A Positive Role for Rhizobitoxine in *Rhizobium*-legume Symbiosis

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Although *Bradyrhizobium elkanii* is a mutualistic symbiont of legumes, it synthesizes a phytotoxin, rhizobitoxine, that causes chlorosis on a variety of legume hosts, giving a pathogenic character to these interactions. No positive role for rhizobitoxine has been previously demonstrated. Interestingly, rhizobitoxine inhibits the rate-limiting step for ethylene biosynthesis, a plant hormone known to inhibit or down-regulate nodule development. We hypothesized that rhizobitoxine plays a positive role in nodule development through its inhibition of ethylene biosynthesis. To test this hypothesis, host plants of *B. elkanii* were screened for a differential nodulation response to the wild-type and rhizobitoxine mutant strains. In *Vigna radiata* (mungbean), the rhizobitoxine mutant strains induced many aborted nodules arrested at all stages of pre-emergent and post-emergent development and formed significantly fewer mature nodules than the wild type. Experiments revealed that nodulation of mungbean plants is sensitive to exogenous ethylene, and that the ethylene inhibitors aminoethoxyvinylglycine and Co²⁺ were able to partially restore a wild-type nodulation pattern to the rhizobitoxine mutants. This is the first demonstration of a nodulation phenotype of the rhizobitoxine mutants and suggests that rhizobitoxine plays a positive and necessary role in *Rhizobium*-legume symbiosis through its inhibition of ethylene biosynthesis.

The symbiotic relationship between nitrogen-fixing rhizobia and legume plants has been studied for over 100 years as a classic example of mutualistic associations in which rhizobia exchange reduced nitrogen for the carbon and energy supplied by the legume host. Contrary to the concept of *Rhizobium*-legume symbiosis as a mutually beneficial association, it was shown 40 years ago that *Bradyrhizobium elkanii* synthesizes a plant toxin, rhizobitoxine, that induces chlorosis on a variety of plants (Johnson et al. 1959). Since then, several reports have appeared on the structure (Owens et al. 1972), biochemical activities (Owens et al. 1968, 1971;

Yasuta et al. 1999), and genetics (Ruan and Peters 1992; Ruan et al. 1993) of this toxin. Nevertheless, no beneficial role for rhizobitoxine in *Rhizobium*-legume symbiosis has been demonstrated and its role in symbiosis has remained an enigma.

One of the early discovered biochemical activities of rhizobitoxine was the inhibition of plant ethylene biosynthesis (Owens et al. 1971), which has been shown to be mediated through inhibition of 1-aminocyclopropane-1-carboxylic acid synthase (Amrhein and Wenker 1979; Yasuta et al. 1999). Inhibition of ethylene biosynthesis by rhizobitoxine is interesting because there are several reports that demonstrate the importance of ethylene in the regulation of nodule formation. Exogenous ethylene was shown to inhibit nodule formation (Grobelaar et al. 1971; Goodlass and Smith 1979; Lee and Larue 1992), and inhibition of ethylene biosynthesis has been shown to enhance nodule formation (Peters and Crist-Estes 1989; Zaat et al. 1989). Plant studies also provided evidence that ethylene regulates nodule number (Fearn and Larue 1991; Penmetsa and Cook 1997), nodule position (Heidstra et al. 1997), and the morphology of nodules formed (Fernandez-Lopez et al. 1998).

These studies indicate that the synthesis of an ethylene biosynthesis inhibitor might modulate the efficiency with which the rhizobial symbiont nodulates its host plant. It was therefore hypothesized that rhizobitoxine plays a beneficial role in nodule formation through its inhibition of ethylene production. To test this hypothesis, mutants in rhizobitoxine biosynthesis were isolated. Attempts to demonstrate the role of rhizobitoxine in nodulation of soybean revealed no apparent difference in nodulation kinetics between the rhizobitoxine mutants and the wild-type strains (Ruan and Peters 1992). Later reports, however, showed that nodulation of soybean is insensitive to ethylene (Hunter 1993; Xie et al. 1996), which explains the insignificant difference observed between the nodulation patterns of rhizobitoxine mutants and the wild-type strain on soybean. These findings led us to investigate the importance of rhizobitoxine in nodulation of other host plants of *B. elkanii*, such as mungbean.

Here we show for the first time that rhizobitoxine is necessary for normal development of nodules on some legume hosts. Evidence is also provided to indicate that the beneficial effect of rhizobitoxine on nodule development is mediated through its ability to inhibit ethylene biosynthesis in legume hosts during symbiotic interactions.

