APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1987, p. 1611–1619
0099-2240/87/071611-09$02.00/0

Vol. 53, No. 7

Split-Root Assays Using *Trifolium subterraneum* Show that *Rhizobium* Infection Induces a Systemic Response That Can Inhibit Nodulation of Another Invasive *Rhizobium* Strain

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Received 18 November 1986/Accepted 15 April 1987

Subterranean clover plants possessing two equally infectible and robust lateral root systems ("split roots") were used in conjunction with several specific mutant strains (derived from *Rhizobium trifolii* ANU843) to investigate a systemic plant response induced by infective *Rhizobium* strains. This plant response controls and inhibits subsequent nodulation on the plant. When strain ANU843 was inoculated onto both root systems simultaneously or 24, 48, 72, or 96 h apart, an inhibitory response occurred which retarded nodulation on the root exposed to the delayed inoculum but only when the delay period between inocula was greater than 24 h. Equal numbers of nodules were generated on both roots when ANU843 was inoculated simultaneously or 24 h apart. The ability to infect subterranean clover plants was required to initiate the plant inhibitory response since preexposure of one root system to non-nodulating strains did not retard the ability of the wild-type strain to nodulate the opposing root system (even when the delay period was 96 h). Moreover, the use of specific Tn*5*-induced mutants subtly impaired in their ability to nodulate demonstrated that the plant could effectively and rapidly discriminate between infections initiated by either the parent or the mutant strains. When inoculated alone onto clover plants, these mutant strains were able to infect the most susceptible plant cells at the time of inoculation and induce nitrogen-fixing nodules. However, the separate but simultaneous inoculation on opposing root systems of the parent and the mutant strains resulted in the almost complete inhibition of the nodulation ability of the mutant strains. We concluded that the mutants were affected in their competitive ability, and this finding was reflected by poor nodule occupancy when the mutants were coinoculated with the parent strain onto a single root system. Thus the split-root system may form the basis of a simple screening method for the ranking of competitiveness of various rhizobia on small seeded legumes.

Rhizobium species are soil bacteria which infect the roots of specific legume plants and induce the formation of nitrogen-fixing nodules. The infection process usually occurs via the penetration of plant root hair cells (17). Several workers have shown that crucial interactions occur during the early stages of infection which determine both the susceptibility to infection of the plant cells and which of the different invasive *Rhizobium* strains in the soil will predominate by outcompeting the other strains and occupying the nodules (4, 12, 13, 18, 26). Studies of the infection of soybean plants have shown that the cells destined to become root hair cells show a high but transient susceptibility to infection by a nodulating strain of *Bradyrhizobium japonicum* (2). In addition, exposure of nodulating *B. japonicum* cells to the newly emerging root hair zone on the roots of soybean plants results in the rapid induction of a regulatory response in the plant (18). This response diminishes the susceptibility to infection of other plant cells which are destined to become root hair cells (i.e., root hair cells which are developmentally younger at the time of inoculation) (18).

After less than 24 h of exposure to soybean root hairs, *B. japonicum* cells can initiate root hair curling and infection of susceptible root hair cells (2). A rapid (20-h) infection of the root hair cells also occurs when the fast-growing *Rhizobium* sp. strain NGR234 is exposed to *Macroptilium atropurpureum* (siratro) plants (19) and when *Rhizobium* sp. strain ANU843 is inoculated onto subterranean clovers (S. Z. Huang and M. A. Djordjevic, manuscript in preparation). Signals which stimulate cortical cell division are rapidly released as the first divisions can be seen after only 12 h of exposure to the plant (4).

Strains within a given species display various capacities for occupying nodules when in the presence of other competitive strains. The failure to fully understand the factors which affect strain competition diminishes the possible commercial application of *Rhizobium* inoculum strains to the soil. For example, soybean nodules in North America are predominantly occupied by *B. japonicum* strains which have a serotype similar to that of strain USDA123 and not by the more desirable nitrogen-fixing strains which have a serotype similar to that of strain USDA110 (13). Kosslak et al. (14) reported that preexposure of less-competitive strains of *B. japonicum* to soybean roots for periods as short as 6 h before the inoculation of a second, more-competitive strain can markedly increase the nodule occupancy rates of the less-competitive strain.

The ability to initiate nodules may not be required by a *Rhizobium* strain to produce a discriminatory response from the plant. Nodulation of a primitive cultivar of peas (Afghanistan) by a *Rhizobium leguminosarum* strain able to induce nitrogen-fixing nodules (strain TOM) can be inhibited by the simultaneous exposure of these plants to *R. leguminosarum* PF2, which is unable to induce nodules on these plants (3). A 24-h delay in the exposure of strain PF2 to the roots of the Afghanistan pea plants is reported to effectively diminish the inhibitory response of this strain to nodulation by strain TOM (26).

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Previous studies have been done using split-root plant systems. Split-root white clovers have been used to study the inhibition of nodulation and nitrogen fixation by nitrate (6), and a split-root system was used to evaluate the effect of salinity on soybean-*B. japonicum* symbiosis (23). Split-root assays are advantageous because the two root systems of the one plant are partitioned in space and time, allowing the effect of the inoculation of one root system to be compared with the effect of a separate inoculum on the second root system. Kosslak and Bohlool (12), using split-root soybean plants, showed that nodulation on one root by a mixture of *B. japonicum* USDA138 and USDA110 could be inhibited by prior inoculation on the other root with strain USDA138 despite the demonstrated superior competitive capacity of strain USDA110. However, the comparative effect of the inoculation of USDA138 on one root and USDA110 on the other was not addressed by these researchers (12). Various studies (15–17) have shown that the appearance of the first group of nodules inhibited further infection and nodule development. These results indicate that the legume plant controls the number of nodules which occur on the roots. Bhuvaneswari et al. (2) and Pierce and Bauer (18) have suggested that the decreased frequency of nodulation of rhizobia in younger regions of soybean roots may result from a rapid regulatory mechanism in the host which prevents excessive nodulation.

