

## NODULATION STUDIES IN LEGUMES

### III. THE EFFECTS OF DELAYING INOCULATION ON THE SEEDLING SYMBIOSIS OF BARREL

MEDIC, *MEDICAGO TRIBULOIDES* DESR.

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[Manuscript received June 16, 1959]

#### Summary

The technique of delayed inoculation with effective, ineffective, or mixtures of effective + ineffective nodule bacteria was used to study seedling symbiosis of barrel medic, *M. tribuloides*, in sand culture.

Nodule production over the first 6 weeks of growth was stimulated in all bacterial series by inoculation delays of from 5 to 25 days. Maximum nodules were placed on primary roots by delays of 14–19 days, on lateral roots by delays of 25 days.

Following delayed inoculation, nodule initiation commenced on existing root-hair-invested portions of the root system. The highest nodule on the primary root usually developed 1–2 cm behind the root tip and more than two lateral primordia below the lowest lateral visible at inoculation.

Nodule frequencies, determined in each delay treatment for numbered laterals and for spaces between laterals on the primary root, were used to describe the "infection potential" of the root system. "Infection centres" were apparently distributed in a regular fashion down the primary root. With long inoculation delays, laterals developed clumps of closely packed nodules exhibiting nodule intensities (nodules/cm) 10–20 times those realized by inoculation at sowing. Results suggested that only a very small fraction of the infection potential was exploited under any inoculation regime.

Serological and visual typing of nodules in delay treatments using mixed inocula showed the ineffective partner competed much more successfully for positions on the primary root than might be expected from a sharing of the infection potential in proportion to the nodulating capacities of the respective strains in single culture.

There was abundant evidence that existing nodules restricted subsequent root growth and nodulation of host seedlings. These restrictive influences were cumulative and characteristic for the invading strain(s). Results were discussed with reference to possible exchange reactions between host plant and nodule tissues.

#### I. INTRODUCTION

The effects of delaying inoculation on the nodulation of agar cultures of red clover were described by Nutman (1949) in his series of investigations on the physiology of nodule development in legumes. Delays in inoculation with an effective *Rhizobium* were found to reduce the time lag between inoculation and the appearance of nodules on the root, and also led to much greater rates of nodule production than could be obtained by inoculation at sowing. Nodule production was not increased if severe nitrogen hunger conditions had developed in seedlings prior to inoculation. No permanent stimulus to infection rate was obtained by delaying inoculation with an ineffective *Rhizobium*.

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The results of the above experiments, together with others on the relationship between lateral root and nodule formation (Nutman 1948) and the effect of excising nodule apices on subsequent root and nodule development (Nutman 1952), were used to support a physiological hypothesis of host-plant interaction in nodule formation. This hypothesis, expanded by Nutman in later publications (Nutman 1956, 1958), proposed that identical meristematic foci were utilized in lateral root and nodule formation and that existing meristematic centres on the root produced inhibitory substances limiting further infection of the root system.

The study of barrel medic detailed in this paper is relevant to several of the issues mentioned above. However, the technique of delayed inoculation was used here primarily for other reasons, namely, to determine the precise site of infection on the root, to test whether competitive abilities of bacterial strains vary with time of inoculation of the seedling root system, and to describe the "infection potential" of different regions of the root. Sand culture was selected for these and other experiments so that the full implications of delayed inoculation could be examined over the complete symbiotic cycle.

## II. MATERIALS AND METHODS

### (a) *Plant Culture and Inoculation Procedure*

Sand culture of barrel medic (*Medicago tribuloides* Desr., strain 173) was carried out under natural illumination in a glass-house in Sydney in May–August, 1957. Enamelled iron pots of 7 in. diameter and 4 l. capacity were filled with acid-washed Nepean R. sand and steam-sterilized prior to sowing. Seeds, refrigerated for 12 hr at 0°C to promote even germination, were surface-sterilized with mercuric chloride and sown 20 seeds to a pot. Each pot was watered to field capacity with distilled water and 300 ml of a standard nitrogen-free culture solution made up as follows: 0.8 g  $\text{CaSO}_4$ , 0.4 g  $\text{K}_2\text{HPO}_4$ , 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , iron as chelate (5 mg Fe/l), 1 ml of trace-element solution per litre of distilled water. The trace-element solution consisted of 2.86 g  $\text{H}_3\text{BO}_3$ , 1.81 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.11 g  $\text{ZnCl}_2$ , 0.05 g  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.025 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  per litre distilled water. Sand cultures watered with culture solution showed a reaction of pH 6.7–7.0.

For the first 10 days after sowing, pots were covered with transparent polythene sheeting to reduce the chance of airborne contamination by foreign nodule bacteria and to prevent seedling injury through excessive drying out of pots.

