Communication

When Does the Self-Regulatory Response Elicited in Soybean Root after Inoculation Occur?¹

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ABSTRACT

The inoculation of soybean (Glycine max L.) roots with Bradyrhizobium japonicum produces a regulatory response that inhibits nodulation in the younger regions of the roots. By exposing the soybean roots to live homologous bacteria for only a short period of time, the question of whether or not early interactions of rhizobia with root cells, prior to infection, elicit this regulatory response has been explored. B. japonicum cells mixed with infective bacteriophages were applied to the roots and then 6 or 24 hours later roots were again inoculated with phage-resistant rhizobia. Mixing of the rhizobia and bacteriophages caused bacterial lysis in 6 to 8 hours and allowed the bacteria to act as live symbionts on the root for only a few hours. However, the interaction of live homologous bacteria with the soybean roots for a few hours did not cause inhibition of nodulation in the younger regions of the roots. Results of these experiments indicate that the self-regulatory response in soybean is not rapidly produced by the early, pre-infection, interactions between rhizobia and the root cells.

In soybean (Glycine max L.), nodulation frequency decreases considerably down in the younger regions of the roots (1, 2). Previous studies (7), involving double inoculation of the roots, showed that the nodulation in the younger regions of soybean root was suppressed by prior inoculation of more mature regions of the root. The suppressive response was not elicited by heterologous or by dead homologous bacteria (7). Based on the short distance between the regions of maximum nodulation and suppressed nodulation, it was inferred that the suppressive response was elicited very rapidly, within an hour or so, by the first inoculum (2, 7). More recent studies (3), however, revealed that the initiation of infection in the younger regions was not suppressed. Instead, it appeared that the process was suppressed at various stages of infection development, including later stages just prior to nodule emergence. This raised the question of whether suppressive responses were triggered by pre-infection events taking place within a few hours after inoculation or by post-penetration events associated with subsequent infection development. In order to address this question, we attempted to provide the soybean roots with short, defined exposure(s) to live rhizobia to learn if early interaction between the root and the rhizobia (first few hours after inoculation) can elicit the suppressive responses. Results of these experiments are reported here.

MATERIALS AND METHODS

Bacterial Culture. Bradyrhizobium japonicum strain USDA-110-ARS (azide, rifampicin, and streptomycin resistant) and its phage-resistant derivative (PR11) were used in all the studies described here. The conditions for their storage and culture have been previously described in detail (5, 6).

Isolation of Bacteriophage and Phage-Resistant Rhizobial Cells. Bacteriophage (NM11), which was capable of specifically killing our standard bacterial strain, B. japonicum I-110-ARS, was isolated from local soil which had been heavily inoculated with rhizobia I-110-ARS, according to the method described by Klowalski et al (4). The bacteriophage remained active for several weeks when stored in water at 4°C. In our experiments, however, phages were stored for no more than 1 week prior to use.

The cells of B. japonicum I-110-ARS which were resistant to phage NM11 were isolated by continuously shaking the rhizobial culture infected with the phage for a number of days. Prolonged shaking of this culture produced resistant cells which were then plated on YEM agar plates to isolate cells from single colonies and later multiplied by liquid shake culture. These phage-resistant rhizobia (PR11) were then tested for their nodulation ability and were found to nodulate as well as the parent phage-sensitive rhizobia (Table I).

Plant Growth. Soybean seeds (Glycine max L. Merr. cv Williams) were purchased from Dewine and Hamma Seed Co,

Table 1. Comparison of the Ability of B. japonicum Strain I-110-ARS and Its Phage-Resistant Isolate, PR11, to Nodulate Soybean Roots

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Average No. of Nodules on the Tap Root</th>
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<tbody>
<tr>
<td></td>
<td>Nodules above RT mark</td>
</tr>
<tr>
<td>B. japonicum I-110-ARS</td>
<td>2.2 ± 0.2*</td>
</tr>
<tr>
<td>Phage-resistant isolate of I-110-ARS (PR11)</td>
<td>2.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Mean ± SE.
Yellow Springs, OH. Seeds were surface-sterilized with NaOCl, washed, germinated on agar, and then seedlings were grown in plastic growth pouches, containing N-free Jensen's nutrient media, as described previously (5, 6).