A criticism of previous studies is that analysis was not conducted with mutant strains and the effect of variability between plants was not assessed. To minimize the number of variables and to gain a better understanding of the factors which influence *Rhizobium* strain competition, we studied the infectibility of subterranean clover plants with two equally robust lateral root systems (i.e., the split root method) using isogenic strains.

In the present study we utilized specific, Tn*5*-induced mutant strains of *Rhizobium trifolii* ANU843 which are subtly impaired in their nodulating ability on subterranean clovers but which retain the ability to induce nitrogen-fixing nodules. The mutations in these strains lie in the *nodFE* or region IV nodulation genes (7). These mutants, like the parent strain, can infect the emerging root hair cells of subterranean clover plants that occur behind the root tip at the time of inoculation. Using subterranean clover plants possessing split roots, we show that the nodulating ability of each of the mutant strains was greatly inhibited when the parent strain was simultaneously inoculated onto an opposing root system. We propose that the genes affected in these strains are involved in the determination of both strain competitiveness and host-specific nodulation ability.

MATERIALS AND METHODS

Strains. Wild-type *R. trifolii* ANU843 (9) and its Sym plasmid-cured Nod$^-$ mutant ANU845 were used (22). Tn*5*-induced mutants of wild-type strain ANU843 were used which were located in *nodD* (ANU851) and the *nodFE* operon (*nodC7*, *nod-258* and *nod-297*) or in a separate locus designated region IV (*nod-251*) (11).

Media. Clover seeds were germinated on modified Bergersen medium (MBM) so that possible seed contamination could be easily identified (21). Seedlings were grown on modified Fahraeus medium (F) (21). Bacterial cultures were grown on tryptone-yeast medium (TY) (21). When appropriate, kanamycin (200 μg/ml) was added to TY medium. Jensen medium (25) was used as the plant medium for subterranean clovers when grown in plastic growth pouches.

Plant manipulations. Seed sterilization was achieved as previously described (21). Axenic subterranean clover seedlings were prepared and transferred to plastic growth pouches. Three plants per pouch, which were sustained initially with 7.5 ml of half-strength sterile Jensen medium (25), were used as described by Bhuvaneswari et al. (2). Seedlings were introduced to the pouches and incubated upright, and aluminum foil was used to ensure that light did not penetrate to the roots. Seedings were checked daily to determine whether the Jensen medium needed to be replenished. Plants were maintained in the growth chambers (light intensity, 550 micro-einsteins/m^2 per s; 24°C). Seedlings were inoculated 4 days after their transfer to the pouches (when the roots were 4 to 5 cm long), and the position of the root tip was marked. Plants were inoculated by flooding the entire root surface with 200 μl of the inoculum suspension (10^9 late-logarithmic-phase bacteria per ml). Plants were maintained for up to 15 days before the nodules were scored. Plants were cut at the root tip (used as a reference point), and the numbers of nodules on the primary root were scored with the aid of a dissecting microscope. Nodulation profiles were constructed by calculating the number of nodules above and below the root tip mark, and at least 60 plants were scored to generate the results presented.

Log-phase cultures of *R. trifolii* strains were used to inoculate sterile water (10 ml) to a concentration of 5×10^8 cells per ml. For each culture, 10 F plates were inoculated with 200 μl of inoculum and then dried in a sterile lamina flow hood. Three subterranean clover seedlings were laid on each plate and incubated upright at a light intensity of 490 to 515 micro-einsteins/m^2 per s and a temperature of 24°C. The onset of nodulation and nodule numbers were recorded for each plant at regular intervals throughout the first 25 days of infection.

Preparation of strains for nodule occupancy studies. Tubes containing sterile 5-ml portions of MBM broth were separately inoculated with either *R. trifolii* wild-type ANU843 or Tn*5* mutant strains. The tubes were then incubated on a shaker overnight at 30°C to comparable optical densities. Aliquots of 25 μl of the ANU843 culture were mixed with 25 μl of each mutant culture. Each mixed-cell suspension was then spread over an F plate on which four subterranean clover seedlings were placed. Plates were incubated for 21 days at 24°C at a light intensity of approximately 500 micro-einsteins/m^2 per s. Nodules were then removed, sterilized in 10% sodium hypochlorite for 10 min, washed in sterile water, and rolled over MBM plates to test the surface sterility. Nodules were crushed aseptically in 100 μl of protoplast dilution buffer (8). Aliquots of 10 μl from each suspension plus 100 μl of sterile protoplast dilution buffer was spread over a MBM plate and incubated at 28°C until single colonies arose. They were then replica plated onto TY and TY supplemented with kanamycin, respectively, to determine the proportions of kanamycin-resistant mutant cells in the nodules. All plates were incubated for 48 th 72 h at 28°C.

Growth curves. Volumes of 3 ml of TY broth cultures, shaken overnight at 30°C, were used to inoculate 25 ml of sterile TY broth preparations in side-arm flasks. Initial optical densities were 40 to 60 Klett units. Cultures were incubated at 30°C, with shaking for 36 h, and density measurements were made at frequent intervals with a Klett-Summerson photoelectric colorimeter (with a green filter).