Two serologically dissimilar strains of nodule bacteria were obtained from the University of Sydney School of Agriculture Collection and used as inoculants. One, *Rhizobium* SU41.237, was ineffective (I), the other, *Rhizobium* SU277.1, was fully effective (E) on the host variety. In experiment 1, a preliminary study of the biology of the two strains, 40 pots of medics were inoculated with I bacteria and another 40 pots with E bacteria to form two series from which periodic samples were taken to study seedling symbiosis following a normal inoculation at sowing.

In experiment 2 the effects of delaying inoculation were studied in three bacterial series:

- (1) I 1–10, where sets of five pots of seedlings were inoculated with ineffective bacteria at 10 selected times in seedling growth.

(2) E 1-10, a similar series using effective bacteria.

(3) E+I 1-10, as above but using a mixed inoculum of effective plus ineffective bacteria at each inoculation time.

The inoculation schedule for the 10 treatments of each series is given in Table 1. Bacteria were applied to all treatments at a rating of more than  $10^6$  bacteria per pot giving an immediate rhizosphere density assumed to be well in excess of that required for maximum nodulation (see Purchase and Nutman 1957).

TABLE 1  
SCHEDULE FOR THE DELAYED INOCULATION OF MEDICAGO TRIBULOIDES SEEDLINGS

Treatment* Number	Time of Inoculation		Seedling Condition at Inoculation†		
	Days from Sowing	Days from Germination	Primary Root Length (cm)	Lateral Roots per Plant‡	Top Development
1	3	0	0.2	0	Cotyledons expanded
2	5	2	1.2	0	
3	7	4	2.3	0	
4	9	6	2.9	0	
5	12	9	5.7	0.4	Spade leaf expanded
6	14	11	7.1	2.0	
7	16	13	9.0	7.8	
8	19	16	11.0	14.2	First trifoliate leaf expanded
9	22	19	12.6	17.3	
10	25	22	14.5	22.1	

\* Each treatment comprised an E, I, and E + I series inoculated respectively with effective, ineffective, and a mixture of effective + ineffective nodule bacteria (E = strain SU277.1, I = strain SU41.237). Five replicate pots, each of 10 plants, were established for each treatment of a series.

† Seedling development determined from a sample of 40 uninoculated plants.

‡ Only those lateral roots projecting 1 mm or more beyond the primary root surface were counted.

At each inoculation time 40 plants were extracted from pots of similarly aged uninoculated seedlings to determine host-plant condition at inoculation and to check that seedlings being inoculated had remained unnodulated (see Table 1). Eventual nodule distribution patterns, determined at harvest, also showed that airborne contamination had not produced nodules prior to artificial inoculation. There was no evidence in the experiment of contamination of the I series by E bacteria or vice versa although this did occur sporadically in long-term experiments using other legumes and strain combinations (see Pate 1958*b*). The success in maintaining controls sterile and preventing later contaminations was attributed to the precautions taken to exclude dust from the glass-house, to the separation of inoculated pots from uninoculated controls, and to the use of sterile equipment when applying bacteria or water to the seedlings.

Post-emergence thinning reduced seedlings to 10 per pot. Pots were watered daily to field capacity with distilled water, and 100 ml of nitrogen-free culture solution was added weekly to each pot.

(b) *Harvesting and Recordings from Samples*

(i) *Experiment 1*.—Samples of 40 plants were taken from both E and I series at 2- or 3-day intervals in seedling development. The following quantities were measured—nodule numbers on primary and lateral roots, average size (fresh weight), and colour (white, red, or green) of nodules on the primary root (see Pate (1958a) for further details of nodule colour classes, sampling procedure, etc.).

(ii) *Experiment 2*.—A single harvest was made 39 days from sowing. Samples of 30 plants were taken from the five replicate pots of each of the 30 treatments. All quantities listed for experiment 1 were recorded. Nodule distribution maps were constructed for each treatment where the frequency of occurrence of nodules was recorded for each numbered lateral root and for the numbered spaces between laterals on the primary root. The average distance below the hypocotyl junction to the first nodule on the primary root was determined for each treatment.

In the E + I series, primary root nodules were classed on a visual basis as either E or I using criteria obtained from experiment 1 (see Section III). Serological typing, using the method of Vincent (1941), was used to check this morphological separation of nodule types. Visual typing was not possible for the populations of young nodules on lateral roots of the E + I series.

### III. RESULTS

(a) *The Biology of the Effective and Ineffective Bacterial Associations with Barrel Medic (Experiment 1)*

The following paragraphs summarize the principal features of the two types of symbiosis over the first 30 days of seedling growth.

Infection of the seedling axis produced the first visible nodule in either series at about 10 days from sowing. The final intensity of nodulation on the primary root was similar for both strains.

Nodule pigmentation on the primary root occurred in both nodule types over the 15–20-day period of seedling growth. All E nodules remained red and in active growth throughout the period of the experiment. In the I series nodule haemoglobin was rapidly decomposed, giving nodules formed by this strain a maximum pigmented life of 5 days. Coincident with haemoglobin disappearance in the I nodule a pronounced collapse of the meristem was observed.

