pattern on PI 417566 (Fig. 2). Nodules produced by strain USDA 110 on PI 417566 were mostly located about 7–10 cm below the root tip mark, and plants had relatively low numbers of nodules (Fig. 2A). Similar nodule distribution studies done with USDA 110 at the 10^5–10^6 cell inoculation levels showed that the majority of nodules were located around 8–10 cm below the root tip mark, although as expected, there were fewer nodules produced and some were scattered farther up the root system (data not shown). In contrast, when 10^4 cells of mutant strain D4.2-5 was inoculated onto PI 417566, there was about a 5-fold increase in the number of nodules, most of which were located near the root tip mark. As expected, while PI 417566 restricted nodulation by USDA 110 at the 10^9 cell level, mutant D4.2-5 efficiently nodulated this host (Fig. 2B). Moreover, the majority of D4.2-5 nodules were located near the root tip mark, indicating relatively early nodulation by this strain.

Both strain USDA 110 and mutant D4.2-5 nodulated the permissive soybean cv. Kasota at the 10^4 cell level (Fig. 2C). While the majority of nodules produced by strain D4.2-5 were located near the root tip mark on cv. Kasota, nodules produced by USDA 110 were scattered along the tap root. With the 10^9 cell inoculum similar nodulation distribution patterns were seen for strain USDA 110 and mutant D4.2-5 (Fig. 2D). However, the number of nodules produced by mutant D4.2-5 at the 10^9 cell level was less than that seen at the 10^4 cell level. The nodule distribution patterns produced by USDA 110 and mutant D4.2-5 on cv. Kasota were similarly centered around the root tip mark at the intermediate inoculation levels of 10^5–10^8 cells (data not shown), suggesting rapid and efficient nodulation of this host. Takats (1986) also reported that the distribution of nodules on the root changes with respect to inoculum dosage and that greater amounts of inoculum suppresses nodulation. Taken together, our result indicate that lower inoculant rates allow USDA 110 to partially overcome nodulation restriction, which is conditioned by PI 417566

