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Rhizobitoxine Production by *Bradyrhizobium elkanii* Enhances Nodulation and Competitiveness on *Macroptilium atropurpureum*

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Application of 1-aminoocyclopropane-1-carboxylic acid, an ethylene precursor, decreased nodulation of *Macroptilium atropurpureum* by *Bradyrhizobium elkanii*. *B. elkanii* produces rhizobitoxine, an ethylene synthesis inhibitor. Elimination of rhizobitoxine production in *B. elkanii* increased ethylene evolution and decreased nodulation and competitiveness on *M. atropurpureum*. These results suggest that rhizobitoxine enhances nodulation and competitiveness of *B. elkanii* on *M. atropurpureum*.

The symbiotic interactions between a legume and (brady) rhizobia result in a unique, nitrogen-fixing plant organ, the nodule. Recent studies have shown that the phytohormone ethylene inhibits nodule formation in some legumes (8, 9, 16, 24, 25). Application of 1-aminoocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, inhibits nodulation in *Medicago truncatula* (24).

Rhizobitoxine [2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid] is an ethylene synthesis inhibitor that is produced by the legume symbiont *Bradyrhizobium elkanii* (15, 17–19, 22, 39). It is thought that production of this compound enhances nodulation of the host legume because of its inhibitory effect on ethylene synthesis. However, some reports have shown that there is not a significant difference in nodule number between plants inoculated with *B. elkanii* USDA61 and plants inoculated with rhizobitoxine-deficient mutants during nodulation of *Glycine max*, *Glycine soja*, *Vigna unguiculata*, and *Macroptilium atropurpureum* (26, 39). Recently, Duodu et al. observed a significant difference in nodule number between plants inoculated with isogenic variants of USDA61 during nodulation of *Vigna radiata* (7). Although these findings do not seem to be consistent with the hypothesis that rhizobitoxine has a positive effect on nodulation, the inconsistency can be explained by differences in the ethylene sensitivity of nodulation among leguminous species; nodulation of *G. max* is generally not sensitive to ethylene (10, 31, 38), while nodulation of *V. radiata* is sensitive (7). The inconsistency could also result from differences in the abilities of the strains used in the experiments to produce rhizobitoxine; strain USDA61 is a weak producer of rhizobitoxine (39).

In addition to *G. max*, the leguminous plant *M. atropurpureum* is a nodulating host for *B. elkanii* and *Bradyrhizobium japonicum* (12, 15). Although the effect of ethylene on nodulation has been studied in many leguminous host plants so far, the effect of ethylene in *M. atropurpureum* is not known. *B. elkanii* was found to be more competitive than *B. japonicum* for nodulation of *M. atropurpureum* in a multistrain environment when a field soil was inoculated with a mixture of several strains isolated from the field soil (21). In general, *B. elkanii* accumulates rhizobitoxine in cultures and in nodules, while *B. japonicum* does not (5, 15, 18, 19). These results led us to investigate the role of rhizobitoxine production on the nodulation and competitiveness of *B. elkanii* on *M. atropurpureum* by using a *B. elkanii* strain that produces high levels of rhizobitoxine, *B. elkanii* USDA94.

Siratro (*M. atropurpureum* Urb. cv. Siratro) seeds were obtained from Yukijirushi Shubyo Co. (Hokkaido, Japan). The seeds were surface sterilized with 70% ethanol for 5 min and then with 3% hydrogen peroxide for 1 min; they were washed 10 times at 1-min intervals with sterile distilled water after each treatment. The surface-sterilized seeds were sown in sterile plastic growth pouches that were watered with a nitrogen-free plant nutrient solution (1) and incubated at 25°C for 2 days in the dark. Two days after sowing, the germinated young seedlings in the pouches were transferred to a chamber and grown by using the following cycle: 14 h of light at 28°C and 10 h of darkness at 23°C.

The bacterial strains and plasmids used in this study are listed in Table 1. *Bradyrhizobium* strains were maintained in HM medium (4) containing 0.1% arabinose and 0.025% yeast extract or in Tris-YMRT (20). *Escherichia coli* strains were maintained in Luria-Bertani medium (29). Before inoculation, *Bradyrhizobium* strains were cultured in HM medium containing 0.1% arabinose and 0.025% yeast extract at 30°C for 6 days. The cells were collected by centrifugation and washed twice with sterile water, and the concentration was adjusted to 10^7 cells · ml^{-1} by direct counting with a Thoma hemocytometer. One milliliter of the bacterial suspension was inoculated onto 2-day-old seedlings in sterile growth pouches.

