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Quantitative and time-course evaluation of nodulation competitiveness of rhizobitoxine-producing *Bradyrhizobium elkanii*

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Abstract

Regression analysis of results from a mathematical competition model showed that rhizobitoxine production by *Bradyrhizobium elkanii* USDA94 gave this strain a nodulation competitiveness about 10 times greater than that of a non-rhizobitoxine-producing mutant strain on *Macroptilium atropurpureum* (Siratro). Rhizobitoxine enhancement of competitive nodulation occurred at a late stage in the time-course of nodulation. All other known rhizobial factors that affect nodulation competitiveness act in the rhizosphere and during the initial interaction with legumes. This unique late action of rhizobitoxine could prove advantageous in inoculant production, because inoculum often fails to nodulate in the latter stages of nodulation kinetics.

Keywords: *Bradyrhizobium elkanii*; Competitiveness; Rhizobitoxine; Nodulation; *Macroptilium atropurpureum*

1. Introduction

Nodulation competitiveness has practical importance in agriculture, because the inoculation of efficient rhizobia is often unsuccessful owing to the presence of more competitive populations of inferior rhizobia in soils [1–3]. Several rhizobial factors that are not required for the establishment of symbiosis with legumes are involved in nodulation competitiveness [2,3]. These include motility [4], rhizopine [5], bacteriocin [6], exopolysaccharides [7] and proline catabolism [8].

Rhizobitoxine (2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid) increases nodulation and competitiveness via the inhibition of endogenous ethylene synthesis in host plants, because ethylene acts as a plant hormone that restricts nodulation in many legumes [9–11]. Duodu et al. [12] reported that non-rhizobitoxine-producing mutants of *Bradyrhizobium elkanii* USDA61 formed fewer mature nodules on *Vigna radiata* (mungbean) than did the wild-type strain. In Japanese lineages of *Amphicarpaea edgeworthii*, efficient nodule development is highly dependent on rhizobitoxine production by *Bradyrhizobium* strain USDA61 [13]. Yuhashi et al. [14] reported that rhizobitoxine production by *B. elkanii* USDA94 significantly enhanced nodulation competitiveness on *Macroptilium atropurpureum* (Siratro), although rhizobitoxine production increased nodule formation only slightly. In the association between *B. elkanii* USDA94 and *M. atropurpureum*, therefore, rhizobitoxine production affects nodulation competitiveness rather than nodulation per se.

In this association between *B. elkanii* USDA94 and *M. atropurpureum* we still do not know the extent to which rhizobitoxine production enhances nodulation competitiveness and the stage of nodulation at which this occurs. To address these questions, we compared the quantitative potency of rhizobitoxine production in nodulation competitiveness and the kinetics of nodule occupancy between a rhizobitoxine-producing strain, *B. elkanii* USDA94, and a rhizobitoxine production-deficient mutant, RTS2, on *M. atropurpureum*.

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2. Materials and methods

2.1. Bacterial strains and growth media

We used *B. elkanii* USDA94; a rhizobitoxine production-deficient mutant, RTS2, of USDA94 [14]; and a *gusA*-marked strain, MA941, of USDA94 [15]. *B. elkanii* MA941 is spectinomycin-resistant, which is derived from omega interposon in mTn*5*SS*gus*A20 [15]. Rhizobial strains were cultured for 14 days at 30°C in HM medium [16] containing 0.1% arabinose and 0.025% yeast extract, and either 100 mg l^{-1} of spectinomycin for MA941 or 150 mg l^{-1} of kanamycin for RTS2. The cells were collected by centrifugation at 5000×*g* for 10 min at room temperature, and washed twice with sterile water. The number of cells was adjusted to 1×10^7 ml^{-1} in sterile water by direct counting with a Thoma hemocytometer (Kayagaki Irika Kogyo, Tokyo, Japan) before inoculation.

2.2. Plant cultivation

M. atropurpureum Urb. cv. Siratro seeds were obtained from Yukijirushi Shubyo (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 with sterile distilled water after each treatment. The surface-sterilized seeds were sown in a 300-ml plant box (CUL-JAR300; Iwaki, Tokyo, Japan) containing sterile vermiculite, watered with a nitrogen-free plant nutrient solution [17], and incubated at 25°C for 2 days in the dark. Two days after sowing, the germinated seedlings were inoculated with 1 ml of inoculum mixed with MA941 and USDA94 or RTS2 (1×10^7 cells ml^{-1}), and grown in a phytotron (KG206 HL-D; Koito Industries, Tokyo, Japan) under 14 h of light (photon flux density 150 μmol m^{-2} s^{-1}) at 28°C and 10 h of darkness at 23°C.