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RESULTS

Based on the ability of rhizobitoxine to inhibit ethylene biosynthesis, we hypothesized that, despite its chlorosis-inducing activity, rhizobitoxine could play a beneficial role in nodule development. As a prerequisite for testing this hypothesis, we have previously isolated Tn5 insertional mutants of *Bradyrhizobium elkanii* that no longer synthesize the phytotoxin (Ruan and Peters 1992). Two strains, RX17E and RX18E, contain insertions in the *rtxA* gene, which catalyzes two of the early steps in rhizobitoxine biosynthesis (Ruan et al. 1993). Host plants of the *B. elkanii* strain USDA61 were screened for a differential nodulation response to the wild-type and rhizobitoxine mutant strains. We found a statistically significant difference in the number of fully differentiated mature nodules formed on the host plant mungbean. Wild-type strain USDA61 formed an average of 21.7 mature nodules per plant while two *rtxA* rhizobitoxine mutants, RX17E and RX18E, formed on average 2.9 and 1.3 nodules per plant, respectively. The reduced number of mature nodules formed by the *rtxA* mutants on mungbean is the first evidence that rhizobitoxine production actually plays a positive role in or is necessary for symbiosis.

The hypothesis that rhizobitoxine is important for nodule development on mungbean because it inhibits ethylene biosynthesis assumes that nodule formation on mungbean is sensitive to ethylene. To test whether nodule development on mungbean is sensitive to ethylene, nodulation experiments were performed in the presence of 2-chloroethylphosphonic acid (ethephon), which spontaneously decomposes into ethylene. Treatment with 100 μ M ethephon resulted in a significant reduction in the number of mature nodules per plant and in the percentage of plants that developed mature nodules, demonstrating that nodule development on mungbean is indeed sensitive to ethylene (Fig. 1). Hunter (1993) reported that nodulation of soybean was unaffected at 100 μ M ethephon, perhaps explaining why these mutants formed as many mature nodules on soybean as the wild-type strain (Ruan and Peters 1992).

We reasoned that if the role of rhizobitoxine in symbiosis is to inhibit ethylene biosynthesis, then inhibition of ethylene biosynthesis or ethylene perception in plants inoculated with rhizobitoxine mutants should restore nodule development to wild-type or near-wild-type levels. Inhibition of ethylene biosynthesis was achieved with a structural and functional analog of rhizobitoxine, aminoethoxyvinylglycine (AVG), that inhibits ethylene biosynthesis by the same mechanism as rhizobitoxine (Yasuta et al. 1999). Growing plants in the presence of 0.01 to 0.1 μ M AVG resulted in statistically significant increases in the mean number of mature nodules per plant, as well as increases in the percentage of plants that developed mature nodules when inoculated with the rhizobitoxine mutant (Fig. 2A and B). At 0.1 μ M AVG, plants inoculated with wild-type *B. elkanii* did not show a statistically significant difference in nodulation. This demonstrates that the rhizobitoxine mutant phenotype can be partially restored by inhibition of ethylene biosynthesis. Plants grown at AVG concentrations of 1.0 μ M showed toxic effects when inoculated with both wild-type and mutant *Bradyrhizobium* strains.

To demonstrate that the partial restoration of nodulation phenotype by AVG was due to inhibition of ethylene biosynthesis rather than an unknown activity of these molecules, we tested the effects of cobalt, an ethylene response inhibitor (Beyer 1976; Lau and Yang 1976), on nodule development in mungbean. Treatment with cobalt had a significant effect on the percentage of plants developing mature nodules. However, because of the large variation in the numbers of mature nodules per plant, the observed increase in mean numbers of nodules was not significant (Fig. 2C and D). These results also indicate that, for the rhizobitoxine mutants, nodule development is limited by plant ethylene.

To determine if the abortion of nodule formation is an active or passive process with regard to the plant, co-inoculation experiments were carried out with the wild-type strain and strain RX18E. When the mutant strain was co-inoculated or inoculated 1 day after the inoculation with wild-type strain,

Fig. 1. Sensitivity of nodulation to ethylene in mungbean. Nodulation response of mungbean plants treated with various concentrations of ethephon. Mature nodules were scored 21 days after inoculation with wild-type strain USDA61. Bars represent mean values of at least 12 plants. Bars with no letter in common are significantly different at the 0.05 confidence level by Fisher's protected least significant difference test.

the mean nodule number was reduced by a statistically significant level from the mean nodule number of the wild type inoculated alone (Table 1). This suggests that plant responses induced by the mutant inhibit nodule formation by the wild-type *Bradyrhizobium* strain.

