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A Rapid Regulatory Response Governing Nodulation in Soybean[1]

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ABSTRACT

The number of nodules which develop on the primary root of soybean seedlings (*Glycine max* L. Merr) after inoculation with *Rhizobium japonicum* is substantially diminished in the region of the root developmentally 10 to 15 hours younger than the region maximally susceptible to nodulation at the time of inoculation. This rapid inhibition of nodulation has been investigated by inoculating soybean seedlings with rhizobia at two different times, 15 hours apart. Living *R. japonicum* cells, but not heterologous rhizobia or UV-killed cells of the homologous bacterium, were capable of eliciting the rapid inhibitory response. Nodulation responses to varying inoculum concentrations showed that bacterial dosages could be superoptimal, resulting in reduced nodulation and reduced inhibition of nodulation. When suspensions of *R. japonicum* were dripped uniformly onto the root surfaces, the degree of inhibition of nodulation in developmentally younger regions of the root was correlated with the number of nodules formed in the older and initially most susceptible region of the root. Nodulation in the developmentally younger region of the root, however, was affected very little if the first inoculum was restricted to contact with root cells in the region initially most susceptible to nodulation. The rapid regulatory response may be an important factor contributing to the clustering of nodules in the crown region of soybean roots in field-grown plants and the sparse nodulation commonly observed in younger regions of the root.

Soybean roots are infected and nodulated by the nitrogen-fixing bacterial symbiont, *Rhizobium japonicum*. Infections take place through root hair cells on the epidermal surface of the root (5, 10, 11). The susceptibility of soybean root hairs to infection and nodulation by the microsymbiont is developmentally restricted and transient (4; Calvert, Pence, Pierce, Malik and Bauer, manuscript submitted). Infections and nodules on the primary root develop most frequently in the region just below the smallest emerging root hairs present at the time of inoculation. Root hairs which are already emerging at the time of inoculation are less susceptible to infection and nodulation. Mature, fully elongated root hairs are not normally susceptible to infection or nodulation. Acropetal development of the root causes root hairs to acquire and then lose their susceptibility to *Rhizobium* within a period of 4 to 6 h (4).

Nodulation frequency (*i.e.* the number of nodules per unit length of root) is not sustained at high levels on the primary root as the root continues to develop in the presence of the bacterial inoculum (4). In regions of the root developmentally just 10 to 15 h younger than the most densely nodulated region, nodulation frequency diminishes to approximately 20% of the maximum frequency. Bhuvaneswari *et al.* (4) suggested that this diminished frequency of nodulation in younger regions of the root might be due to a fast-acting regulatory mechanism in the host which could prevent excessive nodulation. In field-grown soybean plants, nodulation is frequently much diminished in younger regions of the root system in comparison with the crown region (1, 7).

MATERIALS AND METHODS

Cultures of *Rhizobium japonicum* strain I-110 ARS (azide, rifampin, and streptomycin resistant) were obtained from D. Kuykendall, USDA Beltsville, MD, and stored in ampules after freeze-drying. Strain I-110 ARS was cultured in liquid yeast extract-mannitol-gluconate medium as described previously (4). Inocula were prepared from mid-exponential phase cultures by centrifugation, washing with sterile phosphate buffered saline, resuspension in one-tenth-strength phosphate-buffered saline, and dilution with half-strength N-free Jensen's plant growth medium to the desired cell density (4). Approximate cell densities were determined from the absorbance of diluted suspensions at 620 nm ($0.03\ A_{620\ \mathrm{nm}} = 1 \times 10^8$ cells/ml). To test the effects of dead *R. japonicum* cells, suspensions of bacteria were diluted to 1×10^5 cells/ml and exposed in a Petri dish to sufficient UV irradiation to kill more than 99.9% of the cells. Aliquots from such suspensions and from unexposed suspensions of the same bacteria were used to inoculate test and control seedlings, respectively.

Cultures of the heterologous rhizobia *R. leguminosarum* (strain 603) and *R. trifolii* (strain TA1) were obtained from M. Lamborg and B. Rolfe, respectively, and cultured on the yeast extract-mannitol-gluconate medium to mid- or late exponential growth phase as described for *R. japonicum*. Inocula were prepared by dilution of exponential phase cultures of these strains with sterile half-strength N-free Jensen's medium to the desired cell density.