Sixty seedlings, 48 h old, were transferred to a plastic mesh grid set on a deep glass dish; the root tips were always ca. 1 cm above the bottom of the glass dish. These seedlings were collectively inoculated by transferring the plastic grid to a glass dish filled with either rhizobia (I-110-ARS, 10^6 cells/ml) mixed with phage (NM11, 10^7/ml) or phage alone (NM11, 10^7/ml). The seedlings were dipped for 15 min in the inocula, kept in a moist atmosphere for 45 min, and then transferred to plastic growth pouches and the position of the root tips carefully marked on the clear plastic. For the second inoculation of the root, 250 µL of inoculum (PR11) or water was dripped directly on individual roots. Seven days after the second inoculation the nodules were counted on the tap root of individual seedlings as described before (5).

**RESULTS**

Effect of Exposing Soybean Roots to Live Homologous Rhizobia (I-110-ARS) for a Few Hours on Nodulation by a Subsequent Inoculation after 24 h. In this experiment, we dip-inoculated the soybean roots with phage-sensitive rhizobia (10^6 cells/ml) which were mixed with phage (1:1000 units) just before inoculation. These seedlings were transferred to plastic growth pouches and the position of the RT^2 marked on the clear plastic (RT1). The roots were reinoculated 24 h after the first inoculation with the phage-resistant rhizobia (PR11), and the position of the roots at the time of the second inoculation (RT2) was again marked on the pouches. As controls, a second set of seedlings was first inoculated with the phage alone at RT1 and then with the PR11 at RT2. A third set of seedlings was first inoculated with a mixture of rhizobia and phage, as in the case of first set of seedlings, and then for the second time, 24 h later at RT2, the roots were treated with sterile water.

In separate experiments, it was determined that the phages did not cause lysis of most of the bacteria until about 6 h after inoculating. This indicates that the live bacteria would have interacted with the roots for some time, up to approximately 6 h after inoculating the roots with rhizobia mixed with phages.

Figure 1 shows the relative frequency of nodule formation at various positions along the primary root, relative to RT1 and RT2. The nodulation profile shown in Figure 1A, where seedlings were inoculated at RT1 with phages only and at RT2 with PR11, is typical of the previously published profiles (2, 7). It is, however, interesting to note in Figure 1B that, even when the seedlings were first treated with rhizobia mixed with phages, there was no inhibition of nodulation at RT2 (compare nodulation profiles at RT2 of Fig. 1, A and B). When rhizobia were mixed with bacteriophages at the time of inoculation then only a few nodules were produced (see profiles at RT1 of Fig. 1, B and C) which indicates that in our experiments the phages were quite effective in eventually killing the rhizobia.

**Effect of Treating Soybean Roots to Live Homologous Rhizobia for Only a Few Hours on Nodulation by a Subsequent Inoculation after 6 h.** The concept of rapid elicitation of self-regulatory response was based on the observations that inhibition of nodulation starts within 1 to 2 mm below the maximally nodulated region of the root (2). Experiments were therefore conducted to see if inoculation of roots with the live homologous bacteria (I-110-ARS) for a short time period would inhibit nodulation by a second inoculation, conducted 6 h after the first inoculation, with the phage resistant rhizobia (PR11). The ex-

![](image)

**Figure 1.** Effect of exposing soybean roots to live homologous rhizobia for only a few hours upon subsequent nodulation when reinoculated after 24 h. Seeds were germinated in the dark for 48 h on 1% agar made with nitrogen-free nutrient medium. Uniform seedlings were dip-inoculated, as described in "Materials and Methods," and then transferred to plastic growth pouches containing Jensen's nutrient medium. The seedlings were again inoculated after 24 h with the appropriate inocula. The position of the RT at the time of first (RT1) and second (RT2) inoculation were marked on the pouches. The nodules on the primary root were counted, 7 d after the second inoculation, and their relative distance from RT1 and RT2 estimated. In Figure 1A seedlings were first inoculated (RT1) with bacteriophages (10^7 particles/ml) and then reinoculated at RT2 with phage resistant *B. japonicum* cells (10^7 cells/ml). In Figure 1B, a mixture of bacteriophages (10^7 particles/ml) and *B. japonicum* cells (10^7 cells/ml) were applied at RT1 and 24 h later phage resistant *B. japonicum* cells were applied at RT2. In Figure 1C, a mixture of bacteriophages and *B. japonicum* cells (as described above) were applied at RT1 and sterile water at RT2. The direction of root growth is from RT1 to RT2. The number of nodules described are the total nodules counted on sixty seedlings per one relative distance unit (RDU). One RDU is equal to the length of root between RT1 and RT2. Sixty replicate seedlings were used in each treatment and the experiment was repeated twice.