To better understand the phenotype of the rhizobitoxine mutants, we carried out a cytological study of mature nodules induced by wild-type strain USDA61 and mature and aborted nodules induced by mutant strains. Mature nodules induced by the wild-type strain were pink in color and spherical in shape (Fig. 3A). Bright field microscopy of transversely sectioned mature nodules revealed a histology that is typical of determinate nodules (Fig. 4A and B). These nodules possessed a large, circular, central tissue of infected cells, surrounded by a concentric layer of uninfected nodule cortex lacking a distinct

apical meristem. The central tissues were primarily found to contain infected host cells whose cytoplasm was packed with numerous, well-differentiated bacteroids. In addition, small, highly vacuolated, uninfected host cells interspersed among the infected cells could be observed. Most of the cells in the central zone have large nuclei with a prominent nucleolus—an indication of metabolically active cells. Depending on the orientation of the section or the developmental stage of the nodule, single, vascular bundles located at the periphery could be observed in some sections, while others had a bifurcating vascular bundle.

Features similar to those of the wild-type nodules are evident in the transverse section of a normal-looking mature nodule induced by mutant strain RX17E (Fig. 4C and D), although there appear to be larger numbers of uninfected cells in

Fig. 2. Mean nodule number and percentage of plants with mature nodules. Nodulation response of mungbean plants treated with various concentrations of aminoethoxyvinylglycine (AVG) or cobalt. Plants inoculated with the wild-type *Bradyrhizobium elkanii* strain USDA61 are indicated with solid bars; plants inoculated with mutant strain RX17E are indicated with open bars. Mature nodules were scored 21 days post inoculation. Bars represent mean values of at least 12 plants. Bars with no letter in common are significantly different at the 0.05 confidence level by Fisher's protected least significant difference test.

the nodules induced by the mutant. Formation of amyloplasts was very prominent in these nodules. In addition to the few normal-looking mature nodules induced by the rhizobitoxine mutants on mungbean, the mutant strains also induced many more aborted nodule structures that appeared as bumps along the root (Fig. 3B). In these aborted nodules, nodule development was arrested at both pre-emergent and post-emergent stages of development, demonstrating that rhizobitoxine mutant-induced nodules arrest at all stages of development. Collapsed plant cells, which stained dark blue to green with toluidine blue, were seen more frequently in the aborted nodules (Fig. 5B, E, and F). Nodule sections that showed extensive accumulation of collapsed cells contained no amyloplasts (Fig. 5E and F). In addition, no bacteroids could be observed within the cells of most aborted nodules. As indicated in Figure 5B and C, there were instances in which cells with symptoms of degeneration could be observed in the nodule tissue. These types of responses are similar to plant defense responses seen in aborted nodules described on *Medicago sativa* (Vasse et al. 1993).

Table 1. Effect of delayed and co-inoculation on nodulation of mungbean

Strain	Nodules per plant ^z
USDA61	21.7 ± 19.5 a
RX18E	1.3 ± 4.3 b
USDA61 + RX18E (co-inoculated)	8.4 ± 5.5 c
USDA61 ⇒ RX18E (delayed inoculation)	10.0 ± 7.1 c

^z Values presented are means of at least 12 plants, with the standard deviation. Means having no letter in common are significantly different (0.05 confidence level) by Fisher's protected least significant difference test.

Fig. 3. Light micrographs of whole nodules of mungbean. **A**, Whole mount mature nodules induced by the wild-type *Bradyrhizobium elkanii* strain USDA61. **B**, Whole mount aborted nodules induced by *rtxA* mutant strain RX18E.

Since AVG treatment of plants resulted in a significant increase in nodulation with the rhizobitoxine mutant strain, we examined whether the nodules induced in the presence of this compound have normal morphological and anatomical features. Nodules induced by the mutant strain in the presence of AVG that were of a color and size similar to those nodules on wild-type control plants were sectioned for bright field microscopy. Transverse sections of these nodules revealed histological features similar to those of the wild-type control nodules (Fig. 4E and F). The central zone had many infected cells packed with well-differentiated bacteroids. Interspersed within the infected cells were fewer uninfected cells than in mutant strain-induced nodules formed in the absence of AVG.

DISCUSSION

The most striking consequence of the rhizobitoxine mutation on nodule development is the accumulation of visible but arrested nodules. Although rhizobitoxine is best known for its chlorosis-inducing activity on legume plants, the observed nodulation phenotype of the rhizobitoxine mutants demonstrates that rhizobitoxine is required for *B. elkanii* to efficiently nodulate mungbean, thus demonstrating for the first time a positive role of this toxin in mutualistic symbiosis.