Soybean (*Glycine max* L. Merr) seeds of cv Williams were obtained from DeWine and Hamma Seed Company, Yellow Springs, OH. Seeds were surface sterilized, germinated and transferred to plastic growth pouches (Northrup King Seed Co., Minneapolis, MN) as described previously (4). Seedlings were inoculated 2.5 to 3 d after imbibition. At the time of inoculation, the position of the root tip of each seedling was marked with indelible pen on the plastic face of the pouch. The position of the smallest emergent root hairs visible under a dissecting microscope at ×12 was also marked for each seedling at this time. The distance between the root tip and the smallest emergent root hairs at the time of inoculation is referred to as a RDU.[4] The use of RDU rather than simple metric measures facilitates compar-

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[4] Abbreviations: RDU, relative distance unit; RT, root tip.

ison of results within and between sets of plants because it normalizes differences in the rates of root growth and development between plants (4). Seedlings were watered daily with half-strength N-free Jensen's plant growth medium and maintained in an environmentally controlled chamber as described previously (4). Nodulation on the primary root of each seedling was scored 7 to 10 d post-imbibition as described previously (3, 4). Uninoculated control plants did not develop nodules.

Nodulation data in this paper are generally presented in the form of profiles of nodulation frequency. These profiles indicate the average number of nodules which develop within small distance intervals at various relative distances from the mark made at the root tip at the time of inoculation. The averages were obtained by scoring 30 to 100 plants per profile. When seedlings were inoculated twice, the second inoculum was applied 15 h after the first one. RDU were then based on the distance between the smallest emergent root hairs present at the time of the second inoculation and the position of the root tip at this time (RT2). In some experiments, the distance that a root grew in the interval between the first and second inoculations was used as the RDU. In certain instances, the first bacterial inoculum was applied to specific points on the root in droplets of approximately 0.25 μl or of 1 to 2 nl, as described (9).

RESULTS AND DISCUSSION

Effects of Double Inoculation. The diminished frequency of nodulation below the RT mark noted previously (4) might have occurred because the younger regions of the root were not exposed to adequate numbers of rhizobia or because the rhizobia declined in their ability to infect the roots after inoculation. To determine whether either of these explanations might account for the observed decrease in nodulation frequency, test seedlings were exposed to a second inoculum of *R. japonicum* (at RT2) 15 h after exposure to the first inoculum (at RT1). Figure 1 illustrates the nodulation profiles obtained when sets of seedlings were inoculated only at the time of marking RT1 (O), only at the time of marking RT2 (Δ), and at both the time of marking RT1 and the time of marking RT2 (□). Bacteria applied to the root at the time of marking RT2 did not have an observable effect on nodulation in the vicinity of RT1, and had very little effect on the diminished nodulation below RT1, closer to the root tip. Thus, it appears that diminished nodulation below RT1 is due to a regulatory response of the host and is not a result of inadequate exposure to infective rhizobia.

Elicitation by Heterologous or Dead Rhizobia. The regulatory response does not appear to be elicited by exposure of the roots to strains of heterologous rhizobia or to UV-killed cells of the homologous species *R. japonicum*. An initial exposure of soybean seedlings to *R. trifolii* or *R. leguminosarum* at the time of marking RT1, followed 15 h later by inoculation with *R. japonicum* at the time of marking RT2, had no appreciable effect on nodulation in the vicinity of RT2 (Fig. 2). In similar experiments, seedlings were inoculated with 2.5 × 10^4 cells/plant of UV-killed cells of the homologous bacterium, *R. japonicum*, and then inoculated 15 h later with unirradiated *R. japonicum* cells. There was no measurable effect of the initial exposure to irradiated *R. japonicum* cells on nodulation below RT1 (data not shown). We infer from these results that some substance(s) secreted by living *R. japonicum* cells or some specific interaction between *R. japonicum* cells and the host root may be required in order to elicit the regulatory response.