To see if production of ethylene in *M. atropurpureum* inhibits nodule formation by *B. elkanii*, siratro seedlings were inoculated with USDA94 in the presence and in the absence of ACC (Sigma-Aldrich Japan, Tokyo, Japan). It is thought that applying ACC to plants increases the ethylene level because this compound is a precursor of ethylene. ACC Powder was diluted in nitrogen-free plant nutrient solutions to final concentrations of 1 and 10 μM. The nutrient solutions containing ACC were added to plant roots just after inoculation and every day during plant growth. Siratro seedlings that received a nitrogen-free nutrient solution that did not contain ACC were used as controls. For a time course study of nodulation, nodules were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Bacterial strains		
Bradyrhizobium elkanii		
USDA94	Wild type, RT^+	Keyser[b]
RTS2	USDA94 RT^- mutant, *rtxA*::KIXX, Km^r	This study
MA941	USDA94 labelled with *gusA* by mTn*5*SS*gusA20*, Sp^r	This study
Bradyrhizobium japonicum		
USDA110	Wild type, RT^-	Keyser[b]
MA106	USDA110 labelled with *gusA* by mTn*5*SS*gusA20*, Sp^r	This study
Escherichia coli		
JM109	Cloning strain	Toyobo
S17-1 λ-*pir*	Strain used for conjugation and gene disruption	24
Plasmids		
pCR2.1	Cloning vector, Ap^r Km^r	Invitrogen
pUC18	Cloning vector, Ap^r	Toyobo
pUC4-KIXX	Plasmid carrying 1.6-kb *aph* cassette, Ap^r Km^r	Pharmacia
pSUP202	Plasmid used for gene disruption, Ap^r Cm^r Tc^r	20
pmTn*5*SS*gusA20*	Plasmid used for transposon insertion, Ap^r Sp^r	24
pCR7-2950.8	pCR2.1 carrying a 3.0-kb PCR fragment, Ap^r Km^r	This study
pUC7-2950.1	pUC18 carrying a 3.0-kb *Eco*RI fragment from pUC7-2950.8, Ap^r	This study
pUC7-2950::KIXX.15	pUC7-2950.1 carrying a 1.6-kb *Xho*I fragment from pUC4-KIXX, Ap^r Km^r	This study
pSUPrtx::KIXX.7	pSUP202 carrying a 4.6-kb *Eco*RI fragment from pUC7-2950::KIXX.15, Ap^r Cm^r Km^r	This study
pαHD7	pCNTR carrying a 1.2-kb ISB12 (RSα) fragment from *B. japonicum* HRS isolate NK5	4

[a] RT, rhizobitoxine production; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; *aph*, aminoglycoside-3′-*O*-phosphotransferase gene.

[b] Keyser, H. H. Keyser, U. S. Department of Agriculture, Beltsville, Md.

counted by counting the visible nodules with diameters greater than 0.2 mm and large globular nodules with diameters greater than 1 mm. Student's *t* test was used to assess the statistical significance of differences in nodule number at a confidence level of 0.05.