Rapid and extensive infection of lateral roots occurred in the I series resulting in the formation at 26 days of approximately three times as many lateral root nodules as on E-nodulated plants. There is an interesting coincidence between the appearance of size and colour differences in primary root nodules in the responses of the two strains and the establishment of contrasting infection patterns on lateral roots (see also experiment 2).

In summary, the strains exhibit typical effective and ineffective behaviour although the I strain is not totally ineffective since some fixation return might

be expected from the small ephemeral core of haemoglobin-pigmented tissue. The visual characteristics selected from this experiment as criteria for distinguishing E and I nodules in experiment 2 are as follows:

*I Nodules*.—Crater-shaped apex with aborted meristem, brown or green contents, hemispherical outline.

*E Nodules*.—Active meristem, red pigmentation, size at least twice that of contemporary ineffective nodules.

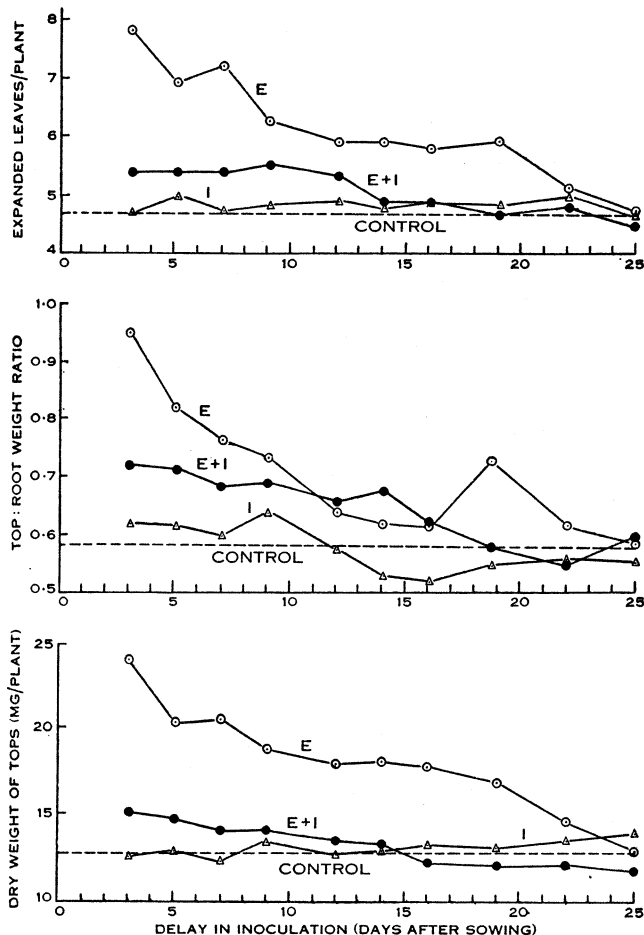


Fig. 1.—Experiment 2: Delayed inoculation of *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective+ineffective (E+I) bacteria. Effects of delayed inoculation on various features of host plant development at 39-day harvest. Each recording is a mean value derived from a sample of 30 plants.

(b) *The Effects of Delaying Inoculation on Seedling Symbiosis of Barrel Medic (Experiment 2)*

Effects of delay in inoculation on host development and nodulation are listed for the three bacterial series in the following subsections.

(i) *Dry Matter Production in Plant Tops, Leaf Development, and Top : Root Weight Ratio (Fig. 1).*—The benefits of early nodulation by E bacteria, and to a lesser extent by E + I bacteria, are seen in greater values in all of the above quantities compared with growth of uninoculated controls or I-nodulated plants. The longer E nodules were present on a root system, the greater the difference from control growth. Quantities recorded for the E + I series at different delay periods approximate more closely to those of the I series than the E series. This is consistent with the observation evaluated later that with all delay times involving mixed inocula a greater proportion of nodules were formed by the ineffective strain. The presence of I nodules on roots clearly detracts from the full benefit of E nodulation.