In further experiments related to the possible secretion of eliciting substances, the moist paper towel wick beneath the roots of inoculated seedlings was carefully cut away below the RT1 mark, 15 h after inoculation. The root of each seedling was rinsed with Jensen's medium just below RT1. The region above RT1 was carefully left undisturbed. A new moistened paper towel wick was added to replace the cut away portion of the original

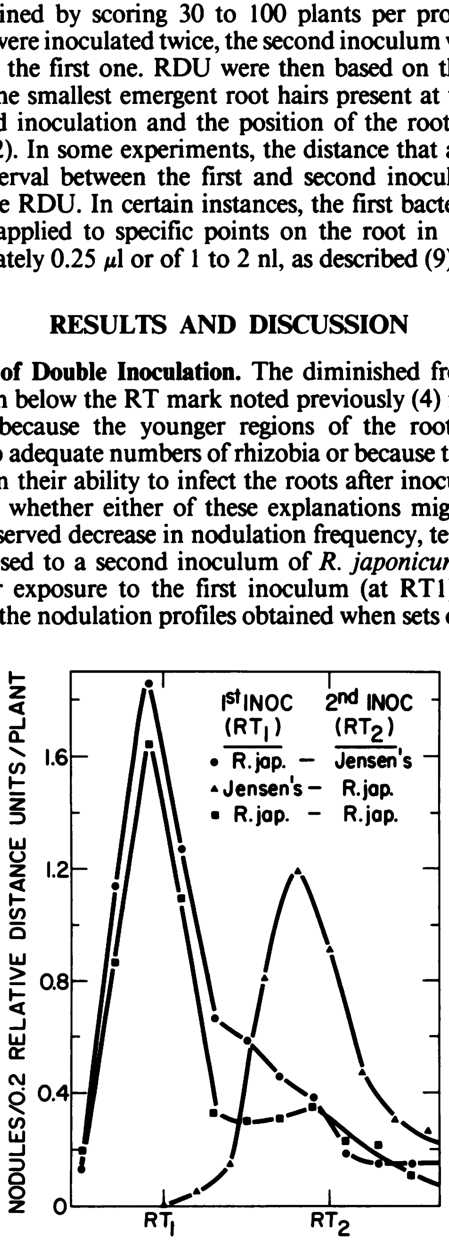

FIG. 1. Double inoculation with *R. japonicum*. Separate sets of 100 seedlings were inoculated at the time of marking RT1 with 250 μl of a suspension of 1 × 10^5 *R. japonicum* cells/ml in half-strength N-free Jensen's medium or with 250 μl of the Jensen's medium. After 15 h, the seedlings were inoculated a second time with either the same number of *R. japonicum* cells or with the Jensen's medium. One RDU is equal to the length of the root between RT1 and RT2. The direction of root growth is from RT1 towards RT2.