Ethylene synthesis was measured by the method of Suganuma et al. (34). After the nutrient solution was removed with paper towels, plant roots were incubated in a 5-ml glass container at 30°C for 2 h (three to five plants per container). The ethylene concentration in the container was measured by using a model GC-7A gas chromatograph (Shimadzu, Tokyo, Japan) equipped with a flame ionization detector and a Porapak Q column (2.2 mm by 2 m; Waters Associates Inc.). We calculated the rate of ethylene synthesis by using the concentrations obtained. As determined in the absence of ACC, the rate of ethylene synthesis in siratro roots 3 days after inoculation with USDA94 (0.79 pmol of ethylene · $plant^{-1}$ · h^{-1}) was less than the rate of ethylene synthesis in uninoculated control roots (5.46 pmol of ethylene · $plant^{-1}$ · h^{-1}). These results suggest that inoculation with *B. elkanii* suppressed ethylene synthesis in the *M. atropurpureum* roots, and they are consistent with previous findings that rhizobitoxine application inhibits ethylene synthesis and the enzymatic activity of ACC synthase in the ethylene synthesis pathway in other plants (23, 40). When 1 μM ACC was applied to USDA94-inoculated siratro roots just after inoculation and during plant growth, the ethylene synthesis rate increased from 0.79 pmol of ethylene · $plant^{-1}$ · h^{-1} (no ACC) to 3.29 (1 μM ACC) or 3.75 (10 μM ACC) pmol of ethylene · $plant^{-1}$ · h^{-1} within 3 days after inoculation. These results indicate that ACC treatment increased ethylene synthesis in *M. atropurpureum* roots inoculated with rhizobitoxine-producing *B. elkanii*. Using 1 μM ACC, we assessed the effect of ethylene on nodulation of *M. atropurpureum* inoculated with *B. elkanii*. The number of nodules formed in the presence of ACC 8 days after inoculation and later were significantly less than the numbers of nodules formed in the absence of ACC (Fig. 1). This finding suggests that in *M. atropurpureum* ethylene-induced inhibition of nodulation is similar to inhibition of nodulation in *Pisum sativum* (8, 16), *Trifolium repens* (8), *Medicago sativa* (25), *Vicia sativa* (9), *M. truncatula* (24), and *V. radiata* (7).

B. elkanii USDA61 is a weak producer of rhizobitoxine (39), so a strain that produces high levels of rhizobitoxine, *B. elkanii* USDA94, was used to construct a rhizobitoxine-deficient mutant. The *rtxA* gene reportedly is responsible for rhizobitoxine

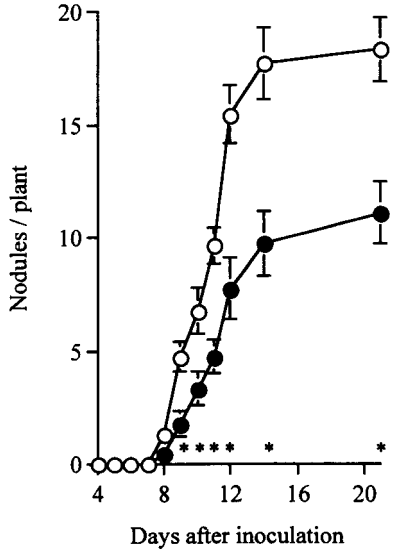

FIG. 1. Effect of ACC on nodulation of *M. atropurpureum* cv. Siratro inoculated with *B. elkanii* USDA94. Each point represents the mean number of nodules per plant. The bars indicate standard errors. Nine plants were treated and assessed for each data point. Symbols: ○, no ACC; ●, 1 μM ACC. Asterisks indicate significant differences in mean nodule number between treatments at a confidence level of 0.05.

production by *B. elkanii* USDA61 (27). This gene encodes two regions similar to a rat serine:pyruvate aminotransferase and a yeast *O*-acetylhomoserine sulfhydrolase (27, 28). The rhizobitoxine-deficient mutant was constructed by homologous DNA recombination by using the homologous *rtxA* gene DNA from USDA94 and a kanamycin resistance gene. To obtain a DNA fragment homologous to the *rtxA* gene, we designed two primers (5′-TAG AAT TCT CCA ACG AGT GAC AGT ATG CGA-3′ and 5′-CTA ACT GAA CAG CCT CAT AAC G-3′) and used them for PCR amplification of total DNA of *B. elkanii* USDA94 with the following temperature program: 94°C for 2 min, followed by 40 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. The PCR products were cloned into pCR2.1 (Invitrogen, San Diego, Calif.). The DNA sequences of the clones were determined by using a model 373A DNA sequencer (Perkin-Elmer Japan, Chiba, Japan). One of the clones obtained contained a 2,948-bp insert whose sequence was 99.6% identical to the DNA sequence of the *rtxA* gene of USDA61. This plasmid was designated pCR7-2950.8. Plasmid pCR7-2950.8 was digested with *Eco*RI, and a 2.9-kb *Eco*RI fragment was cloned into pUC18. The plasmid obtained was designated pUC7-2950.1. Plasmid pUC7-2950.1 was digested with *Xho*I, and a 1.6-kb fragment containing the aminoglycoside 3′-phosphotransferase (*aph*) gene from pUC4-KIXX (Amersham-Pharmacia Biotech, Uppsala, Sweden) was inserted. The resulting plasmid was designated pUC7-2950::KIXX.15. Plasmid pUC7-2950::KIXX.15 was digested with *Eco*RI. A 4.6-kb *Eco*RI fragment from pUC7-2950::KIXX.15 was ligated into pSUP202 (33). The resulting plasmid was designated pSUPrtx::KIXX.7 (Fig. 2A). The restriction map of the region around *rtxA* in USDA94 is shown in Fig. 2B. These cloning experiments were performed by using *E. coli* JM109.