Split-root clover assays. The root tips of ca. 12-mm-long subterranean clover seedlings (1 to 2 days old) were squeezed with forceps. After another 5 days of incubation at

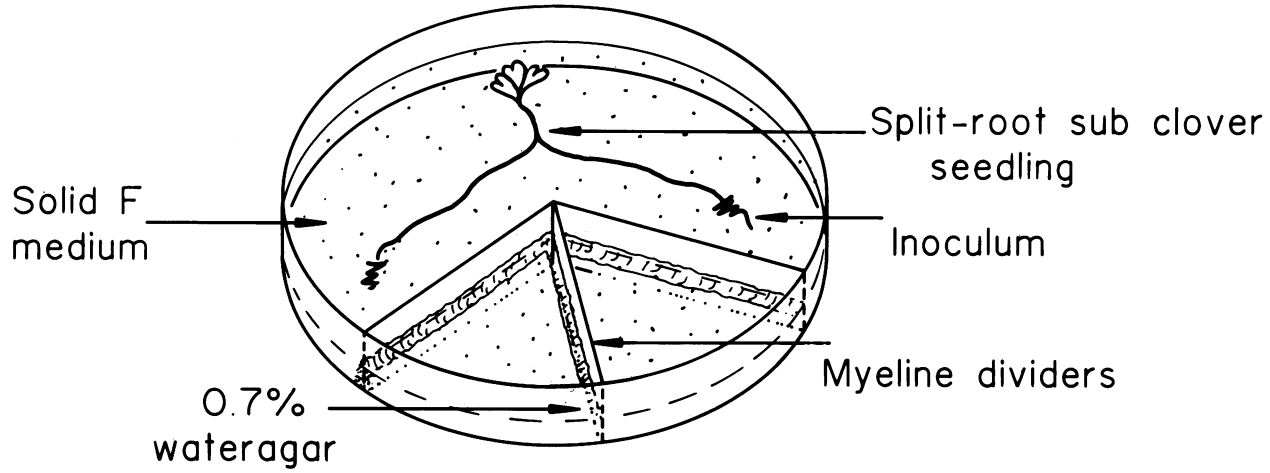


FIG. 1. A diagrammatic representation of the split-root subterranean clover plate assay described and used in this paper.

24°C in the plant growth chamber at a light intensity of 500 micro-einsteins/m^2 per s, the damaged plants formed lateral roots. Seedlings with two or more lateral roots of comparable length and robustness were used for split-root clover assays. These were mounted on F plates, and the roots were separated with myeline strips (60 by 11 mm) and pressed into the agar (Fig. 1). Dividers were held firm with 0.6% water-agar.

Inoculations were made just below the root tip of plants, using fresh *Rhizobium* cultures (i.e., in the late-logarithmic-growth phase) grown on TY plates. Seedlings were incubated upright at 24°C with a light intensity of 500 to 550 micro-einsteins/m^2 per s. Any condensation which accumulated in the plates was drained away to minimize cross-contamination between roots of the same plant.

To show that diffusion through or around the myeline barriers did not occur, bromcresol green (M_r, 698.05) and bromcresol purple (M_r, 540.24) were added to the media on one side of the myeline barriers. Neither of these dyes showed any visible diffusion under, around, or over the dividers.

Root staining and examinations. Seedlings were rinsed in water with 0.01% methylene blue for 10 min, rinsed again, and then destained in water for an hour by the method of Vasse and Truchet (24). The roots were examined by light microscopy with high (×40)- and low (×10)-power objectives. Root hair curling responses were recorded as in the system of Yao and Vincent (27, 28).

RESULTS

Quantitative comparison of the nodulation ability of strain ANU843 compared to that of nodulation-defective mutants. The quantitative comparison of the nodulation efficiency and the capacity of strain ANU843 and several nodulation-defective mutants of ANU843 (derived by Tn*5* mutagenesis) was achieved by using standard inocula (200 μl of a suspension of 10^9 bacteria per ml) to infect subterranean clover plants grown under standard conditions in plastic growth pouches. Over 50% of the total number of nodules induced on the subterranean clover plants by strain ANU843 occurred in an area 20 mm above and below the root rip mark with a distinct peak of nodulation developing just 2 mm behind the root tip mark at the same time of inoculation (Fig. 2A).

Mutants located in the *nodFE* operon (*nod-297*, *nod-258*, and *nodC7*) consistently gave nodulation responses on subterranean clovers grown in plastic growth pouches which were similar to those induced by the parent strain ANU843 (Table 1), with the peak nodulation response occurring close to the root tip mark. The total number of nodules, however, was diminished by 35 to 45% (Fig 2B). The nodule response of the region IV mutant (*nod-251*) was essentially identical to that shown by strain ANU843. The total number of nodules was in fact slightly higher than that induced by the parent strain under the same conditions (Fig. 2C).

Subterranean clovers grown on F plates and inoculated with *R. trifolii* ANU843 (wild type), the *nod-258* and *nodC7* (*nodFE*) mutants, or the *nod-251* region IV mutant were observed regularly over the first 20 days of infection. It was observed that the *nodC7* mutant was slower to nodulate and nodulated fewer plants than did the wild type (Fig. 2D). The *nod-258* and *nod-251* mutants did not show a detectable delayed nodulation response. Nodules appeared at the same time as strain ANU843 nodules (3 days after inoculation) and on equivalent numbers of inoculated plants. When the average number of nodules per plant was recorded (over a 25-day period), it was observed that the *nodFE* mutants and the region IV mutant produced almost the same number of nodules as did the wild type (per plant) over the first 10 days of incubation but that by 25 days the number of nodules induced by ANU843 had increased to 11 per plant, whereas the number of nodules induced by the mutant strains remained much the same as at the 10-day level (Fig. 2D and E).