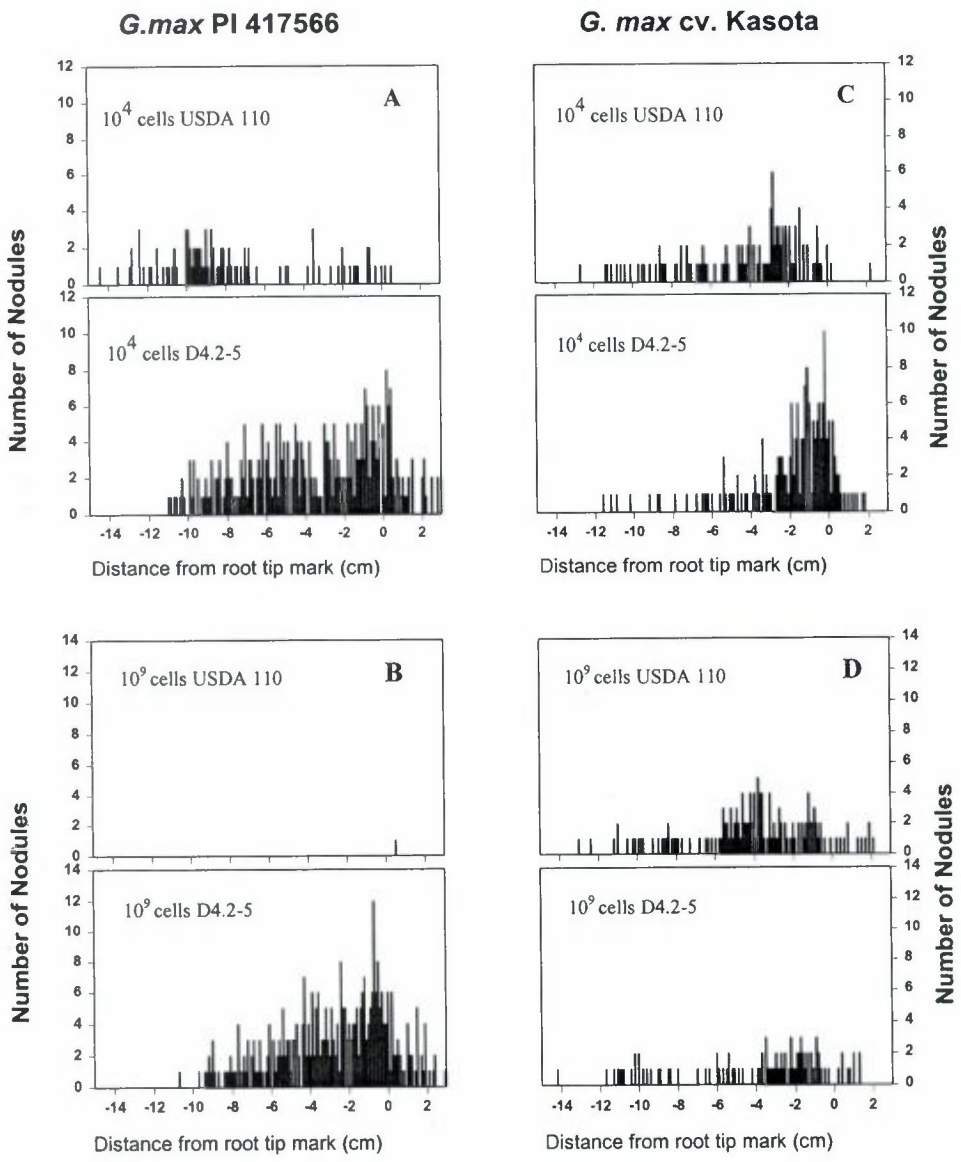

Figure 2. Distribution of nodules on tap roots of *Glycine max* PI 417566 and (A and B) cv. Kasota (C and D) following inoculation with 10^4 or 10^9 cells of *Bradyrhizobium japonicum* strain USDA 110 or mutant strain D4.2-5. Values are expressed as distance in cm from the root tip mark made at time of inoculation.

at the higher inoculum levels. However, nodulation is delayed relative to a nodulation permissive strain, such as mutant D4.2-5.

We previously reported that USDA 110 produces few nodule primordia, and limited numbers of mature nodules on PI 417566, suggesting that nodulation restriction in this PI genotype could be the result of aborted infections occurring after initial plant-microbe interactions (Lohrke et al., 1995; Sadowsky et al., 1995). At this time, the physiological and genetic reasons for this response were unknown. However, Vasse et al. (1993) indicated that in alfalfa the abortion of infection threads was linked to activation of a hypersensitive plant response.

Phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) are enzymes involved in the biosynthesis of phenylpropanoid compounds. PAL and CHS are encoded by a gene family (Estabrook and Sengupta-Gopalan, 1991). PAL is one of the first enzymes in the pathway, and catalyses deamination of phenylalanine to cinnamic acid. CHS, which is further downstream in the pathway, subsequently condenses three molecules of malonyl-CoA with cinnamyl-CoA to produce chalcone (Estabrook and Sengupta-Gopalan, 1991), a flavonoid. Plant pathogenic and symbiotic microorganisms have been shown to induce host defense responses, including the production of phenylpropanoid compounds (Nap and Bisseling, 1990; Yang et al., 1992) and increase the level of PAL and CHS transcripts (Estabrook and Sengupta-Gopalan, 1991; Grosskopf et al., 1994). Similarly, Pazdernik and coworkers (1997) reported that higher levels of expression of the plant defense gene, phenylalanine ammonia lyase (PAL) in PI 437153A, occurred during ineffective nodulation by *B. japonicum* UMR 161, as compared to levels when PI 437153A was inoculated with a compatible strain. These results suggest that plant defense-related responses, including the induction of genes involved in phenylpropanoid metabolism, may be activated by inoculation of legumes with inappropriate rhizobia and may be involved in the incompatibility of USDA 110 with PI 417466.