Plasmid pSUPrtx::KIXX.7 was used to transform *E. coli* S17-1 λ-*pir* (37) for conjugative transfer to *Bradyrhizobium* cells. The transformant was grown at 37°C overnight in Luria-Bertani medium containing 50 mg of kanamycin per liter, 50 mg of ampicillin per liter, and 20 mg of tetracycline per liter. *B. elkanii* USDA94 was grown in HM medium at 30°C for 6 days. Cells of the *E. coli* transformant and *B. elkanii* USDA94 were collected, washed with sterile 0.8% NaCl, and resuspended in sterile 0.8% NaCl containing 0.01% Tween 20. The suspension was applied to a sterile filter and incubated at 30°C overnight. The cells on the filter were suspended in sterile water, spread onto HM medium containing 150 mg of kanamycin per liter and 50 mg of polymyxin B per liter, and incubated at 30°C. Kanamycin- and polymyxin B-resistant colonies were selected and maintained in HM medium containing 150 mg of kanamycin per liter and were used for Southern analysis. The probes used in the Southern analysis were the 3.0-kb *Eco*RI *rtxA* homologue fragment from pCR7-2950.8, the *Eco*RI-digested pSUP202 plasmid vector (length, 7.8 kb), the 1.6-kb *Xho*I kanamycin-resistant *aph* gene of pUC4KIXX, and the 1.2-kb *Bam*HI fragment of pαHD7 containing *Bradyrhizobium* insertion element RSα (11, 14) for DNA fingerprinting. One of the appropriate strains, a kanamycin-resistant mutant that was designated RTS2 and was obtained from USDA94, was used in this study. Rhizobitoxine concentrations in cultures were determined as described by Yasuta et al. (40).

Southern analyses of RTS2 performed with the pSUP202 plasmid vector, the *aph* gene fragment of pUC4KIXX, and RSα probes revealed that the mutant produced unique signals characteristic of both the plasmid vector and the *aph* gene and produced the same signal pattern as the parent strain when the RSα probe was used (data not shown). The mutant contained a DNA insertion with a kanamycin resistance cassette downstream of the *rtxA* gene in the genomic DNA (Fig. 2C). The