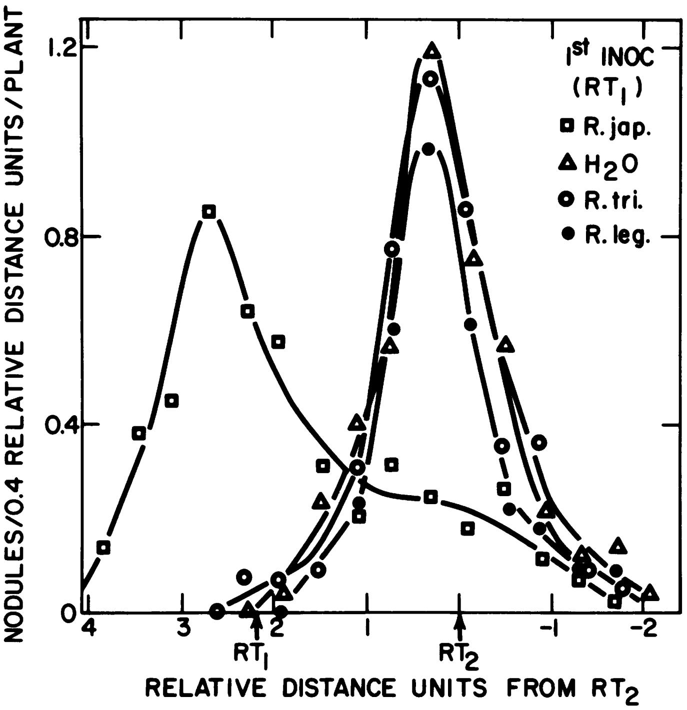

FIG. 2. Effects of initial exposure to heterologous rhizobia. Separate sets of 40 to 50 plants were inoculated at the time of marking RT1 with 250 μl of: (□), 1 × 10^5 cells/ml *R. japonicum*; (Δ), half-strength N-free Jensen's medium; (O), 1 × 10^5 cells/ml *R. trifolii*; or (●), 1 × 10^5 cells/ml *R. leguminosarum*. RDU are based on root hair emergence at the time of marking RT2. The direction of root growth is from RT1 towards RT2.

wick. The entire 3 or 4 cm of the root above the tip (RT2) was then reinoculated. The results of these experiments are shown in Table I. It was found that rinsing of the root and removal of the old paper towel wick had no appreciable effect on either nodulation above the RT1 mark or on the inhibition of nodulation below this mark. This result seems to indicate that any substances which might be secreted by *R. japonicum* and present on the paper towel wick or root surface 15 h after inoculation do not contribute to elicitation or expression of the regulatory response. It remains possible that substances secreted by *R. japonicum* are effective at earlier times.

Effects of Inoculum Dose on Elicitation. Sets of seedlings were inoculated with either 0, 7.9 × 10^1, 2.5 × 10^4, or 7.9 × 10^6 *R. japonicum* cells per plant at the time of marking RT1. The same plants were then inoculated 15 h later, at the time of marking RT2, with the normal dosage of 2.5 × 10^4 bacteria per plant (Fig. 3). It is evident that an initial exposure of the host roots to 7.9 × 10^1 *R. japonicum* cells did not elicit a strong regulatory response in the region below RT1. This result appears to rule out the possibility that the inhibition is triggered to full expression by just a few rhizobia. Maximum inhibition of nodulation below RT1 was elicited by prior exposure of the roots to the normal inoculum dose of rhizobia (2.5 × 10^4 cells/plant).

Table I. *Effect of Rinsing Roots in the Vicinity of RT2 on the Inhibition of Nodulation*

Fifteen h after inoculation with 2.5 × 10^4 *R. japonicum* cells/plant and marking of RT1, the paper towel wick was removed from below RT1, the root of each test plant below RT1 was briefly rinsed, a new wick was inserted, and the root was uniformly inoculated again with the same number of bacteria at the time of marking the new position of the root tip (RT2). Nodulation on the primary root was scored 1 week later, as described (3, 4).

Treatment	Average No. of Nodules/Plant		Plants Treated
	Above midpoint[a]	Below midpoint	
Two inoculations, no rinse	4.27 ± 0.30[b]	2.11 ± 0.18	66
Two inoculations, rinse in between	4.36 ± 0.42	1.72 ± 0.27	39
One inoculation following rinse	0.03 ± 0.03	3.58 ± 0.36	33

[a] Midpoint = position on the root halfway between RT1 and RT2.
[b] Mean ± SE.