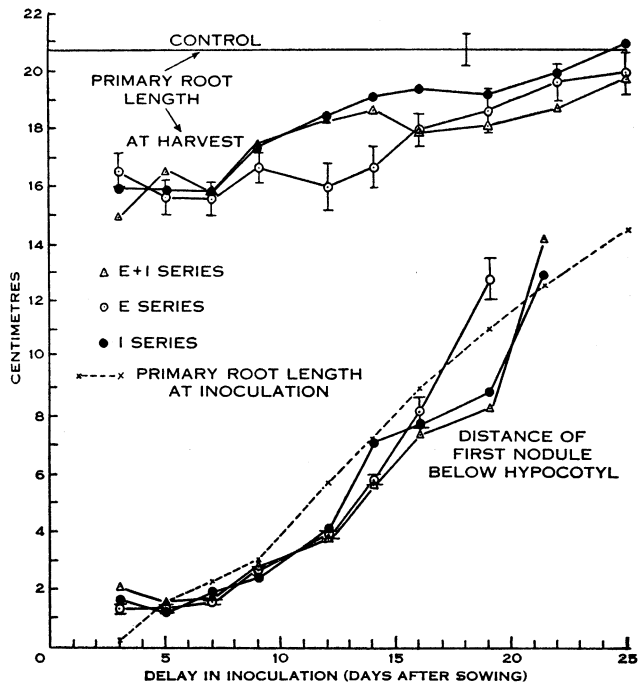


Fig. 2.—Experiment 2: Delayed inoculation of *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective+ineffective (E+I) bacteria. Primary root extension in the three bacterial series and in control uninoculated plants at 39-day harvest. In the lower half of the figure distances below the hypocotyl to the highest nodule are compared with primary root length at inoculation. Experimental variability is indicated by standard errors of means of treatments of the E series.

(ii) *Primary Root Extension and Lateral Root Production (Figs. 2 and 3).*—In all series there is a marked reduction in both primary root growth and lateral formation when performances of inoculated plants are compared with uninoculated root growth. The maximum reduction in these quantities is obtained by inoculation at germination (3 days after sowing) and shows a root approximately 5 cm shorter and bearing 15 fewer laterals than similarly-aged uninoculated plants. At greater delay intervals in the three series the degree of reduction is proportional to the length of time that nodule bacteria have had access to the primary root (see Figs. 2 and 3).

(iii) *Nodule Production* (Fig. 4).—The overall effect of delaying inoculation in all three bacterial series is to promote the production of substantially greater numbers of nodules at harvest than in the plants inoculated at germination. A two- or three-fold increase in the nodule numbers was obtained in the period of the experiment with the longest delay treatment used.

*Primary root infection.*—With increasing delay interval, primary root nodules increase slightly but significantly in all series, with maxima attained by withholding the inoculum for 14–19 days (primary root 7–11 cm at inoculation). Longer delay led to the production of fewer nodules as sites on the upper part of the root became closed to infection and as seedlings entered a severe nitrogen-hunger condition.

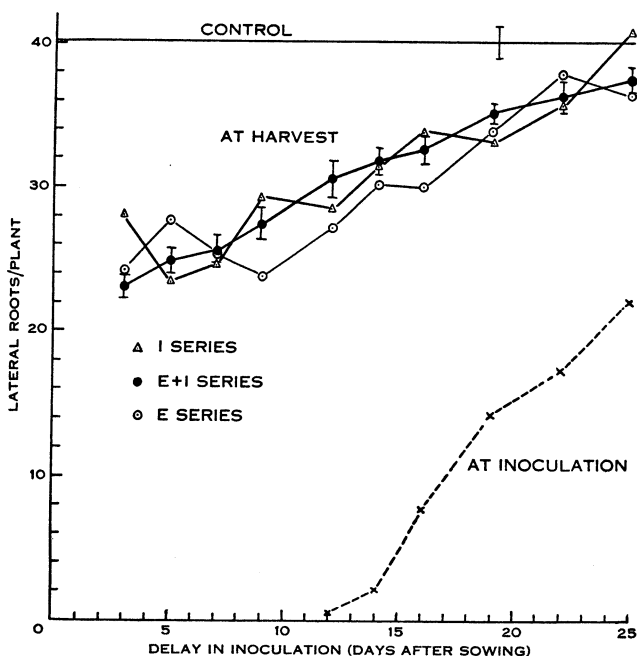


Fig. 3.—Experiment 2: Delayed inoculation in *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective+ineffective (E+I) bacteria. Lateral root production in the three bacterial series and in control uninoculated plants at 39-day harvest. Laterals visible on plants at inoculation are shown in the lower half of the diagram. Experimental variability is recorded as standard errors of means of treatment of the E+I series.

*Lateral root infection.*—The more substantial increase in lateral root nodulation with increasing delay in inoculation shows maxima in the three series of some 60 nodules per plant with the longest delay period of 25 days. It is probable that if even longer delay periods had been used, nitrogen starvation of the root would have restricted nodulation in a similar manner to that described for red clover by Nutman (1949), and reproduced in delayed-inoculation studies of other legumes (Pate and Dart, unpublished data).

The curves of lateral root nodulation against inoculation delay in Figure 4 show two response phases. Firstly, over the delay range increasing from 3 to 16

days there is a gradual increase in nodule numbers, with evidence of a differential response between strains. In this phase the I series nodulation is consistently about double that of the E series. The E + I response yields intermediate nodule numbers, with a closer approximation to the E response with short delays but tending to