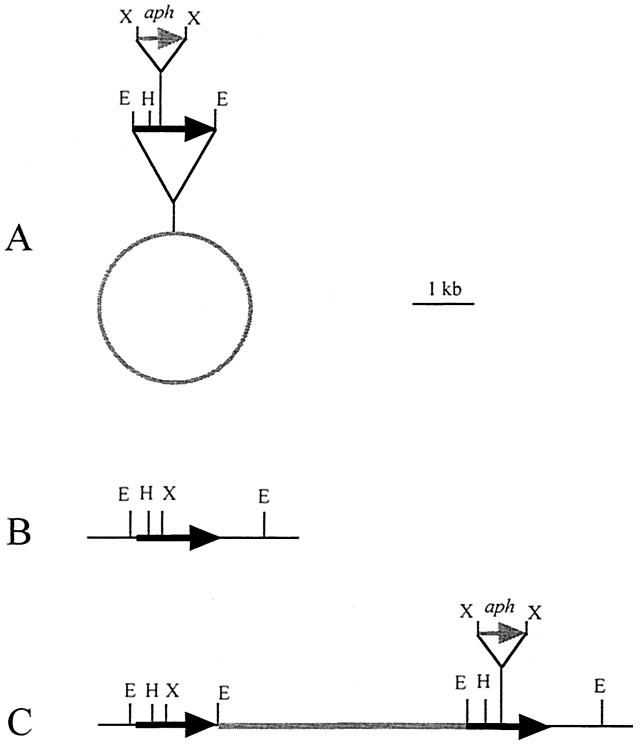

FIG. 2. Construction of rhizobitoxine production-deficient mutant RTS2. RTS2 was derived from *B. elkanii* USDA94 by homologous recombination with a 2.9-kb PCR product (Table 1). (A) Plasmid pSUPrtx::KIXX.7. This plasmid contains DNA homologous to *rtxA* from USDA61 (length, 2.9 kb) and a 1.6-kb kanamycin resistance cassette (*aph*). The vector region of pSUP202 is 7.8 kb long. (B) Restriction map of the region around *rtxA* in the USDA94 genome. (C) Restriction map of the region around *rtxA* in the RTS2 genome. Black arrows, *rtxA*; gray arrow, *aph*; gray line, pSUP202; E, *Eco*RI site; H, *Hin*dIII site; X, *Xho*I site.

DNA insertion might have occurred through a single crossover recombination event involving the USDA94 genome and the introduced plasmid pSUPrtx::KIXX.7 in the downstream region of *rtxA*. The rhizobitoxine concentrations in the mutant RTS2 and wild-type strain USDA94 cultures were compared. The limit of the detection was 0.01 μM rhizobitoxine. No rhizobitoxine was detected in the mutant RTS2 culture (concentration, <0.01 μM), whereas the rhizobitoxine concentration in the wild-type strain USDA94 culture was 17.5 μM. Inoculation with mutant RTS2 did not induce foliar chlorosis in *G. max* cv. Lee (data not shown), a finding similar to a previous finding for a rhizobitoxine mutant of *B. elkanii* USDA61 (26). Because the single-crossover mutant RTS2 did not produce rhizobitoxine, at least one additional gene (downstream of *rtxA* and in the same operon) may be required for rhizobitoxine production.

Using isogenic variants of rhizobitoxine-deficient mutant RTS2 and wild-type strain USDA94, we assessed the effect of rhizobitoxine production by *B. elkanii* on ethylene synthesis in *M. atropurpureum*. Ethylene synthesis in plant roots was measured as described above by examining plants on days 6 and 23 after inoculation. On days 6 and 23 after inoculation in the absence of ACC, the rates of ethylene synthesis in siratro roots inoculated with USDA94 were less than the rates of ethylene synthesis in uninoculated control roots (Fig. 3). When siratro was inoculated with RTS2, the ethylene synthesis rate was greater than the rate of synthesis in roots inoculated with USDA94 and equivalent to the rate of synthesis in uninocu-

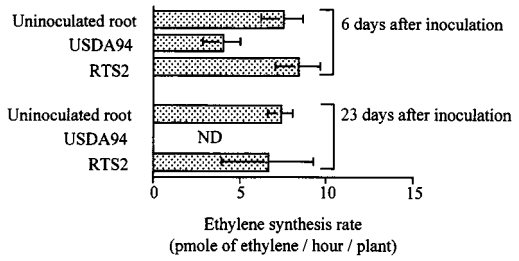

FIG. 3. Effects of *B. elkanii* strains on ethylene synthesis in *M. atropurpureum* roots. Plants were inoculated with *B. elkanii* USDA94 and rhizobitoxine production-deficient mutant RTS2. For each treatment, the mean ethylene evolution rate was determined 6 days after inoculation (15 plants) and 23 days after inoculation (9 plants). The bars indicate standard errors. ND, not detected.

lated roots. The lack of rhizobitoxine production by *B. elkanii* USDA94 resulted in a loss of ethylene synthesis suppression in siratro, indicating that rhizobitoxine production by *B. elkanii* suppressed ethylene synthesis in *M. atropurpureum*. When we examined siratro roots inoculated with USDA94 in the absence of ACC, the ethylene synthesis rate was 3.24 ± 0.68 pmol/h/plant on day 6 after inoculation, while on day 23 after inoculation ethylene synthesis was not detectable, suggesting that more rhizobitoxine accumulated over time in the presence of *B. elkanii*. Rhizobitoxine is produced in nodules and is transported to the roots and shoots (17, 39). Because the number of nodules on day 23 after inoculation with USDA94 was about 13 times more than the number of nodules on day 6 after inoculation (data not shown), the increase in nodule number should have resulted in a higher concentration of rhizobitoxine in plants inoculated with *B. elkanii*.