To determine if host defense mechanisms and specific physiological responses may be involved in limiting infection and nodulation by USDA 110 on PI 417566, we examined inoculated plants for the induction of PAL, chalcone synthase (CHS), and the activation of genes encoding early and late nodulin genes. Seeds of cv. Kasota and PI 417566 were surface-sterilized (Vincent, 1970) and germinated for 2–3 days at 28°C in a sterile mixture of vermiculite and perlite (3:1). After germination, seedlings were transferred to sterile plant growth pouches (Mega International, Minneapolis, MN) and Leonard jars. Two seedlings of each genotype was aseptically transferred to each sterile growth pouch. Pouches were watered with 10 ml of nitrogen-free nutrient solution (Keyser and Cregan, 1987). Leonard jars were planted with three seedlings of PI 417566 or cv. Kasota as described (Sadowsky et al., 1987; Sadowsky at al., 1991) and thinned to two seedlings per Leonard jar, 3 days after emergence. Mid-log phase, AG-grown, cultures of wild-type USDA 110 and mutant D4.2-5 were diluted to 10^8 cells per ml in sterile AG-medium and 1.0 ml of culture was used to

inoculate each seedling. Fifteen growth pouches and 10 Leonard jars were inoculated with each strain. Uninoculated (uninfected) plants served as a negative control. Plants were incubated at 20°C in a plant growth chamber as described (Sadowsky et al., 1995). Plants were alternately watered with nutrient solution or water, as needed. Plants in growth pouches were harvested 0, 2, 4, and 8 days after inoculation and Leonard jar-grown plants were harvested after 35 days. Harvested plant tissue and nodules were immediately frozen in liquid nitrogen and RNA was isolated as described (De Vries et al., 1988).

Total nodule and root tissue RNA (2 and 5 μg/well) from all host X strain combinations and RNA from uninfected root tissue were dot-blotted onto precut nitrocellulose filters (BRL), using a Hybri-Dot Manifold (BRL), as described (Boundy-Mills et al., 1994; Sambrook et al., 1989). Filters used for the PAL and pcdc2-S5 probes (cdc-2, a cell cycle gene) had 10 and 20 μg of RNA/well. Filters were hybridized to ^{32}P-labelled nodulin, and PAL gene probes as described (Sambrook et al., 1989). The CHS, PAL (Estabrook and Sengupta-Gopalan, 1991), E10, and E41 clones were obtained from Champa Sengupta-Gopalan, New Mexico State University, Las Cruces, NM and the nodulin clones [pNOD25 (nodulin-23), pNOD26 (nodulin-26), pcdc2-S5, pLB23 (leghemoglobin), E10, E41, and pCPGS2 (cytosolic glutamine synthetase)] were obtained from Dr. D.P.S. Verma, Ohio State University, Columbus, OH. Purified and ^{32}P-labeled insert DNA from PAL, pNOD25 cDNA, pNOD26 full length cDNA, pcdc2-S5, pLB23 (near full length soybean LB cDNA clone), E10, E41, and pCPGS2 (cytosolic glutamine synthase cDNA clone) were used as probes. Duplicate northern blots were analyzed and quantified by using an Ambis Radioanalytic Imaging system (San Diego, CA).

When RNA from root tissue of PI 417566 and cv Kasota plants that had been inoculated with USDA 110 or D4.2-5 were probed with the cDNA clone corresponding to the PAL gene, a differential response between compatible and incompatible interactions was noted. Generally speaking, with PI 417566, a higher level of PAL mRNA in infected root tissue was found when strain USDA 110 was the inoculum than when the compatible strain, D4.2-5 was used. When PI 417566 was inoculated with USDA 110, maximal PAL activity was detected 2 days post inoculation and declined after this. There was a 15% increase (range of 9–22%) in PAL mRNA levels when PI 417566 was inoculated with USDA 110 as compared to strain D4.2-5. At 4 days post inoculation, however, PAL expression for plants inoculated with USDA 110 and mutant D4.2-5 were approximately equal, about 32% higher (range of 20–55%) than an uninoculated control. The level of PAL mRNA in uninoculated plants of both genotypes was maximal at 2 days after planting and remained at a near constant, but low level over the incubation period. There was little difference in total PAL mRNA

levels in cv. Kasota plants inoculated with either *B. japonicum* strain and that found with uninoculated plants.