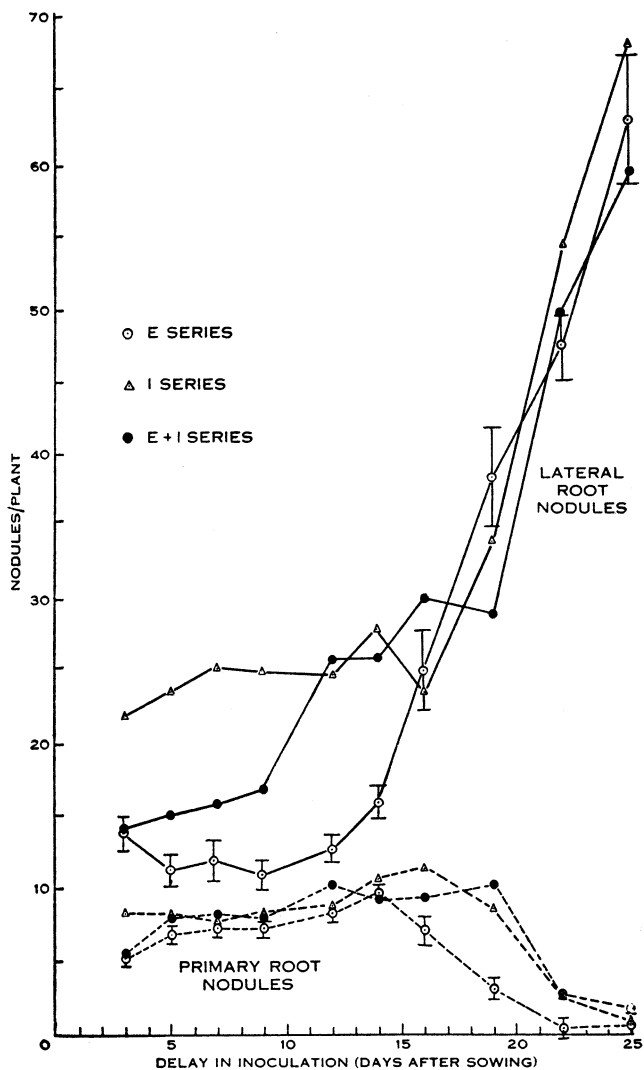


Fig. 4.—Experiment 2: Delayed inoculation in *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective + ineffective (E+I) bacteria. Nodule production at 39-day harvest. Standard errors of means of the treatments of the E series are included as an indication of experimental variability.

approach I values with longer delays. This last result can be correlated with the observation (described later) that the percentage of I nodules developing from mixed inocula increases the longer inoculation is delayed (see Section IV).



The second phase of response (16–25 days delay) witnesses a marked increase in lateral nodulation with delay. In this phase no strain differences were established during the time of the experiment.

(iv) *Nodule Distribution at Different Delay Treatments (Fig. 5 and Plate 1).*—Four delay intervals of 3, 12, 16, and 22 days are selected to illustrate nodule frequencies in the three series in relation to their spatial occurrence on the root system. Nodule distributions are expressed in the form of histograms where the frequency of occurrence of nodules is recorded for each lateral root numbered from the hypocotyl and for each numbered space between laterals on the primary root (see Fig. 5). The three series show remarkably similar distribution patterns in response to increasing delay in inoculation:

- (1) Inoculation at sowing or with a delay of up to 9 days allows successful bacterial exploitation of any space between laterals on the primary root. There is a slight preference in all series for infection to occur in positions on the primary root between and above the first three laterals below the hypocotyl, i.e. a slight tendency towards crown nodulation. Similarly, there is a tendency for the upper laterals to be more abundantly nodulated, although here it must be borne in mind that older and longer laterals are likely to carry larger populations of nodules.
- (2) With delay periods of more than 12 days upper regions of the primary root become closed to infection and in consequence the main nodule-bearing zone becomes placed further and further down the root as inoculation is progressively delayed. There is a concomitant increase in lateral root nodulation particularly on those laterals opposite the upper nodule-free portions of the primary root.
- (3) In the E + I series, primary root nodule populations show an increasing proportion of I nodules with delay in inoculation.
- (4) Distal portions of roots develop “clumps” of nodules where inoculation is delayed for more than 16 days. This “clumping” effect is illustrated in the photographs of individual root systems (see Plate 1). The histograms of Figure 5 also depict the overall increase in nodule intensity on the infected portion of the primary root and on secondary roots with increasing inoculation delay.

(v) *The Site of Infection on the Primary Root as Determined by Delayed Inoculation.*—The lower portion of Figure 2 compares average primary root length at inoculation with the average distances down the primary root to the first nodule with different inoculation treatments. In the delay range up to 5 days, a root grows some 1 or 2 cm after inoculation before producing a nodule. With delays of more than 7 days but less than 20 days portions of the primary root present at inoculation appear open to infection, with the highest nodule usually developing in a region 0.5–2 cm shorter than the primary root length at inoculation.