The effect of rhizobitoxine-deficient strain RTS2 on nodulation of siratro was also examined. When we compared inoculation with mutant RTS2 and inoculation with wild-type strain USDA94, we found no difference in the percentages of nodulated plants that had the first visible nodules (Fig. 4A). The same trend was observed for the percentages of nodulated plants having the first large nodules (data not shown). These results suggest that a lack of rhizobitoxine production in *B. elkanii* does not result in a delay in nodulation of *M. atropurpureum*, which is consistent with the findings of Ruan and Peters (26), who used *B. elkanii* USDA61 and isogenic rhizobitoxine mutants. A lack of rhizobitoxine production did not affect emergence of the first nodules, but the numbers of nodules were significantly different over time after inoculation when the isogenic variants of USDA94 were used (Fig. 4B). When the numbers of nodules were compared, the numbers of visible nodules on siratro roots after inoculation with the different isogenic variants were not different before day 6 after inoculation. From 8 days after inoculation, however, the numbers of visible nodules on siratro roots inoculated with RTS2 were less than the numbers of visible nodules on roots USDA94. Fewer nodules after inoculation with RTS2 were also observed when the numbers of large nodules were compared (data not shown). These results suggest that rhizobitoxine production by *B. elkanii* enhances nodulation of *M. atropurpureum*. A similar effect of rhizobitoxine production was described by Duodu et al. (7), who examined ethylene-sensitive nodulation of *V. radiata*. The reason(s) for the delayed effect of rhizobitoxine production on the number of siratro nodules is not clear. One possible explanation is that a higher concentration of rhizobitoxine in plants is necessary before there is a visible effect on nodulation. This seems logical because there was no difference in the time of appearance of the first nodules when inoculation with the rhizobitoxine mutant and inoculation with the wild type were compared (Fig. 4A). The conclusion that rhizobitoxine production by *B. elkanii* enhances nodulation of *M. atropurpureum* seems to contradict the data obtained with the isogenic variants of USDA61 (26). The latter data might have resulted from differences in the ability to produce rhizobitoxine; Xiong and Fuhrmann (39) reported that USDA94 produced more rhizobitoxine than USDA61 produced in plants.

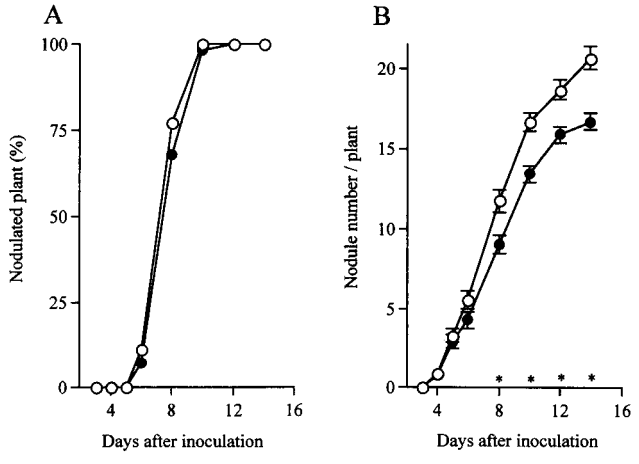

FIG. 4. Nodulation of *M. atropurpureum* cv. Siratro inoculated with *B. elkanii* USDA94 and rhizobitoxine production-deficient mutant RTS2. Each point represents a mean based on six independent experiments. (A) Time course for percentages of nodulated plants. (B) Time course for number of nodules per plant. Symbols: ○, plants inoculated with *B. elkanii* USDA94 (73 plants); ●, plants inoculated with rhizobitoxine production-deficient mutant RTS2 (72 plants). The bars indicate standard errors. The asterisks indicate significant differences in mean numbers of nodules between the treatments at a confidence level of 0.05.