Determination of the nodule occupancy rates with mixed *Rhizobium* inocula. Before mixed-inoculation experiments were conducted, the growth rates of all strains used in these studies were compared to that of the parent strain (ANU843). When several types of laboratory media were used (including MBM and TY), no discernable differences could be detected in the growth rate of the parent strain, *R. trifolii* ANU843, or any of the Tn*5*-induced nodulation-defective mutants used.

As a conrol experiment, strain ANU843 was coinoculated onto subterranean clover roots with equal numbers of Tn*5*-induced kanamycin-resistant Nod$^-$ mutant ANU851 (Tn*5* located in the *nodD* gene) to estimate the extent of coinfection. Various numbers of kanamycin-resistant cells were found to co-occupy about 11% of the nodules induced (Table 2). This proportion increased to approximately 25% in some

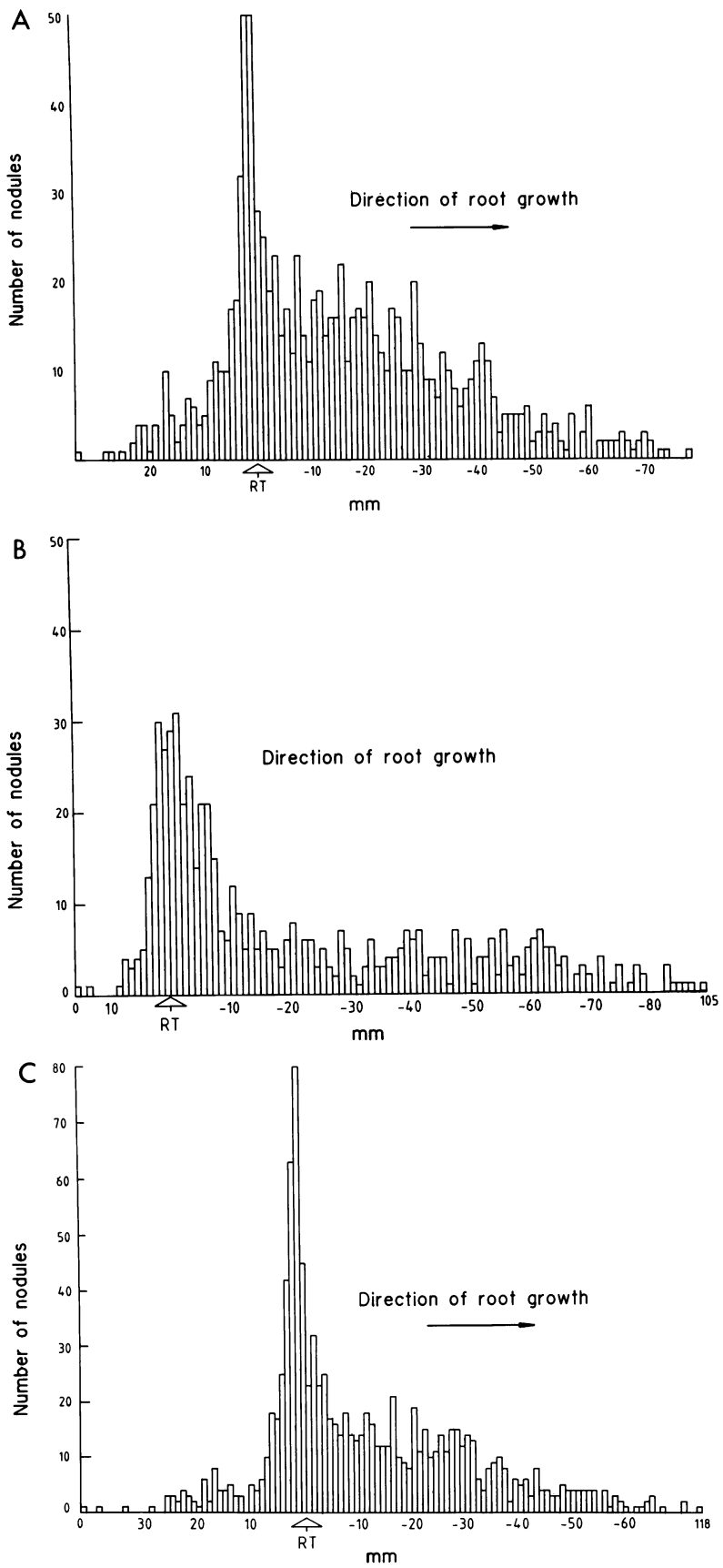
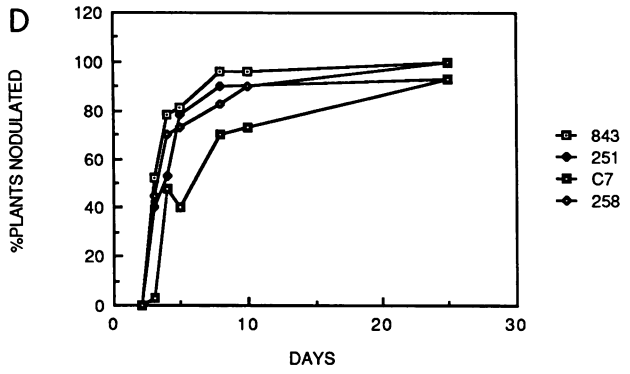
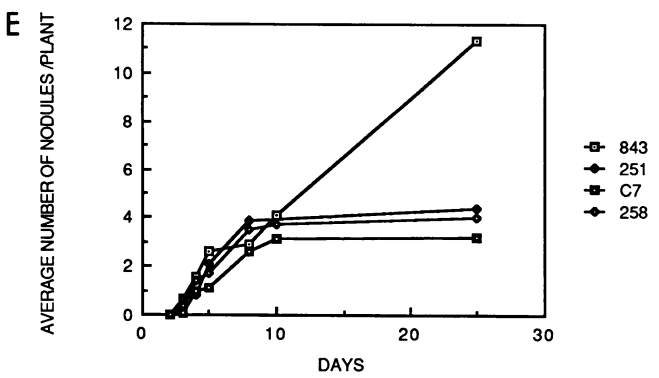