However, PI 417566 plants inoculated with the incompatible strain USDA 110 had PAL mRNA levels on day 2 that were about 68% higher (range of 61–76%) than cv. Kasota plants inoculated with the same strain. There were no differences seen in the expression of CHS in either plant genotype inoculated with USDA 110 or D4.2-5.

Results seen here with both the PAL and CHS probes are very similar to that reported by Pazdernik et al. (1997) when PI 437153A was inoculated with an ineffective strain. Similarly, soybean inoculated with incompatible rhizobia or fungal pathogens and *R. meliloti* inoculated with a Fix$^-$ mutant resulted in higher levels of PAL or CHS mRNA relative to controls (Estabrook and Sengupta-Gopalan, 1991; Groskopf, et al., 1994). The "plant defense related" induction of PAL and CHS has been also observed during fungus (*Phytopthora sojae*) infection of *Glycine max* (Habereder et al., 1989). Results of our studies suggest that nodulation-incompatible host/strain combination result in increased accumulation of PAL messages, suggesting that host-defense responses may be activated by these strains and this response might limit nodulation ability by specific *B. japonicum* strains.

All of the nodulin probes hybridized to both 2 and 5 μg of nodule RNA from all host-strain combinations, but failed to hybridize to RNA from uninfected PI 417566 and cv Kasota roots (data not shown). Generally speaking, there was no difference in the hybridization signal when RNA was isolated from nodules produced by the nodulation restricted strain, USDA 110, or mutant D4.2-5. However, PI 417566 and cv. Kasota inoculated with mutant D4.2-5 had nodule RNA which gave a stronger hybridization signal with the nodulin-26 probe than did the same genotypes inoculated with USDA 110. A similar result was seen when the glutamine synthase probe was used. In addition, nodulin probes E10 and E41 hybridized more strongly to all of the RNAs than did the other nodulin gene probes used, regardless of the source of the RNA. Taken together, these results indicate that despite the incompatibility between strain USDA 110 for nodulation of PI 417566, the few nodules that are produced by the interaction have a full complement of early and late nodulin mRNAs, including leghemoglobin.

In conclusion, it appears that relatively low inoculum levels suppress host-controlled restriction of nodulation in the soybean-*B. japonicum* symbiosis. Our results, however, also indicate that it is necessary to examine the relationship between rhizobia and host plants under ecologically relevant conditions. In some respects, our results are similar to those reported by Takats (1986) and Pierce and Bauer (1983), who reported that there is an optimal inoculum level which results in maximum nodulation. While their studies were done with nodulation-permissive, host-strain combinations, the end result appears to be

the same. Similarly, our studies and those of Ferrey et al. (1994), show that the distribution of nodules on the root is influenced by inoculum level, suggesting that the density of rhizobia influences the efficiency and timing of nodulation. This may be due to a type of autoregulatory response (Pierce and Bauer, 1993), similar to that reported by Takats (1986) for relatively high doses of *B. japonicum* strains on compatible soybean genotypes. Results from our studies also suggest that plant defense responses, particularly the induction of the PAL gene, may be involved in limiting nodulation by specific *B. japonicum* strains. While our data, at this time, is correlative, such responses have been previously noted in the interaction of *B. japonicum* strain USDA 123 with *G. soja* (Parniske et al., 1990) and in the interaction of an ineffective *B. japonicum* strain, UMR 161, with PI 437153A. Future studies will determine whether other host defense-related mechanisms are activated during the interaction between nodulation-restricting soybean genotypes and incompatible *B. japonicum* strains and whether these responses limited nodulation.

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