To investigate competitiveness for nodulation in the wild-type and rhizobitoxine-deficient strains, mTn*5*SS*gusA20* (37) was used to label *B. elkanii* and *B. japonicum* strains. Recently, *gusA*-marked transposons, including mTn*5*SS*gusA20*, have been developed (37), and using these transposons has some advantages over other techniques (2, 3, 6, 13, 30, 36). The *gusA* marking system results in marked (*Brady*)*rhizobium* cells with competitive ability indistinguishable from the competitive ability of the parent cells; this makes screening for the competitive ability of strains of interest simple and rapid (32). *Bradyrhizobium* strains marked with *gusA* by using mTn*5*SS*gusA20* were constructed as described by Yuhashi et al. (41). In this study, 3 weeks after coinoculation nodules whose diameters were greater than 1 mm were harvested and used in a GUS assay performed as described by Yuhashi et al. (41). Each of the harvested nodules was cut in half with a razor blade. The hemispheric nodule segments were immersed in a GUS assay solution (50 mg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid [X-Gluc] per liter, 2 g of sodium dodecyl sulfate per liter, 20% methanol, 20 mM sodium phosphate buffer [pH 7.0]), subjected to a vacuum for 15 min, and incubated at 30°C overnight. Samples were fixed with 0.5% glutaraldehyde–0.2 M sodium cacodylate (pH 7.2) for 1 h, washed twice with distilled water, and observed with a stereoscopic microscope. When uniform GUS activity was observed in the infected region of a nodule, the nodule was considered occupied only by a *gusA*-marked strain. When there was no GUS activity in the infected region of a nodule, we assumed that the nodule was formed only by the unmarked strain. When GUS activity was observed as a spattered pattern in the infected region of a nodule, the

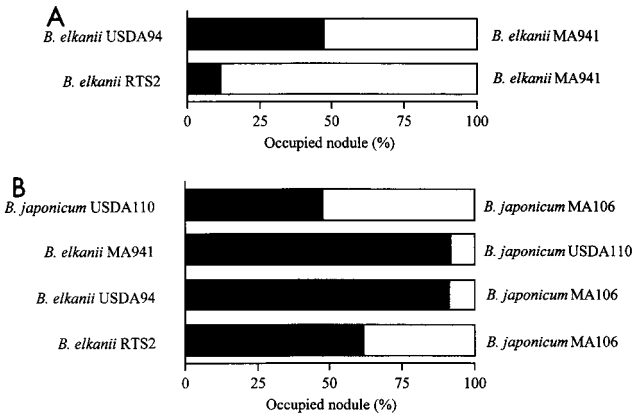

FIG. 5. Effect of rhizobitoxine production-deficient phenotype on competitiveness of *B. elkanii* USDA94 during nodulation of *M. atropurpureum* cv. Siratro. A chi-square test was used for statistical analyses at a confidence level of 0.05. (A) Competition between *B. elkanii* USDA94 and RTS2. *B. elkanii* MA941 is a *gusA*-marked USDA94 variant. RTS2 is a rhizobitoxine production-deficient mutant of USDA94. In the first experiment 228 nodules from 18 plants that were coinoculated with USDA94 and MA941 were used (47.4% USDA94 and 52.6% MA941). The data were consistent with the hypothesis that the occupied nodule ratio was 1:1. In the second experiment 159 nodules from nine plants that were coinoculated with RTS2 and MA941 were used (11.6% RTS2 and 88.4% MA941). The data were not consistent with the hypothesis that the occupied nodule ratio was 1:1. Statistical analysis showed that the difference in nodule occupancy values between USDA94 and RTS2 is significant. (B) Competition between *B. elkanii* USDA94 and *B. japonicum* USDA110. *B. japonicum* MA106 is a *gusA*-marked USDA110 variant. In the first experiment 141 nodules from 17 plants that were coinoculated with USDA110 and MA106 were used (47.5% USDA110 and 52.5% MA106). The data were consistent with the hypothesis that the occupied nodule ratio was 1:1. In the second experiment 141 from nine plants that were coinoculated with MA941 and USDA110 were used (91.5% MA941 and 8.5% USDA110). In the third experiment 193 nodules from 17 plants that were coinoculated with USDA94 and MA106 were used (91.2% USDA94 and 8.8% MA106). In the fourth experiment 177 nodules from 18 plants that were coinoculated with RTS2 and MA106 were used (61.9% RTS2 and 38.1% MA106). The MA941-USDA110 values and the USDA94-MA106 values are not significantly different. All other pairs of values are significantly different.

nodule was considered cooccupied by both strains. In the case of cooccupation, each strain was scored as if it occupied one-half of a nodule in order to calculate nodule occupancy values. The chi-square test was used to assess the statistical significance of differences in the numbers of occupied nodules at a confidence level of 0.05.