**Table II.** Effect of Pretreating Soybean Roots with Live Homologous Rhizobia for Only a Few Hours upon Subsequent Nodulation when Reinoculated after 6 h

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Time between inoculation</th>
<th>Nodules/Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>First inoculation (RT1)</td>
<td>Second inoculation (RT2)</td>
<td>Total Nodules</td>
</tr>
<tr>
<td>h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. japonicum + NM11</td>
<td>6 PR11</td>
<td>1.90</td>
</tr>
<tr>
<td>B. japonicum + NM11</td>
<td>6 Water</td>
<td>0.51</td>
</tr>
<tr>
<td>NM11</td>
<td>6 PR11</td>
<td>0.97</td>
</tr>
</tbody>
</table>

* Mean ± se.

Abbreviation: RT = root tip.
per experimental procedure and the treatments were same as in the above experiment except that the time duration between the two inoculations was reduced to 6 h.

In treatment number one of these experiments, plants were first treated, at RT1, with the rhizobia (I-110-ARS) mixed with the phages (NM11), and 6 h later plants were again inoculated, at RT2, with the phage resistant rhizobia (PR11). In treatment number two, plants were treated only with the rhizobia (I-110-ARS) mixed with the phages at RT1 and were not inoculated again after 6 h at RT2. In treatment three, however, plants were only inoculated at RT2 with the phage resistant rhizobia (PR11), and did not receive prior inoculation with the rhizobia at RT1. Thus in a sense, treatments two and three were combined in treatment one. It was our hypothesis, that if application of rhizobia (I-110-ARS) did not elicit inhibitory response for the second inoculation at RT2 by phage resistant rhizobia (PR11), then the total number of nodules produced on plants by treatment one would be equal to the combined number of nodules produced on plants of treatments two and three. This proves to be true for these experiments as indicated by the results presented in Table II.

DISCUSSION

There is a little doubt that nodule formation in the younger regions of soybean roots is inhibited if the roots had been previously inoculated with homologous rhizobia. This phenomenon has been consistently seen in our experiments and has been reported (2, 7). However, the earlier hypothesis (2, 7) that the elicitation of an inhibitory response is accomplished in a matter of a few hours after inoculation could not be supported by our results presented here. As can be seen in Figure 1 and in Table II, initial inoculation of soybean roots with rhizobia mixed with the bacteriophage could not inhibit nodulation in the younger regions of the roots inoculated with the phage resistant rhizobia (PR11). The younger regions of the roots were inoculated 6 or 24 h after the first inoculation. Since the lysis of the *Bradyrhizobium* (I-110-ARS) by the phage (NM11) takes 6 to 8 h, we can safely assume that in our experiments, where rhizobia were mixed with the phages at the time of inoculation, the interaction between root cells and the live homologous rhizobia continued for at least 3 to 6 h. However, this interaction between live homologous rhizobia and the infectable root cells could not elicit an inhibitory response to suppress nodulation in the younger regions of the soybean root (Fig. 1 and Table II). Thus the self-regulatory response in soybean that inhibits nodulation in the younger regions of roots (2, 3, 7) must be produced at later stages of infection development. These results are supported by the finding of Calvert et al. (3) who could not detect inhibition of infections in the region of the root where nodulation frequency was inhibited, which also indicates that the self-regulatory response is perhaps produced by the maturing infections or developing nodules.

All in all, the results presented here demonstrate that the self-regulatory response in soybean is not rapidly elicited, within a few hours of inoculation, as was proposed earlier (2, 7). It is not yet clear, however, at what stage of infection development this regulatory response is produced. Isolation of *B. japonicum* mutants capable of developing infections only to specific stages without developing into nodules on the root will be helpful in further defining the stage at which the self-regulatory response is produced in soybean.

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LITERATURE CITED