A second description of the site of infection can be made by comparing secondary root development at inoculation with the eventual spacing of primary root nodules between numbered secondary roots on the seedling axis. In the frequency

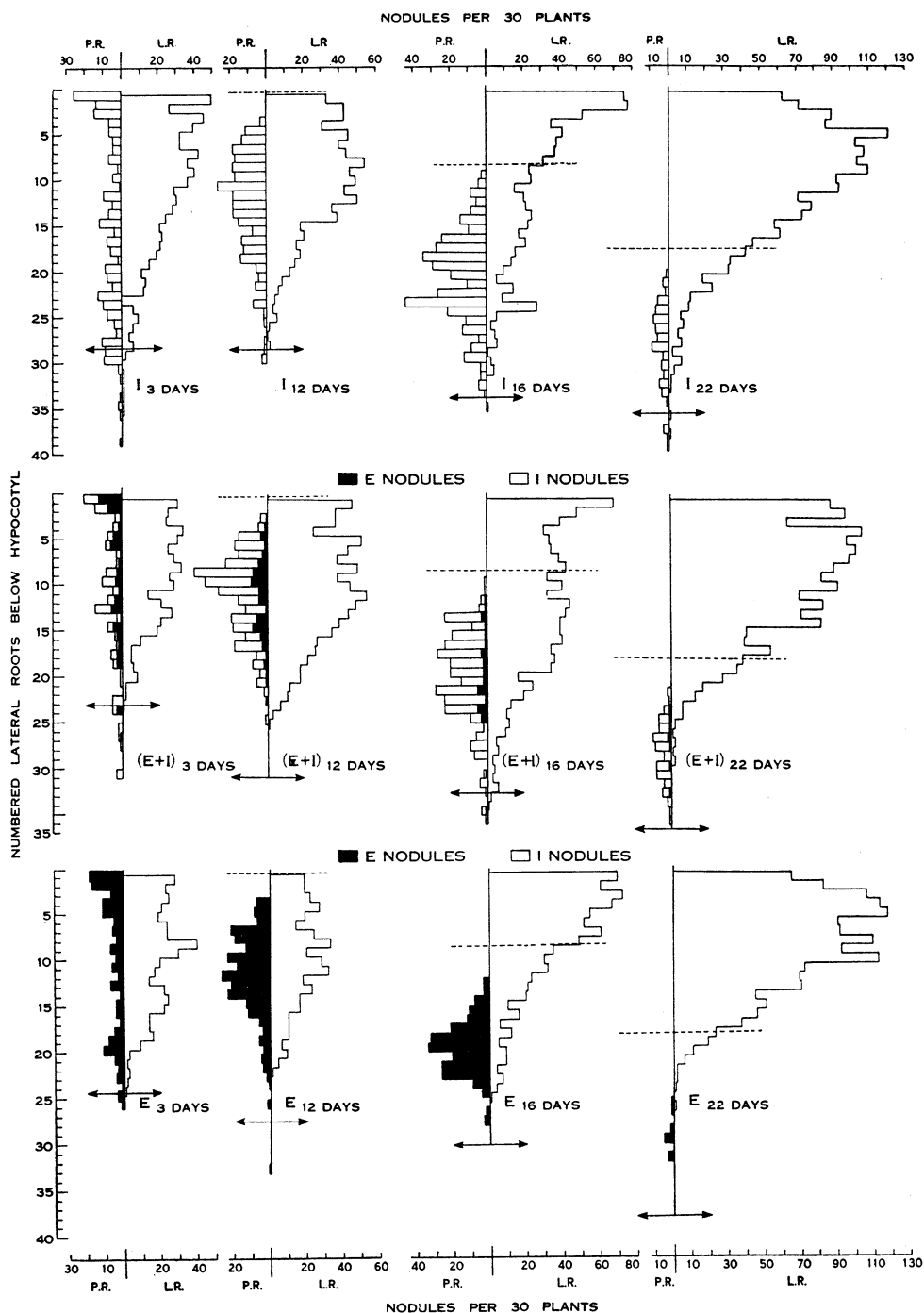


Fig. 5.—Experiment 2: Delayed inoculation in *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective+ineffective (E+I) bacteria. Histograms of nodule distribution are recorded for the three bacterial series at delay treatments of 3, 12, 16, and 22 days. Nodule

[For continuation see opposite page.]

distribution patterns of Figure 5 there is clearly a space difference representing some 2–10 secondary roots between average secondary root number at inoculation (broken line across top of histogram) and the first space recorded as being likely to be occupied by a nodule.

#### IV. DISCUSSION

The present study of delayed inoculation on barrel medic provides information on the site of infection and utilization of the nodule-forming potential of the root under different inoculation regimes. In addition quantitative interactions between symbionts are evident which are particularly relevant to current theories on the nature, origin, and activity of the legume nodule.

##### *(a) The Site and Intensity of Nodulation*

Application of nodule bacteria at any time during primary root extension in barrel medic usually leads to infection of existing portions of the root. A root-hair-invested zone some 1–2 cm behind the root tip and more than two lateral primordia below the lowest lateral visible at inoculation is prescribed as the region where the highest nodule on the primary root is likely to develop. The nodule-distribution patterns following different delays in inoculation suggest that bacterial entrance in barrel medic is restricted to zones of the root showing healthy root hair development.