Our experimental results demonstrate that the difference in nodulation phenotype between the wild-type and rhizobitoxine mutants is mediated by ethylene. This conclusion is supported by the fact that nodulation of mungbean was efficiently suppressed with addition of 100 µM ethephon, and that wild-type nodulation can be partially restored to rhizobitoxine mutants by the addition of AVG or cobalt. The data show that nodulation of mungbean is more sensitive to ethylene than that of soybean (Hunter 1993), but not as sensitive to ethylene as other legumes such as pea and clover (Grobbelaar et al. 1971; Goodlass and Smith 1979; Lee and Larue 1992).

As reported in this study, the co-inoculation and delayed inoculation experiments on mungbean resulted in a significant reduction in the number of mature nodules formed by the wild-type strain (Table 1). A possible explanation is that ethylene is induced following inoculation by *Bradyrhizobium* strains. The wild-type strain can locally inhibit ethylene biosynthesis through the biosynthesis of rhizobitoxine. However, ethylene biosynthesis is not inhibited by the rhizobitoxine mutant such that ethylene can diffuse from the site of infection and inhibit nodule development at adjacent sites of infection. Although the exact mechanism of ethylene induction by the infecting rhizobia is unknown, it has been shown that the lipochitin-oligosaccharides or Nod factors can induce ethylene and ethylene-like responses in the plants (van Spronsen et al. 1995). Rhizobial infection itself may cause the host plant to induce production of ethylene, as shown for vetch and alfalfa (Zaat et al. 1989; Caba et al. 1993; van Workum et al. 1995).

Although the above data provide convincing evidence that the nodulation phenotype of the rhizobitoxine mutants is mediated through ethylene, it is not yet known how ethylene can block nodulation, leading to arrested nodule development. Ethylene is known to have many effects on plant development, including effects on root hair cell development (Masucci and Schiefelbein 1996), inhibition of cell division, DNA synthesis (Apelbaum and Burg 1972), and induction of

host defense-related genes (Ecker 1995). Each of these effects of ethylene by itself is antagonistic to nodule formation and would lead to arrested or aborted nodule development. Because many infections proceed beyond the stage of initial in-

fection of the root hair, we conclude that the primary effect of ethylene is not at the initial stages of root hair infection.

We suspect that ethylene affects abortion of the developing nodules through a combination of inhibition of cell division

Fig. 4. Bright field micrographs of 8- μ m transverse sections of mungbean nodules stained with toluidine blue. **A**, Whole section of a mature nodule induced by the wild-type *Bradyrhizobium elkanii* strain USDA61. **B**, Enlargement of marked region in **A**, containing a large, circular, central tissue of infected cells packed with well-differentiated bacteroids (ic) and few uninfected cells (ui). Nodule vascular bundle (NVB) and nodule cortex (NC) are also indicated. **C**, Whole section of a mature nodule induced by rhizobitoxine mutant strain RX17E. **D**, Enlargement of marked region in **C**, containing relatively fewer infected cells (ic), a large number of accumulated amyloplasts, and a larger number of uninfected cells relative to nodules induced by wildtype. **E**, Section of a whole, normal-appearing nodule induced by mutant rhizobia in the presence of aminoethoxyvinylglycine (AVG). **F**, Enlarged portion of the marked region of **E**, showing numerous infected cells (ic) containing well-differentiated bacteroids.

and induction of plant defense-like responses. Although in this study no data on cell division were collected directly, most nodules showed arrested development at the meristematic stage with no bacteroids observed in host plant cells. Several observations suggest a defense-like response including the failure of bacteroids to differentiate and the presence of collapsed plant cells that stain dark blue-green or green with

toluidine blue, suggesting an accumulation of polyphenolic compounds (Faria et al. 1987). Similar observations have been made during the ineffective interaction between a normal plant and a symbiotic bacterial mutant, such as that between *R. meliloti* *exo* mutants of alfalfa (Niehaus et al. 1993) or between *R. leguminosarum* *LPS* mutants of pea (Perotto et al. 1994). This kind of response is also similar to plant defense