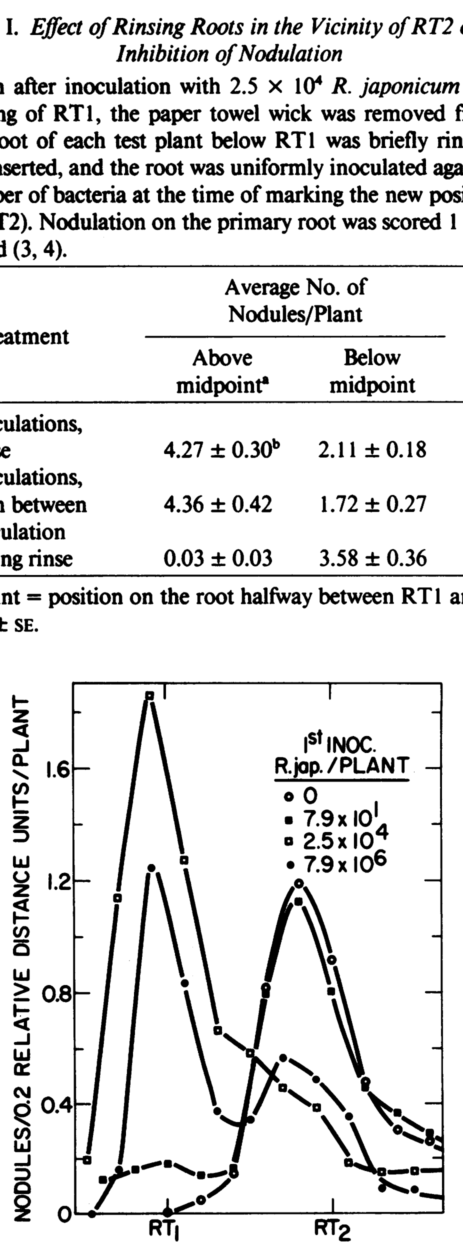

FIG. 3. Effects of inoculum dose on inhibition on nodulation. Separate sets of 100 plants were inoculated (250 μl) at the time of marking RT1 with: (○), the half-strength N-free Jensen's medium; (■), 7.9 × 10^1 *R. japonicum* cells/plant; (□), 2.5 × 10^4 *R. japonicum* cells/plant; or (●), 7.9 × 10^6 *R. japonicum* cells/plant. All plants were inoculated 15 h later (at RT2) with 250 μl of 1 × 10^5 *R. japonicum* cells/ml. One RDU is equal to the distance between RT1 and RT2.

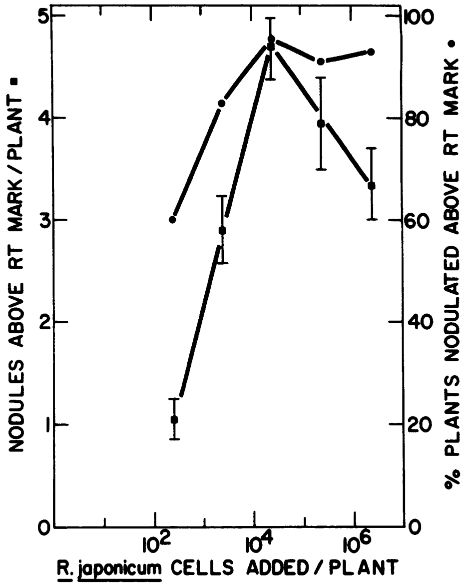

FIG. 4. Effect of inoculum dose on nodulation above the root tip mark. Separate sets of 50 seedlings were inoculated with 250 μl aliquots of *R. japonicum* suspensions from a dilution series. Different sets of seedlings received inoculum doses ranging from 2.5 × 10^2 bacteria/plant to 2.5 × 10^6 bacteria/plant. Nodulation above the RT mark made at the time of the single inoculation was scored 1 week later. The dependence of nodulation on the logarithm of the inoculum dose is shown for both the average number of nodules above the RT mark/plant (■) and the percentage of plants with nodules above the RT mark (●).

An unanticipated result of these experiments was that prior exposure of the root to a larger than normal dosage of rhizobia (7.9 × 10^6 cells/plant) did not elicit a stronger than normal regulatory response below RT1 (Fig. 3). Also unexpectedly, fewer nodules were generated above the RT1 mark following inoculation with 7.9 × 10^6 bacteria per plant than following inoculation with 2.5 × 10^4 bacteria per plant (Fig. 3). Additional experiments were performed in order to determine whether this diminished frequency of nodulation above RT1 might be an artifact of the double inoculation technique. Sets of test seedlings were inoculated a single time with *R. japonicum* at dosages ranging from 2.5 × 10^2 to 2.5 × 10^6 bacteria per plant. Subsequent scoring of nodulation on these plants revealed that the average number of nodules above the RT mark decreased significantly at inoculum doses larger than 2.5 × 10^4 (Fig. 4). This is in agreement with the diminished nodulation obtained above RT1 in Figure 3 after an initial exposure to 7.9 × 10^6 rhizobia.