FIG. 2. Nodulation profiles of subterranean clovers 9 to 10 days after inoculation with ANU843 (average number of nodules per plant was 16.2) (A), *nodC7* mutant (average number of nodules per plant was 9.4) (B), and *nod-251* mutant (average number of nodules per plant was 16.7) (C). RT, Root tip mark at the time of inoculation. (D) Plot of number of subterranean clovers nodulated versus days of incubation. (E) Plot of average number of nodules per plant versus days of incubation (average number of nodules per plant = total number of nodules/total number of plants).





experiments (data not presented). In nodules containing both inoculant strains, the mutant ANU851 constituted more than 50% of the total nodule *Rhizobium* population. That none of the kanamycin-resistant (mutant) cells were able to nodulate clovers after subsequent reinoculation of these cells into clover plants indicated that genetic transfer between strain ANU843 and the Nod$^-$ mutant strain had not occurred. Thus the co-occupation of the nodules by strain ANU851 was most likely due to a passive helping (intracellular complementation) or carrying of this strain into the nodules by the parent strain, presumably via the same infection thread. No evidence of genetic transfer was detected in all subsequent mixed-inoculation experiments.

When wild-type strain ANU843 and either of the two *nodFE* mutants (*nod-258* or *nodC7* mutants) were coinoculated onto subterranean clover plants, the mutants were present in about 41 to 44% of the nodules (Table 2). In nodules containing the *nod-258* or *nodC7* mutants the mutant organisms constituted the majority (50 to 100%) of the bacteria present. The region IV *nod-251* mutant was present in only 25% of the nodules resulting from coinoculation of subterranean clovers with wild-type strain ANU843 and the *nod-251* mutant. In nodules containing the *nod-251* mutant, the mutant strain constituted 100% of the bacteria present since no kanamycin-sensitive bacteria were found.

Assessment of root infectibility using split root assays. Several control assays were done to compare the infectibility of *Rhizobium* strains on split roots and normal root systems and to ascertain whether any cross-contamination could be detected by split-root assays. First, a fresh culture of the wild-type strain ANU843 (containing 10^8 cells per ml) were serially diluted to 10^0, 10^{-2}, and 10^{-4}; one of the split roots of a subterranean clover seedling was inoculated with a given dilution, while the other was left uninoculated. Six replicas per dilution were then prepared. After 21 days each root was scored for nodulation, and representative roots were stained and examined for root hair curling and the production of infection threads. For all dilutions, only inoculated roots bore nodules and the nodule number decreased with increasing dilution. Inoculated roots all showed distinct hair curling and infection thread formation, whereas roots not exposed to rhizobia did not show any of these features. These results indicate both that the number of nodules per root was comparable to the number induced by a wild-type strain on a normal root system and that no evidence of cross-contamination could be detected.

Nodulation ability on one root can be affected by the presence of a nodulating strain on the second root system. The nodulating ability of strain ANU843 was unaffected when the second root system was inoculated simultaneously with the Sym-plasmid-cured Nod$^-$ strain ANU845. In addition, when the Nod$^-$ strain ANU845 was inoculated onto one root system and the inoculation of strain ANU843 was delayed by up to 4 days on the second root system, the total number of nodules induced by strain ANU843 was comparable to that produced when there was no delay in the inoculation time. As expected, the position of the nodules was farther down the root, and only a few nodules were initiated in the mature root hair zone.

When the two separate roots of the one plant were inoculated with strain ANU843 at the same time (or up to 24 h apart), both roots produced nodules at equivalent rates and

TABLE 1. Nodulation of subterranean clover plants relative to the root tip mark on primary roots inoculated with various *R. trifolii* strains

Inoculum	% Total nodules around the root tip mark	
	10 mm above and below mark	20 mm above and below mark
Wild-type ANU843	41	63
Region III mutants		
nodC7	50	61
nod-258	61	70
nod-297	43	59
Region IV mutant (*nod-251*)	51	68

TABLE 2. Nodule yields from subterranean clovers 21 days after inoculation with *R. trifolii* strains

Inoculum	No. of plants	No. of nodules analyzed	No. of nodules with some kanamycin reistance	% Nodules with mutant strain
ANU843 + *nod-851* mutant	8	28	3	10.7
ANU843 + *nodC7* mutant	4	22	9	40.9
ANU843 + *nod-258* mutant	7	46	20	43.5
ANU843 + *nod-251* mutant	8	56	14	25.0