The *gusA*-marked strains *B. elkanii* MA941 and *B. japonicum* MA106 were obtained. When MA941 was coinoculated with parent strain USDA94 onto siratro (cell ratio, 1:1), the nodule occupancy value for the marked strain was almost the same as the value for the parent (47.4% USDA94 and 52.6% MA941) (Fig. 5A). Similar results were obtained when *B. japonicum* MA106 and USDA110 were coinoculated (47.5% USDA110 and 52.5% MA106) (Fig. 5B). These findings indicate that the competitive ability of the *gusA*-marked strains was indistinguishable from that of the parent strain.

When rhizobitoxine production-deficient mutant RTS2 was coinoculated with MA941 (a *gusA*-marked USDA94 derivative) onto siratro roots, the nodule occupancy values for RTS2 and MA941 were 11.6 and 88.4%, respectively (Fig. 5A). Compared to the results obtained after coinoculation of USDA94 and MA941, the loss of rhizobitoxine production by USDA94 resulted in lower nodulation competitiveness in siratro roots. This suggests that rhizobitoxine production enhances the competitiveness of *B. elkanii* during nodulation of *M. atropurpureum*. As summarized by Triplett and Sadowsky (35), previous research has indicated that several phenotypes of (*Brady*)*rhizobium* strains play significant roles in nodulation competitiveness; these phenotypes include antibiosis, cell surface characteristics, motility, speed of nodulation, and symbiotic effectiveness. Among the phenotypes involved in nodulation competitiveness, rhizobitoxine production is unique because it suppresses ethylene synthesis in inoculated plants.

When *B. elkanii* MA941 and *B. japonicum* USDA110 were coinoculated onto siratro roots, the nodule occupancy values were 91.5 and 8.5%, respectively (Fig. 5B). Similar results were obtained when *B. elkanii* USDA94 and *B. japonicum* MA106 were coinoculated (91.2% USDA94 and 8.8% MA106) (Fig. 5B). In our preliminary studies, similar results were also obtained when *gusA*-marked strains of *B. elkanii* USDA76 and USDA31 were used in coinoculation experiments involving USDA110 (data not shown). These results suggest that *B. elkanii* exhibits greater competitiveness than *B. japonicum* in *M. atropurpureum* roots. This is consistent with the observation of Minamisawa et al. that the nodule occupancy value for *B. elkanii* was greater than the nodule occupancy value for *B. japonicum* when siratro was inoculated with a field soil and a mixture of several strains isolated from the field soil (21).

When we examined competitive nodulation by using the rhizobitoxine production-deficient mutant *B. elkanii* RTS2 and *B. japonicum* MA106 (a *gusA*-marked USDA110 derivative), the nodule occupancy values were 61.9% RTS2 and 38.1% MA106 (Fig. 5B). Compared with the results obtained in experiments performed with the parent strains (91.5% MA941 and 8.5% USDA110; 91.2% USDA94 and 8.8% MA106) the competitiveness of RTS2 with USDA110 was less than the competitiveness of wild-type strain USDA94. These results suggest that a lack of rhizobitoxine production affects the competitiveness of *B. elkanii* and *B. japonicum* during *M. atropurpureum* nodulation. During *M. atropurpureum* nodulation, rhizobitoxine production by *B. elkanii* is one of the factors that contribute to high occupancy values for the species that are in competition with *Bradyrhizobium* field strains.

In leguminous plants in which ethylene-induced inhibition of nodulation occurs, rhizobitoxine production is an effective strategy for enhancing competitive nodulation. However, the mechanism that results in a higher occupancy value for a rhizobitoxine producer is still unclear. In our preliminary analysis, the rate of ethylene evolution in siratro roots that were coinoculated with *B. japonicum* USDA110 and *B. elkanii* USDA94ΔNOD (42) lacking $nodD_1D_2KABC$ genes was significantly lower than the rate of ethylene evolution in siratro roots inoculated with USDA110 alone but similar to the rate of ethylene evolution in siratro roots inoculated with USDA94 (3 and 6 days after inoculation) (data not shown). Lower rates of ethylene synthesis could be expected in whole roots in a multistrain environment containing rhizobitoxine-producing rhizobia and non-rhizobitoxine-producing rhizobia. One possible explanation for a higher occupancy value for a rhizobitoxine producer in a multistrain environment is the local effect of the rhizobitoxine produced at infection sites of the producer.

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