These results suggest the existence of another regulatory phenomenon affecting nodulation in soybean. Bhuvaneswari *et al.* (4) previously pointed out that the logarithmic (rather than arithmetical) dependence of nodulation on inoculum dose implies the existence of one regulatory effect. In addition to this regulatory effect, the results in Figure 3 and 4 demonstrate that a further regulatory phenomenon is expressed at high inoculum doses. This effect reduces nodulation below the normal log-linear response levels (Fig. 4). The superoptimal dosage effect appears

Table II. *Effect of Restricted Inoculation on Expression of the Regulatory Response in Younger Regions of the Root*

In the experiments of set A, test seedlings were inoculated with a 0.25-µl droplet of 1 × 10^8 cells/ml of *R. japonicum* in half-strength, N-free Jensen's medium. The droplet was carefully placed on the root at 0.8 RDU from the tip of each seedling at the time of marking RT1. After 15 h, these seedlings were inoculated a second time by dripping 250 µl of a bacterial suspension containing 1 × 10^5 cells/ml uniformly over the lower 2 to 4 cm of the root. The position of the root tip was marked again (RT2) at the time of this second inoculation. Control seedlings were drip inoculated with 250 µl of the bacterial suspension or the Jensen's medium, as indicated. In the experiments of set B, test seedlings were inoculated with four 1- to 2-nl droplets of a bacterial suspension containing 1 × 10^8 cells/ml at 0.8 RDU from the tip (RT1) and then reinoculated 15 h later (RT2) with 250 µl of a suspension containing 1 × 10^5 cells/ml dripped onto the root. Nodulation was scored 1 week after inoculation, as described (3, 4).

Inoculations	Average No. of Nodules/Plant		Plants Treated
	Above midpoint[a]	Below midpoint	
Experiment Set A			
1st: 0.25 µl 10^8 Rj/ml 2nd: 250 µl 10^5 Rj/ml	2.21 ± 0.24[b]	4.18 ± 0.31[c]	56
1st: 250 µl 10^5 Rj/ml 2nd: 250 µl 10^5 Rj/ml	4.27 ± 0.30	2.11 ± 0.18[c]	66
1st: 250 µl Jensen's 2nd: 250 µl 10^5 Rj/ml	0.33 ± 0.13	4.86 ± 0.60	21
Experiment Set B			
1st: 4 nl 10^8 Rj/ml 2nd: 250 µl 10^5 Rj/ml	3.68 ± 0.38	6.58 ± 0.44[d]	50
1st: 250 µl 10^5 Rj/ml 2nd: 250 µl 10^5 Rj/ml	5.41 ± 0.42	2.74 ± 0.36[d]	34
1st: 4 nl Jensen's 2nd: 250 µl 10^5 Rj/ml	0.24 ± 0.10	6.03 ± 0.48	33

[a] Midpoint = position on the root halfway between RT1 and RT2.

[b] Mean ± SE.

[c,d] Nodulation averages designated by the same superscript letter are significantly different from each other by the ± test at $P < 0.01$.

to influence the expression of the rapid regulatory response below RT1 as well as nodulation above RT1 (Fig. 3).

Restricted Inoculation. Preliminary studies have been made to explore the question of whether the rapid regulatory response is elicited in the region that is maximally susceptible at the time of inoculation or in the younger region of the root where the inhibition of nodulation is expressed. The zone of maximum susceptibility to *Rhizobium* lies between 0.5 and 1.0 RDU from the root tip (4). Accordingly, sets of seedlings were inoculated with small droplets of bacterial suspension placed carefully at a position 0.8 RDU from the root tip of each test seedling. The effects of this localized or restricted inoculation on nodulation in younger regions of the root following a second, unrestricted inoculation were determined.

In one set of experiments, an inoculum droplet of 0.25 µl containing 1 × 10^8 bacteria/ml was delivered with a 1-µl syringe to the desired position at 0.8 RDU from the root tip of each test seedling. After 15 h, the roots were inoculated a second time with an equal number of bacteria. The second inoculum (250 µl of a 1 × 10^5 bacteria/ml suspension) was applied uniformly in large drops over the root surface from below RT2 to above RT1. As controls, separate sets of plants were inoculated initially with 250 µl of either the Jensen's medium or a suspension of *R. japonicum* containing 1 × 10^5 bacteria/ml. This inoculum was not restricted to the initially susceptible portion of the root, but was applied uniformly over the lower 2 or 3 cm of the root.