The site of infection following a very short inoculation delay reveals that the top few centimeters of root below the hypocotyl junction but above the first lateral root are normally unsuited to bacterial exploitation. Root hairs are absent or poorly developed in this region. The sparse nodulation of the base of primary roots following long delay in inoculation suggests that the nitrogen-hunger state of the seedling induced by prolonged absence of external or symbiotic sources of nitrogen renders roots fit for only occasional nodulation.

Nodule-distribution maps for primary roots depict nodulation as occurring in any space between laterals, the spaces exploited most fully depending on the infection circumstances. In Table 2 the maximum nodulation frequency per space between laterals on the primary root is recorded for each series from pooled observations of the 10 delay treatments. 26 nodules per 30 plants per space between adjacent laterals is obtained as the mean of the maximum frequencies recorded in any series. The uniform figures recorded for the top 30 spaces between laterals on the primary root suggest the existence of regularly distributed infection foci down the primary root (see also Nutman 1952, 1958). In this connection the distribution maps of Figure 5 stress that the marked increase in nodule intensity (nodules/cm) on the lower primary root with increasing delay in inoculation is clearly effected through a fuller use of the maximum nodulating potential of adjacent spaces between laterals rather than abnormally high nodule frequencies in any one or more of these spaces.

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frequencies for the 30 plants of a treatment sample are recorded for each lateral root numbered from the hypocotyl and for the numbered spaces between laterals on the primary root. Effective and ineffective nodules are distinguished in the primary root frequency distributions of the E+I series. ---- Average laterals on root at inoculation. ←→ Average laterals on root at harvest.

Tetraploid cells in the root cortex are implicated as predetermined nodule-forming initials in the studies of Wipf and Cooper (1940), and Fujita and Mitsuishi

TABLE 2  
DELAYED INOCULATION OF *MEDICAGO TRIBULOIDES*  
Maximum nodule frequencies recorded for the numbered spaces between laterals  
on the primary root

Space Number*	Maximum Nodule Frequency (nodules per space per 30 plants)†			
	E Series	I Series	E + I Series	Combined Series‡
1	19	26	22	26
2	20	19	16	20
3	22	20	13	22
4	15	15	12	15
5	18	11	15	18
6	17	13	19	19
7	21	18	17	21
8	19	17	23	23
9	13	18	40	40
10	23	17	34	34
11	18	26	27	27
12	26	25	16	26
13	21	18	27	27
14	23	31	29	31
15	16	17	19	19
16	25	13	15	25
17	15	24	29	29
18	21	27	26	27
19	32	34	22	34
20	32	29	26	32
21	28	19	14	28
22	26	25	28	28
23	26	26	29	29
24	10	44	24	44
25	14	21	15	21
26	9	16	24	24
27	10	15	11	15
28	5	20	22	22
29	7	16	27	27
30	5	12	22	22
Average for 30 spaces	$19 \pm 1.3$	$21 \pm 1.3$	$22 \pm 1.2$	$26 \pm 1.2$

\* Spaces numbered from hypocotyl down root, e.g. space 1 is region above 1st lateral, space 9 between 8th and 9th laterals, etc.

† Frequencies selected from pooled observations of the 10 treatments of a series.

‡ Maximum frequencies for any series.

(1953). On such an assumption the present study would favour that initials would be present on a root far in excess of the normal demands of nodulation. Indeed a

condition approaching a full utilization of nodule foci in a particular region of the root might be encountered only in special infection conditions, e.g. the high nodule intensities on distal roots following excessive delay in inoculation. Furthermore, full exploitation of all infection foci in a root segment may require the use of an abundantly nodulating bacterial strain, which might be pictured as being particularly efficient in the fulfilment of the sequences leading to the location and stimulation of a nodule initial in the root cortex. It is quite clear that only a very small fraction of the infection potential of the seedling root system is exploited under any particular inoculation regime.

*(b) Strain Competition for Access to Roots and Delayed Inoculation*

Multiple infection of a host legume is a well-known natural phenomenon and has also been reproduced in culture by inoculating legumes with two or more invasive bacteria (e.g. see work of Nicol and Thornton 1941; Erdman and Means 1951; Vincent and Waters 1953). It is generally conceded that the host plant exerts the selective influence which may lead to a differential uptake of bacteria in root nodulation. Differently timed applications of contrasting bacterial strains have been used to study whether antecedence by one strain confers immunity against subsequent invasion by a second strain (e.g. results of Lohnis 1930; Chen 1941; Virtanen and Linkola 1947; Burton and Allen 1949). It is clear from these studies that both the root growth pattern of the host plant and the number and type of existing nodules influence the subsequent course of infection.