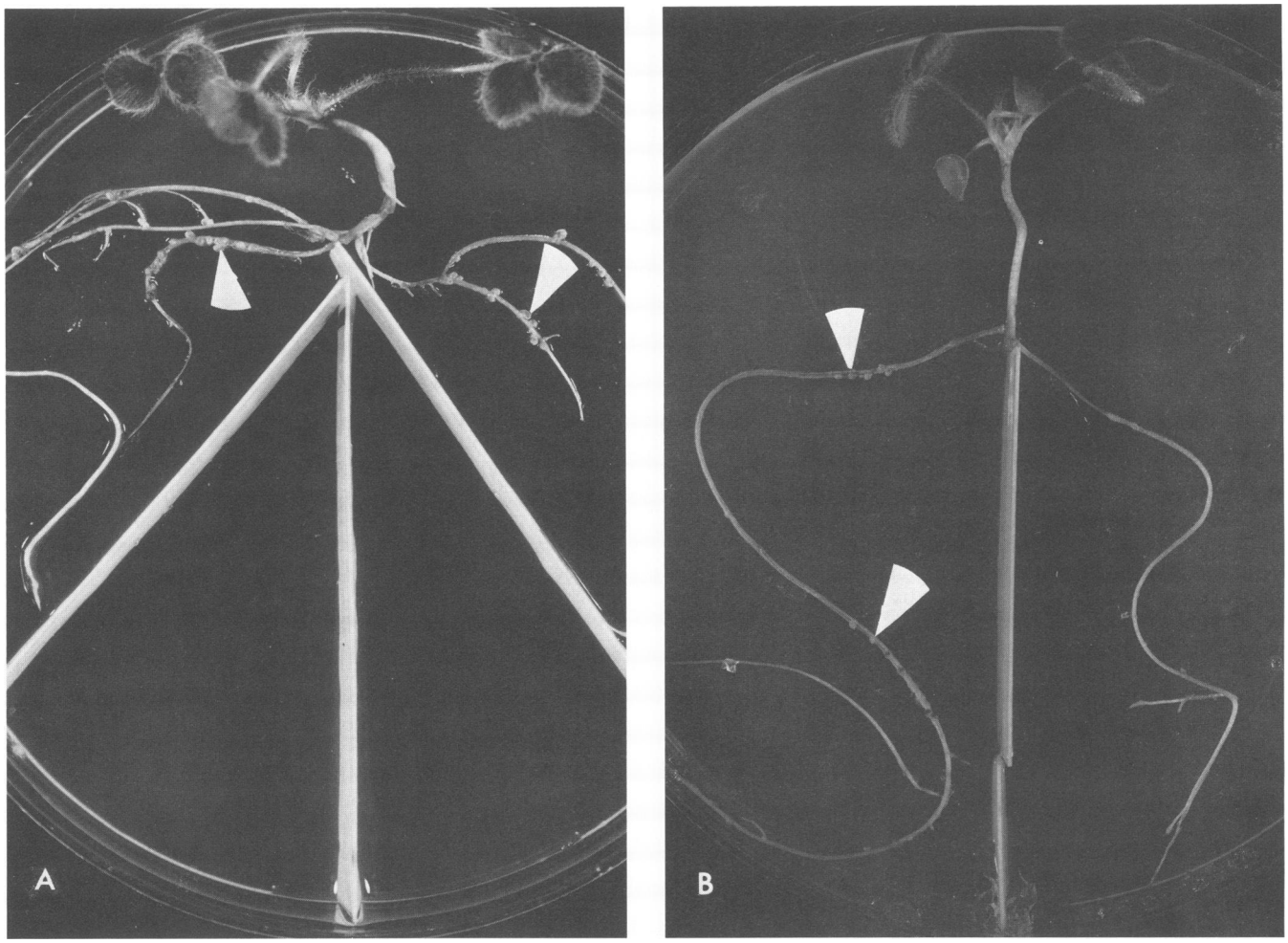


FIG. 3. Photographs of split-root subterranean clovers after 3 weeks of incubation following simultaneous inoculation of alternate root systems with ANU843 and ANU843 (A) and ANU843 and a *nodFE* or region IV mutant (B).

numbers. However, if one root was delayed in its inoculation with strain ANU843 by 2 to 4 days, the root inoculated first gave a normal response but the second root gave a depressed response. The depressed response showed by the delayed inoculum was more pronounced as the delay period was increased. Up to 15 to 20% of the roots on seedlings exposed to the delayed inoculum displayed a complete lack of nodulation when the delay was 2 days. This increased to 40% of the split roots when the delay was 4 days. The nodulation response on the root inoculated with strain ANU843 first was constant, regardless of the presence, absence, or delay period of any subsequent inoculation on the second root system of the one plant.

Effect of inoculating specific nodulation mutants on the second root system. Alternate roots of subterranean clover seedlings possessing split-root systems were inoculated with strain ANU843 and with a specific nodulation-defective mutant derived from strain ANU843 by Tn*5* mutagenesis. A *nodD* mutant strain (ANU851) was inoculated onto the second root system at the same time as an ANU843 inoculum was introduced onto the first root system. The Nod^- mutant did not affect the nodulation response of strain ANU843. The presence of the parent strain ANU843 on the opposing root system did not stimulate the Nod^- mutant to induce any detectable nodulation response.

As shown above, the nodulation profiles of the *nodFE* mutants of strain ANU843 and in particular, the region IV mutant (*nod-251*) was not substantially different from that induced by the parent strain ANU843 when inoculated alone onto the subterranean clover plants (Fig. 2). Thus it was surprising that the inoculation of the *nodFE* or a region IV mutant on one root at the same time as strain ANU843 was inoculated on the opposing root system resulted in the almost complete retardation of nodulation on the roots inoculated with any of the mutant strains (Fig. 3 and Table 3).

Examination of the root system inoculated with the mutants showed that the root hair curling (Hac^+) and infection thread formation (Inf^+) still occurred but that the ability to initiate nodules (Noi^-) was affected by the inoculation of ANU843 on the other root system. The nodulation and infection ability of the parent strain was not detectably affected by the presence of the mutant strain on the opposing root system.

Since it had been shown previously that the phenotype of *nodFE* mutants was different from that of the region IV mutant (*nod-251*), the *nodC7* mutant was inoculated onto one root system of a split-root subterranean clover, and the region IV *nod-251* mutant was inoculated on the other. Under the light and temperature conditions used here, the

nodC7 mutant inhibited nodulation of the region IV mutant. The nodulation and infection ability of the *nodC7* mutant appeared to be uninhibited by the presence of the *nod-251* mutant on the other root system (Table 3).