2.3. Quantitative evaluation of nodulation competitiveness

We quantified the competitive nodulation capabilities of *B. elkanii* USDA94 and the rhizobitoxine-production-deficient mutant RTS2 on *M. atropurpureum* cv. Siratro using *gusA*-marked MA941 as a reference strain. We prepared seven inoculant mixtures, in which the proportions of the test strains (*B. elkanii* USDA94 and RTS2) vs. the reference strain ranged from 1:1000 to 1000:1. One milliliter of mixed inoculum was added to the 2-day-old seedlings. After 30 days of cultivation, nodules more than 1 mm in diameter were sampled. Nodule occupancy analysis was carried out by a GUS assay using X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt, Wako Pure Chemical Industries, Osaka, Japan) as a substrate, as described previously [15]. When GUS activity was observed as a spattered pattern in the infected region of a nodule, the nodule was considered occupied by both strains. In the case of co-occupation, each strain was scored as if it occupied one half of a nodule in order to calculate nodule occupancy values [14,15]. Approximately 100 nodules from three or four plants were examined by GUS assay in each inoculation treatment.

2.4. Time-course evaluation of nodulation competitiveness

We compared the time-courses of competitive nodulation of the rhizobitoxine-producing strain *B. elkanii* USDA94 and the non-producing strain RTS2 during nodule development using *B. elkanii* MA941 as a reference strain. USDA94 or RTS2 was mixed with MA941 at a concentration of 1×10^7 cells ml^{-1} of each strain (cell ratio, 1:1). We then inoculated 1 ml of each mixture on to 2-day-old Siratro seedlings in sterile growth pouches (Northrup King, Minneapolis, MN, USA). After 7, 9, 11, 13, 15, or 21 days of cultivation, all nodules greater than 1 mm in diameter were collected, and the nodule occupancy ratios of USDA94 and RTS2 against MA941 were determined by GUS assay. The χ^2 test was used for statistical analysis at a confidence level of 0.05. To determine the viability of each inoculant strain, 0.1 ml of hydroponic cultures was sampled from growth pouches on 7, 9, 11, 13, 15, or 21 days of cultivation. After serial dilution, hydroponic cultures were plated on HM agar containing 0.1% arabinose and 0.025% yeast extract with or without 250 mg l^{-1} of spectinomycin. After 7 days incubation, spectinomycin-resistant colonies were counted as MA941 and the numbers of USDA94 or RTS2 were determined as the fraction of spectinomycin-sensitive colonies.

3. Results

The nodule occupancy rates of the test strains were strongly dependent on the inoculum cell ratios between the test strains and the reference strain (Fig. 1). When *B. elkanii* USDA94 and MA941 were mixed in the inoculum at a 1:1 ratio, both strains formed similar numbers of nodules (Fig. 1A). This indicates that *gusA* insertion of MA941 did not affect the nodulation competitiveness of the parent strain USDA94. When the rhizobitoxine mutant RTS2 was co-inoculated with reference strain MA941 on to Siratro roots at a 1:1 ratio, the nodule occupancy rate of RTS2 was 16% (Fig. 1A). A 10:1 ratio of USDA94 cells to the reference strain resulted in approximately 94% nodule occupancy by USDA94 cells, whereas a 10:1 ratio of RTS2 cells to the reference cells resulted in 69% nodule occupancy by RTS2. When the test strain to reference strain ratio was 1:10, the nodule occupancy rates of USDA94 and RTS2 were only 5.7% and 0.6%, respectively. To attain 100% nodule occupancy, the ratio of test strain to reference strain needed to be more than 1000:1 in the case of both USDA94 and RTS2.