Fig. 5. Bright field micrographs: transverse sections of aborted nodules induced by the rhizobitoxine mutant strain RX17E. **A**, Cross section of an aborted mutant nodule with few infected cells and a large number of amyloplasts (circled). Some cells in the central tissue show degeneration of the cytoplasm (arrow). **B**, Traverse section (5 µm) of an aborted nodule showing accumulation of darkly staining cytoplasmic contents and collapsed cells. **C**, Whole section of an aborted nodule devoid of infected cells. Root vascular bundle is indicated (R). **D**, Enlargement of the marked region in C. Darkly staining host cells do not contain bacteroids. Arrow indicates shrinkage of cytoplasm away from cell wall. **E**, Whole section of an aborted nodule showing collapsed plant cells. **F**, Enlargement of the marked region in E. Darkly stained cells are collapsed plant cells (arrows). Vascular bundle (VB) is indicated.

responses seen at the sites of aborted infections when alfalfa is inoculated with its regular symbiont (Vasse et al. 1993), and in the incompatible plant-pathogen interactions described for the tomato root (Beswetherick and Bishop 1993). Given that some but not all aborted nodules showed symptoms of degeneration, we suggest that cell degeneration is not necessarily the primary cause of abortion, but may rather follow senescence of the aborted nodule.

Ethylene has been shown to be an important regulator of nodule number, suggesting that it would be advantageous for rhizobia to evolve or adapt mechanisms to limit plant ethylene biosynthesis or perception during nodule development. However, apart from *B. elkanii*, no other species of rhizobia are known to have mechanisms to limit plant ethylene biosynthesis. Since several legume hosts are sensitive to ethylene, we may expect to find that other rhizobia have evolved as yet undiscovered alternative mechanisms to limit ethylene induction or perception.

MATERIALS AND METHODS

Bacterial and plant materials.

Bacterial strains used were the wild-type, rhizobitoxine-producing *Bradyrhizobium elkanii* strain USDA61 and two previously described strains, RX17E and RX 18E (Ruan and Peters 1992), mutated in rhizobitoxine biosynthesis due to Tn5 insertions in the *rtxA* gene (Ruan et al. 1993). The host plant used was *Vigna radiata* cv. Berken (mungbean).

Plant cultivation.

Three- to 5-day-old mungbean seedlings were transferred to autoclaved, modified Leonard jars constructed from Magenta boxes (Sigma Chemical, St. Louis, MO) containing vermiculite. The vermiculite was saturated with Jensen's nitrogen-free salts medium (Vincent 1977). Plants were inoculated with approximately 10^8 cells per plant of strain USDA61 or mutant strain RX18E (Ruan and Peters 1992). Plants were grown in a growth chamber with a 24-h photoperiod at 27°C and 70% relative humidity with roots of plants shielded from light by placing the Leonard jars in black cloth jackets. Mature nodules were scored 21 days post inoculation under a dissecting microscope.

For co-inoculation experiments, plants were grown as described above and inoculated individually or in combination. In one instance, RX18E was inoculated 3 days following inoculation with wild-type strain USDA61.

To test the sensitivity of mungbean to ethylene, plants were inoculated and grown as described above, except that 2-chloroethylphosphonic acid (ethephon) was added to the Jensen's medium at concentrations from 1 to 1,000 μ M. Mature nodules were scored 21 days post inoculation under a dissecting microscope. Data presented are a compilation of data from two separate experiments.

For experiments with cobalt chloride, ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; Kebo, Stockholm), plants were grown in modified Leonard jars as described above. Co^{2+} was added to the plant growth medium 2 days after inoculation at concentrations ranging from 0.0 to 1.0 μ M. In order to ensure that the growth medium had the desired concentration, the Leonard jars were flushed twice from the top with 50 ml of Jensen's nitrogen-free salts medium containing the desired concentrations of CoCl_2 .

The effect of aminoethoxyvinylglycine (AVG; Sigma) on nodulation was assayed in a liquid medium with dark brown bottles, a method developed by A. A. N. van Brussel (Leiden University, The Netherlands). Mungbean seedlings with 1- to 2-cm-long roots were transferred to 330-ml, dark brown bottles such that the roots grew into the mouth and the shoots grew up from the bottle. Seedlings were held in place with aluminum foil that also helped to maintain sterility. The liquid medium was Jensen's nitrogen-free salts and plants were inoculated with approximately 10^6 cells and various concentrations of AVG.

Microscopy.

Transverse, serial, 8- μ m sections were made of wax-embedded nodule tissues with a Leitz microtome (Leica Mikroskopi, Heidelberg, Germany). Sections were stained with toluidine blue and photographed with a Leica MPS camera on a Leitz DAS microscope (DM RB/E) at magnifications of 2.5 to 20. Transverse, 5- μ m sections were made from plastic-embedded nodule tissue with a Leitz ultramicrotome and glass knives.

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