DISCUSSION

Infection of clover plants is characterized by the production of a large number of infection threads (7, 8, 17, 20). Strain ANU843, for instance, can induce between 50 and 200 infection threads per white clover plant but induces only 15 to 20 nodules (7, 8). This indicates that although many infection events occur, only a few (~10%) are permitted to induce nodule formation. The results presented here show that, as in other legumes (1, 19), the root hair cells which occur immediately behind the root tip on subterranean clovers at the time of inoculation are the most susceptible to infection by *R. trifolii*. Furthermore, as the inoculated plant grows and produces new emerging root hair zones, the infectibility of the newly emerging zones diminishes. The typical nodulation profiles shown for other legume species also occur for subterranean clover plants.

When strain ANU843 is inoculated at the same time or 24 h apart onto both roots of a split-root clover plant, no apparent retardation of nodulation occurs on either root system. However, substantial retardation of nodulation ability occurs if the delay period is increased from 24 h to 48 or 96 h after inoculation of the first root system. As the delay period increases so does the degree of retardation. This suggests that about 24 h after inoculation of subterranean clover plants with strain ANU843 the plant responds by inhibiting nodule initiations on the second root system as well as on the original one. This newly induced plant factor(s) becomes quickly transmitted through the plant as its release results in the decrease in the infectibility which occurs on both root systems. In addition, the ability to infect the plant root is required to induce this systemic response since the preexposure of one root system to an inoculum of the Sym-plasmid-cured (Nod^-) strain ANU845 or the non-nodulating (*nodD*) mutant strain ANU851 did not affect the nodulation response of ANU843 on the second root system

TABLE 3. *Rhizobium* strain interactions in subterranean clover split-root assays

Inoculant (side one)[a]	Phenotype with inoculant (side two)			
	ANU843	*nodC7* mutant	*nod-258* mutant	*nod-251* mutant
ANU843				
Nod^+	Nod^+			
Nod^+		Nod^-		
Nod^+			Nod^-	
Nod^+				Nod^-
nodC7 mutant				
Nod^-	Nod^+			
Nod^+		Nod^+		
Nod^+			Nod^+	
Nod^+				Nod^R
nod-251 mutant				
Nod^-	Nod^+			
Nod^R		Nod^+		
NT			NT	
Nod^+				Nod^+

[a] Phenotypes: Nod^-, non-nodulation; Nod^R, nodulation markedly reduced; Nod^+, nodulation positive. NT, not tested.

(even when the delay in the inoculation of ANU843 was 4 days after the inoculation of the Nod^- mutants). It is not known at what stage of the infection process the systemic response is initiated, but it is clear that the ability to infect root hairs is required. We cannot, however, exclude the possibility that another early nodulation event, nodule induction, may also be required to elicit this inhibitory systemic response, as proposed from microscopy studies by Calvert et al. (4).

Strains with mutations located in the *nodFE* operon (previously known as region III) have been shown to possess a differential capacity to efficiently nodulate several legume species (7, 8). These mutants (i) nodulate white clover species poorly with an appreciable delay compared to the parent strain (ii) possess a nodulation response on subterranean clovers which is more akin to the parent strain (which has been quantified here), and (iii) unlike the parent strain ANU843, can induce nodules on both temperate and Afghanistan peas (8). The results presented here verify the relatively good infective capacity of *nodFE* mutants on subterranean clover plants. When the *nodFE* mutant strains are mixed with the parent strain and inoculated onto subterranean clover plants, the number of nodules co-occupied by both strains or exclusively by the mutant strain varies considerably. The proportion of nodules containing mutant cells ranged from 25% for the *nod-251* mutant to ~44% for the *nod-258* mutant. These values could in some cases be artificially high because the parent strain can passively help a Nod^- strain (ANU851) to enter nodules so that the mutant is present in ~11% of the nodules tested. However, the nodules containing kanamycin-resistant mutant cells were often predominantly populated by these cells. Nodulation profiles of these mutants show a distinct peak around the root tip mark at the time of inoculation, indicating that infection events are initiated in this region shortly after exposure of the plants to these mutants; otherwise the peak of nodulation would shift to developmentally younger areas. Except with the *nodC7* mutant, no appreciable delay in the nodulation response could be detected with the mutant strains. This indicates that early infection initiation events occur at comparable rates for both the mutant and wild-type strains.

When the subterranean clover split-root assay is used, inoculation of strain ANU843 on one root system and the *nod-258*, *nodC7*, or *nod-251* mutant on the second results in a severe inhibition of the nodule-forming ability of the mutant strains. Since root hair curling and infection thread formation can still be observed on the root inoculated with either of the mutant strains, it is likely that the ability to initiate nodule formation (Noi^+) is retarded. When nodulation was monitored over time, it was observed that the *nodFE nodC7* mutant was about 24 h slower to nodulate than the wild type and other mutant strains (Fig. 2D). Although the measurement of the kinetics of nodulation failed to detect minor disparities between the nodulation rates of the *nod-258* and *nod-251* mutants and the wild type, experiments using the more-sensitive split-root clover plate assay suggest that *nod-258* and *nod-251* mutants may be subtly affected in some early nodulation step(s). The differences between the phenotypes of *nod-258* and *nodC7* mutants may be explained if the Tn*5* insertion of *nodC7* lies in the *nodF* gene and the *nod-258* insertion lies in the *nodE* gene.