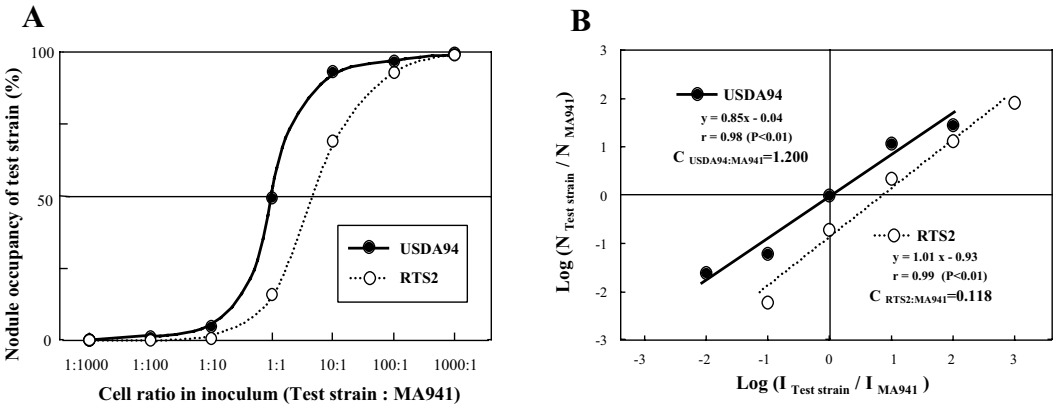

Fig. 1. A: Relationship between percentage nodule occupancy and cell ratio in inoculum. *B. elkanii* USDA94 or rhizobitoxine mutant RTS2 was co-inoculated with MA941, a *gusA*-marked USDA94, on *M. atropurpureum* cv. Siratro at different cell ratios. Each data point represents the mean of about 100 nodules per treatment. B: Relationships between the logarithm of the ratio of the number of nodules formed by two strains ($N_{\text{Test strain}}/N_{\text{MA941}}$) and the logarithm of the ratio of the numbers of cells of the two strains in the inoculum ($I_{\text{Test strain}}/I_{\text{MA941}}$).

To quantify the competitive nodulation capability of *B. elkanii* strains, we used the mathematical model reported by Amarger and Lobreau [18]. In the model, a linear relationship exists between the logarithm of the ratio of the numbers of nodules formed by each of two strains (N_A/N_B) and the logarithm of the ratio of the numbers of cells of the two strains in the inoculum (I_A/I_B): $\log (N_A/N_B) = \log C_{AB} + k \log (I_A/I_B)$.

Amarger and Lobreau proposed the intercept of the regression line, the C_{AB} value, as an index representing the competitiveness of strain *A* in relation to that of strain *B*, and considered it to be equal to the ratio of the number of nodules formed by strain *A* to the number of nodules formed by strain *B* when the two strains were present in the inoculum in equal quantities. Thus, C_{AB} should be close to 1 for two strains of equal competitiveness.

The relationships between the logarithm of the ratio of the numbers of nodules formed by the two strains ($N_{\text{Test strain}}/N_{\text{MA941}}$) and the logarithm of the ratio of the numbers of cells of the two strains in the inoculum ($I_{\text{Test strain}}/I_{\text{MA941}}$) are shown in Fig. 1B. For competitive nodulation with both test strains, the regressions between log ($N_{\text{Test strain}}/N_{\text{MA941}}$) and log ($I_{\text{Test strain}}/I_{\text{MA941}}$) were significant at a confidence level of 0.01. The *k* values – the slopes of the regression lines – were not significantly different between *B. elkanii* USDA94 and RTS2 (Fig. 1B). The *C* values for *B. elkanii* USDA94 and RTS2, $C_{\text{USDA94:MA941}}$ and $C_{\text{RTS2:MA941}}$, were estimated as 1.200 and 0.118, respectively. These values were significantly different at a confidence level of 0.01. This indicated that *B. elkanii* strains USDA94 and RTS2 differed significantly in their nodulation competitiveness. Moreover, rhizobitoxine production conferred approximately a 10-fold competitive advantage in forming nodules on Siratro, because $C_{\text{USDA94:MA941}}/C_{\text{RTS2:MA941}}$ was 10.2.

To examine the stage of nodulation at which rhizobitoxine production enhances nodulation competitiveness, we followed the time-course of nodulation competitiveness. Total nodule appearance with time showed similar patterns when USDA94 and RTS2 were used as test strains (Fig. 2). However, the kinetics of nodule occupancy were significantly different between the two strains. The propor-