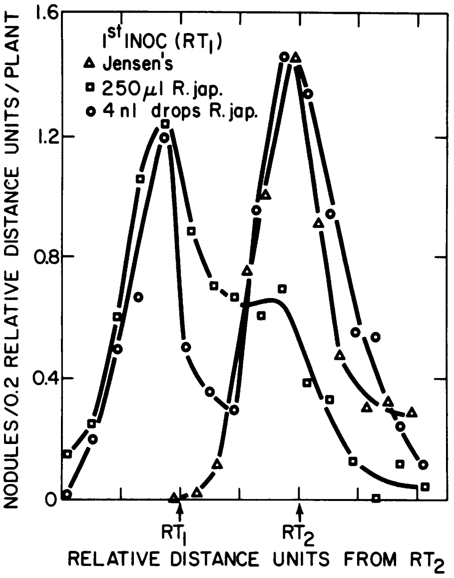

FIG. 5. Effect of initial nanoliter spot inoculations in the region of maximum susceptibility. Sets of 50 to 60 plants were inoculated at the time of marking RT1 with: (△), 250 µl of the half-strength N-free Jensen's medium; (□), 250 µl of 1 × 10^5 *R. japonicum* cells/ml; or (○), four droplets of approximately 1 nl each containing 1 × 10^2 *R. japonicum* cells/nl. The nanoliter droplets were placed at positions on the sides of the roots at 0.8 RDU from the tip. All plants were inoculated 15 h later (at RT2) with 250 µl of 1 × 10^5 *R. japonicum* cells/ml. RDU are based on root hair emergence at the time of marking RT2.

Restricted inoculation with 0.25-µl droplets in the region of maximum susceptibility resulted in a peak of nodulation above the RT1 mark which was approximately half the size of the peak of nodulation generated by unrestricted inoculation (Table II). This peak, however, was sharper and slightly displaced toward the older region of the root (data not shown). Seedlings receiving the restricted inoculum at the time of marking RT1, however, developed about twice as many nodules below RT1 as seedlings receiving an unrestricted inoculum of *R. japonicum* at RT1 (Table II). These results suggest that the rapid regulatory response may not be elicited effectively by interactions with root cells in the region of maximum susceptibility at 0.5 to 0.8 RDU above the tip.

The validity of this conclusion depends in part on how cleanly the restricted, 0.25-µl inoculum was confined to the initially susceptible portion of the root. To determine how effectively the 0.25-µl inoculum was restricted, 50 plants were inoculated with the 0.25-µl droplets as before, but were not inoculated a second time. Spreading of the restricted inoculum to root cells below RT1, as evidenced by the presence of nodules in the RT2 region, occurred in 20% of the plants.

As an alternative method of restricted inoculation, the nanoliter droplet technique (9) was used to place four very small (1-2 nl) droplets of a bacterial suspension (1 × 10^8 cells/ml) onto the root of each test plant at 0.8 RDU from the tip. The droplets were placed on the sides of each root and the positions marked with ion exchange beads as previously described (9). After 15 h, the plants were uniformly drip inoculated with 250 µl of a suspension containing 1 × 10^5 bacteria/ml as before. It was found that the nanoliter droplet inoculation also generated somewhat less nodulation above RT1 then the unrestricted inoculation (Fig. 5; Table II). Nodulation in the vicinity of RT2 appeared to be unaffected by early exposure of the root to the spot inoculations. In a separate experiment, 7 of 52 (13%) of the test plants inoculated at RT1 with the nanoliter droplets developed nodules below the midpoint between RT1 and RT2.

Summary and Conclusions. The rapid regulatory response in soybean is reproducibly elicited by *R. japonicum* but is not elicited to a discernible extent by interactions with either heterologous rhizobia or dead homologous rhizobia (Figs. 1 and 2). If substances secreted by living *R. japonicum* cells are responsible for eliciting the rapid regulatory response, such substances are likely to have their effect in less than 15 h after inoculation (Table I). When the second inoculum is added 48 h after the first inoculum, rather than 15 h after, only a very small peak of nodulation is generated in the vicinity of RT2 (Malik and Bauer, unpublished). This indicates that the rapid regulatory response is strongly expressed for a considerable distance and length of time in the primary root.