A model can be proposed in which the *nodFE* and region IV (*nod-251*) mutants studied here are subtly affected in an early nodulation step which results in these strains being marginally slower to nodulate than the wild-type strain

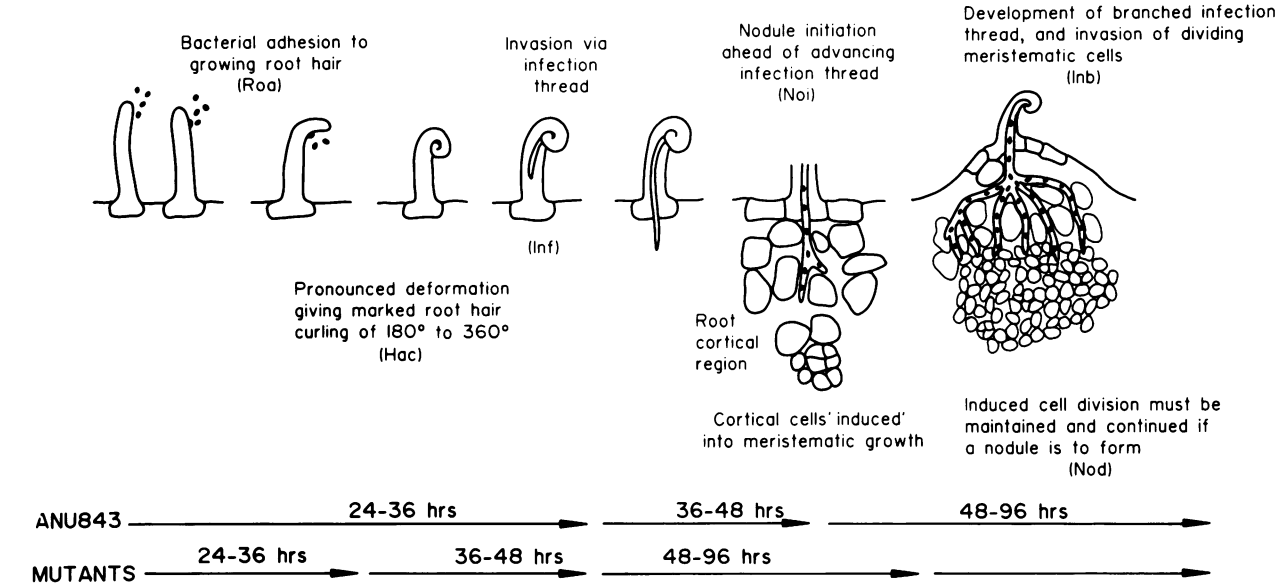


FIG. 4. A diagrammatic representation of initial infection stages of a clover root hair by rhizobia. Relative times taken by ANU843 and region III and IV mutants are indicated beneath, showing mutants to be 24 to 48 h slower than ANU843 in the early stages of infection.

ANU843 (Fig. 4). This could be due to a mild but adverse response by the plant to infection by these mutants. Strains which are only a few hours slower may continually fail to nodulate because the infection threads they initiate are repressed by the inhibitory systemic response induced in the plant by the faster strain. Thus, whereas the parent strain may infect and initiate a nodule meristem in, say, 15 h the mutant strains for reasons that we do not understand may take a longer period of time. This means that the nodule-forming ability of the mutant strains may always be repressed by the systemic response induced first by the parent strain on the opposing root system, a regulatory mechanism which controls the number of nodules produced on the whole palnt. It is therefore possible that highly competitive strains are ones which are simply faster to compete for the highly infectible root hair cells and so rapidly infect them. Furthermore, it is likely that the *nodFE* and region IV genes play an important role in the ability of the bacteria to nodulate efficiently and competitively.

Region IV mutants nodulate white and subterranean clovers, as well as the wild-type strain, under ideal conditions. When the plant is stressed by high light or temperature conditions, the nodulation capacity of these mutants is particularly poor when compared to the parent strain (B. G. Rolfe, unpublished observations). In addition, region IV mutants show no extended host range capacity characteristic of the *nodFE* mutants. The nodulation profile of the region IV mutant presented here is indistinguishable from that of the wild-type strain on subterranean clover plants. It is, therefore, clear that the *nod-251* mutant can still initiate a plant regulatory response controlling nodulation. However, examination of nodule contents of subterranean clover plants coinoculated with the region IV mutant and the wild-type strain ANU843 shows a distinct bias towards the parent strain. Inoculation of the region IV *nod-251* mutant on one root and the wild-type strain on the other results in retardation of nodulation of the mutant strain, even though studies of nodulation rates detected no delay in nodulation compared to the wild type. The *nodC7* mutant can also inhibit nodulation of the *nod-251* mutant when these two mutant strains are inoculated simultaneously on alternate root systems of a split-root clover. The region IV mutant does not exert any apparent reciprocal inhibition of nodulation by the *nodC7* mutant.

Recently, the sequences of the *Rhizobium meliloti nodFEG* genes were determined. The *R. meliloti nodFEG* mutants were subtly affected in their ability to nodulate alfalfa plants. The predicted gene product of the *nodG* gene (which occurs downstream from *nodE*) was shown to possess homology to the ribitol dehydrogenase gene of *Klebsiella aerogenes*, and the *nodFE* genes are highly homologous to those sequenced in other *Rhizobium* species (6). Debelle and Sharma (6) postulate that the *nodG* gene product is responsible for the catabolism of a plant-derived polyol during infection. It is possible that the *nod-251* gene is similarly involved in establishing a nutritional relationship with the plant host and that the defect induced affects the ability of this mutant to grow in the plant milieu.

If strains which differ marginally in their efficiency of nodulation can be distinguished by using the split-root system, then this system may form the basis of a simple screening method which can effectively grade one strain compared to another and so be used to rank the competitive nature of various strains.

ACKNOWLEDGMENTS

This work was supported in part by the Australian Livestock and Meat Research Council. B.G.R. is a recipient of an Agrigenetics grant. M.A.D. is funded by the Australian Wool Board Trust, and L.S. is a recipient of a Commonwealth Postgraduate Research Award.

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