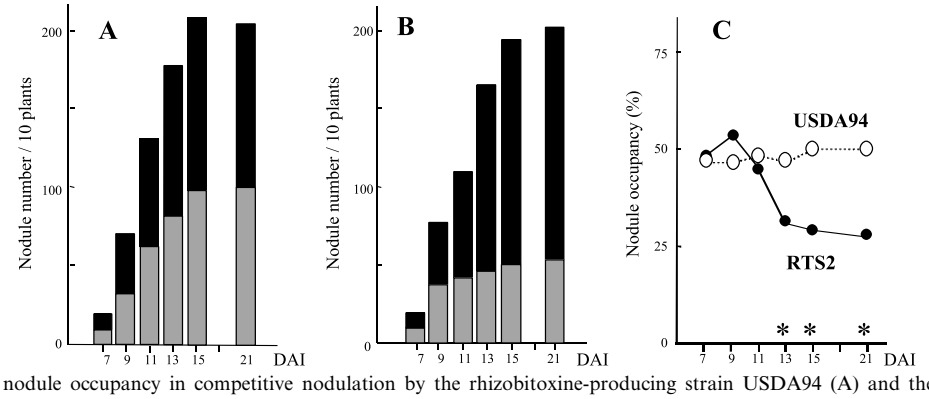

Fig. 2. Time-course of nodule occupancy in competitive nodulation by the rhizobitoxine-producing strain USDA94 (A) and the non-rhizobitoxine-producing mutant RTS2 (B), as compared with the reference strain, MA941. Black bars show the numbers of nodules derived from the reference strain MA941, a *gusA*-marked *B. elkanii* USDA94. Gray bars indicate numbers of nodules derived from the test strains, *B. elkanii* USDA94 (A) and RTS2 (B). C: Fluctuation of nodule occupancy percentage during nodulation time-course. Statistical analysis to compare nodule occupancy between USDA94 and RTS2 at all stages. Asterisks show significant differences in nodule occupancy values between USDA94 and RTS2 by the χ^2 test ($P = 0.05$).

tion of nodules occupied by *B. elkanii* USDA94 was almost 50% throughout the nodulation time-course (Fig. 2A,C). However, when RTS2 was co-inoculated with the reference strain MA941 on to Siratro roots, the proportion of nodules occupied by RTS2 decreased with the time-course of nodule accumulation (Fig. 2B,C). From days 7 to 11 after co-inoculation, the nodule occupancy rates of the two test strains were not significantly different (Fig. 2C). However, after day 13 the accumulation of RTS2-derived nodules almost stopped (Fig. 2B), whereas the proportion of USDA94-derived nodules increased with time (Fig. 2A). The nodule occupancy rate of RTS2 decreased continuously from days 9 to 21 after inoculation, resulting in a 28% occupancy rate at the end of the experimental period. These results indicate that enhancement of nodulation competitiveness by rhizobitoxine occurs at a later stage of nodule accumulation and development.

As shown in Fig. 3, the inoculant strains USDA94, RTS2 and MA941 survived in the hydroponic cultures at a similar cell density, ranging from 10^6 to 10^7 cells ml^{-1}, until 21 days after inoculation. In particular, the populations of RTS2 are similar to that of reference strain MA941 during the assay (Fig. 3A). The ratios of the two strains were confined within 1.5-fold, which was observed 11 days after inoculation (RTS2, 5.0×10^6 cells ml^{-1}; MA941, 3.4×10^6 cells ml^{-1}) (Fig. 3A). These results indicate that the delay effect of rhizobitoxine on nodulation competitiveness was not due to changes in viable cell number in the hydroponic culture.

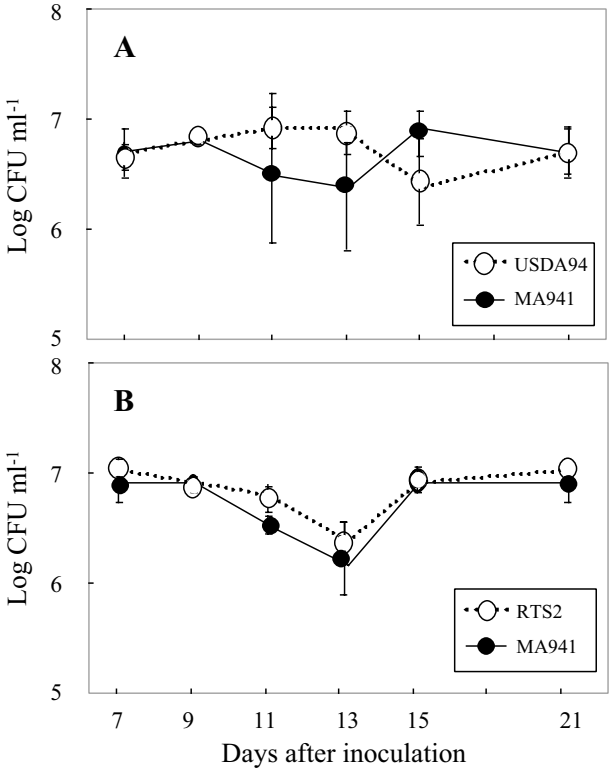

Fig. 3. Time-course of viable cell numbers in hydroponic culture in growth pouches with Siratro plants inoculated with (A) MA941 and USDA94 or (B) MA941 and RTS2 at a concentration of 1×10^7 cells ml^{-1} of each strain. Values represent means of three replications with standard deviations (bar).