The regulatory response was not elicited at full strength by interactions with a few *R. japonicum* cells (Fig. 3). Nor, on the other hand, was the rapid regulatory response elicited and expressed in simple proportion to the number of *R. japonicum* cells. Instead, so long as the inoculum was spread evenly over the lower 2 to 3 cm of the root, expression of the rapid regulatory response was correlated with the number of nodules generated above RT1 by the initial inoculum. The observed correspondence between the number of nodules that develop above the mark and the degree of inhibition of nodulation expressed below the mark seems reasonable if the function of this regulatory effect is to prevent the formation of more nodules than is beneficial to the host.

The results in Table II and Figure 5 appear to indicate that *R. japonicum* cannot effectively elicit the rapid regulatory response in root cells approximately 0.5 to 1.0 RDU above RT1 (the zone of maximum susceptibility at the time of inoculation). However, it should be noted that the restricted inoculations generated fewer nodules above RT1 than the unrestricted inoculations, and may thus have generated significantly fewer infections in this region. We do not know the extent to which elicitation of the rapid regulatory response in soybean depends on the number of infections which are generated by the inoculum. Therefore, the weak expression of the regulatory response in the vicinity of RT2 following restricted inoculations at 0.8 RDU above RT1 may be a reflection of fewer infections or incipient infections near the site of inoculation rather than any inability of root cells at this site to respond to elicitation. Further experimentation is required to determine the number of infections in various regions of the root following restricted inoculation and to identify the earliest cytological events associated with expression of the rapid regulatory response.

We have recently obtained microscopic evidence that the regulatory response is expressed long before nodules develop on the root. Segments of roots excised from test plants inoculated for the experiments illustrated in Figure 1 were fixed, embedded in paraffin, sectioned serially, stained and analyzed with the light microscope to determine the position and stage of development of every infection (Calvert, Pence, Pierce, Malik and Bauer, manuscript submitted). Examination of this root material revealed that considerably fewer interactions reached the stage of infection thread formation in the region below RT1 than in the region just above RT1. Previous cytological studies have shown that *R. japonicum* penetration and infection thread formation occur approximately 24 h after inoculation and that nodules do not appear until 5 or 6 d after inoculation (6, 10, 11). Clearly then, the regulatory response is elicited and expressed in the interval between inoculation and 24 h after inoculation, as originally inferred from the developmental age of the affected region of the root. This means that although expression of the rapid regulatory response is correlated with the density of nodulation above the mark, the presence of nodules above the mark does not directly determine expression of the inhibitory response. Instead, it seems likely that some much earlier events or interactions determine both the extent of nodulation above the mark and the extent of inhibition of nodulation below the mark.

Soybean plants grown in soil normally show a clustering of nodules near the crown of the root (1, 7). Variations in the pattern of nodulation for different soybean cultivars planted in the same soil (7) provide evidence that the phenomenon of crown nodule clustering is primarily a host-determined regulatory effect rather than an environmental or *Rhizobium* distribution effect. Thus, it seems consistent with the available evidence to suggest that the crown clustering phenomenon is a consequence of the effects of the rapid regulatory response examined above.

Earlier studies of nodulation profiles for cowpea and alfalfa indicated the possible existence of a rapid regulatory response in these legumes as well as soybean (2). The studies described in this paper strengthen the likelihood that the nodulation profiles for these other legumes demostrate a real and agriculturally relevant regulatory effect. Nutman (8) has made detailed microscopic studies of the rates and locations of infection and nodule development in clover. These studies revealed no evidence for the operation of a rapid regulatory effect in any of the clover species examined, at least no effect that could be considered comparable to the regulatory response described above for soybean. However, the pattern of infection and nodulation in clover is considerably more complex than in soybean (2, 8), and it is possible that the operation of a rapid regulatory response is simply obscured in this host.

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