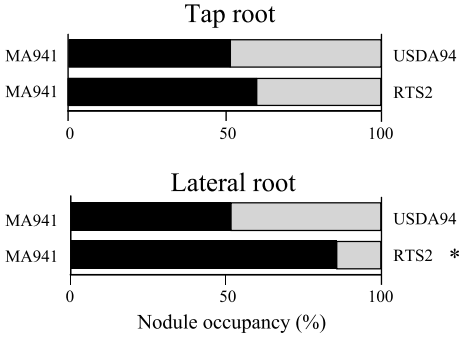

Fig. 4. Nodule occupancy rates of *B. elkanii* strains USDA94 and RTS2 on primary and lateral roots, as compared with those of MA941. Statistical analysis was carried out to compare the rates of nodule occupancy by test strains USDA94 and RTS2. Asterisk shows a significant difference in nodule occupancy values between USDA94 and RTS2 by the χ^2 test ($P=0.05$).

The position of nodules on the tap and lateral roots often provides valuable information that helps us to understand rhizobial nodulation characteristics [19,20]. Therefore, we compared nodulation sites between the rhizobitoxine mutant and parent strains. In Siratro, as in other legumes, the primary root elongates first and then the lateral roots emerge. Under the conditions in the growth pouches used in this study, nodules emerged mainly from the primary root before day 9 after inoculation. From day 9 onwards the numbers of nodules on the lateral roots increased, and by day 21 the Siratro plants had formed an average of 11.9 and 8.5 nodules on the primary and lateral roots, respectively. There was no significant difference between USDA94 and RTS2 in the nodule occupancy rates on primary roots (Fig. 4). However, on the lateral roots, the proportion of nodules occupied by RTS2 was significantly lower than that occupied by USDA94. This result suggests that rhizobitoxine production had a delayed effect on competitiveness in Siratro nodulation.

4. Discussion

The high *C* value ratio of USDA94 against RTS2 clearly demonstrated that rhizobitoxine production enhances competitive nodulation on Siratro plants. Amarger and Lobreau [18] conducted inoculation experiments with *Rhizobium leguminosarum* strains that possessed different competitiveness and found *C* values ranging from 0.064 to 1.78 in competition with rhizobia indigenous to the soil. The *C* value ratio of any two combinations of strains used in their inoculation experiments was at most 8.9 (1.78/0.20), although the *C* values were not calculated in some cases because the regression was not significant. In the present work, we used genetically defined *B. elkanii*

strains to identify the advantages of rhizobitoxine production in nodulation competitiveness on Siratro. Rhizobitoxine production gave *B. elkanii* USDA94 a nodulation competitiveness about 10 times greater than that of a non-rhizobitoxine-producing mutant strain on Siratro (*C* value ratio = 10.2) (Fig. 2). To our knowledge this is the first mathematical determination of nodulation competitiveness for a specific phenotype. Further quantitative evaluations of the many phenotypes and genes involved in nodulation competitiveness could provide an index of the dominant determinants of competitive nodulation in a given association between rhizobia and legumes.

It should be noted that *C* values would probably differ in other host plants according to the plants' susceptibility to rhizobitoxine in nodule formation. The difference between the *C* values of USDA94 and RTS2 might increase in plants that require rhizobitoxine for effective nodulation – such as *V. radiata* (mungbean) and *A. edgeworthii* – because rhizobitoxine is required for normal nodulation in these legumes [12,13].

Since rhizobitoxine production did not change rhizobial populations in the rhizosphere (Fig. 4), the mechanism by which rhizobitoxine production enhanced nodulation competitiveness at a mid to late stage of nodulation kinetics remains unresolved so far. One possible explanation is that in Siratro the crucial ethylene-mediated nodulation restriction is canceled by rhizobitoxine production from the middle of the nodulation time-course. Split root experiments and delayed inoculation experiments have shown that legumes regulate the formation of new nodules through a systemic negative feedback mechanism [21–23]. Ligero et al. demonstrated that increased amounts of ethylene were released from inoculated roots of *Medicago sativa* coincident with the time of nodule development and the beginning of nitrogen fixation [24]. Therefore, it is likely that in this period endogenously produced ethylene mediates a crucial negative feedback on nodulation, and that rhizobitoxine cancels this feedback via inhibition of ethylene biosynthesis.

The known rhizobial factors that affect nodulation competitiveness, including motility [4], rhizopine [5], bacteriocin [6], and exopolysaccharides [7], probably function in the rhizosphere and in the initial interactions between rhizobia and legumes. In contrast, rhizobitoxine enhancement of competitiveness occurs at a later stage of nodulation. This is a unique and promising feature that could prove very advantageous in inoculant production, because inoculum often fails to nodulate in the later stages of nodulation kinetics.

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