Results here add further to present knowledge by demonstrating that bacterial acceptance for nodule formation from mixed inocula varies with the time of inoculation of the host-legume root system. Table 3 shows that the proportion of ineffective nodules in the E + I barrel medic series increases four-fold as inoculation is delayed from 3 to 25 days. The effect bears little relationship to the performances of the respective strains in pure culture in response to delay. This can be seen by comparing the observed values for the nodulation of the two strains in mixed culture with comparable values for the nodulation of the strains in pure culture. The marked and significant divergence of observed from expected values with the longer delay periods reveals that the ineffective partner appears to compete more successfully for lower positions on the primary root than might be expected from a sharing of the nodule-forming potential in proportion to the nodulation capacities of the strains in pure culture. This effect may reflect a differential response of the two strains to a changing biochemical condition in the host root following continued root expansion in nitrogen-deficient conditions. A full evaluation is not possible until it is known whether discrimination between strains takes place at the root hair or subsequent to bacterial invasion.

*(c) Nodule-Host Plant Interactions*

In the establishment of any legume the first-formed symbiotic organs and considerable portions of root and shoot system develop at the expense of cotyledon reserves. Therefore it is not surprising to find in a small-seeded legume like barrel medic that early host plant development is greatly influenced by the time and type of nodulation.

(i) *An Increase over Control Uninoculated Growth in Leaf and Dry Matter Production with Effective Symbiosis.*—The extent of this normal response to effective symbiosis obviously depends on the number and duration of activity of nodules on the seedling root. The higher top : root weight ratio in E as opposed to I symbiosis indicates that the early products of fixation are directed towards top development (see similar results obtained for other annual legumes (Pate 1958a, 1958b)). It is noted below that nodulation actually restricts root growth in the seedling legume: this effect will also tend to favour elevation of the top : root ratio. With continued nitrogen fixation one might expect an ultimate benefit to both root growth and further nodulation. Such a stimulus was not observed within the time limits of this experiment but was obtained in other delayed-inoculation experiments.

TABLE 3  
DELAYED INOCULATION OF *MEDICAGO TRIBULOIDES*

Comparison of primary root nodulation using effective (E), ineffective (I), and a mixture of effective + ineffective (E+I) bacteria

Treatment Number*	% E Nodules on Primary Root (observed)† with 99% Confidence Limits (E+I Series)	% E Nodules on Primary Root (expected)‡	Treatment Number*	% E Nodules on Primary Root (observed)† with 99% Confidence Limits (E+I Series)	% E Nodules on Primary Root (expected)‡
1	45.4 ± 5.51	39.3	6	16.2 ± 2.48	47.5
2	31.8 ± 3.03	45.5	7	11.4 ± 1.65	38.4
3	34.4 ± 2.76	48.0	8	13.1 ± 2.48	26.3
4	31.0 ± 2.20	46.1	9	11.0 ± 2.76	14.1
5	24.8 ± 2.48	48.3	10	—	17.8

\* See Table 1 for inoculation schedule.

† The means of the 30 individual plant percentage E values in each E+I treatment. E and I nodules were distinguished on a visual and serological basis for all treatments except the small nodules produced following the longest delay interval of 25 days (treatment 10).

‡ E nodules in pure culture ÷ (I nodules in pure culture + E nodules in pure culture) expressed as a percentage. This expected value assumes that nodulation in mixed culture is proportional to the performances of the respective strains in pure culture.

(ii) *A Reduction below Control Uninoculated Growth in Primary Root Extension and Lateral Production associated with the Presence of Nodules on the Primary Root.*—The degree of reduction in these quantities bears an inverse relationship to inoculation delay. No differences in reaction between the three bacterial series were observed within the time limits of the experiment, suggesting that nodule-induced suppression of root growth in the seedling legume is not dependent on continued nitrogen fixation in the nodule (Figs. 2 and 3).

*(d) Nodule-Nodule Interactions*

Two features of the nodulation responses to delayed inoculation suggest an interaction between nodules. The first of these is the apparent suppression of lateral root nodulation by existing primary root nodules. The intensity of this effect is related to the position of the primary root nodule zone, and in the short delay treatments a differential strain response is evident where an E nodule on a primary root appears to exercise a more restrictive influence than an I nodule. The second feature is the higher nodule numbers and intensities associated with long delays in inoculation. Both bacterial strains exhibited these responses although greater stimulation of nodule numbers with delayed inoculation occurred in the effectively nodulated series.

The following assumptions are introduced to interpret the observed responses of barrel medic to delayed inoculation:

- (1) Nodule initiation on a particular segment of root is dependent on a combination of the following conditions in the host plant: (i) the presence of young and healthy root hairs; (ii) the presence beneath the root hair zone of suitable infection centres in the cortex for bacterial exploitation; (iii) a suitable biochemical-physiological condition in the root for nodule formation. Any of these prerequisites may limit nodulation. Thus, the patterns of infection with different inoculation delays expose the essentiality of condition (i); situations are suggested above (see Section IV(a)) where requisite (ii) might limit nodulation; condition (iii) might restrict nodulation in nitrogen-deficient tissues, or, as is suggested in paragraphs below, if existing nodules were to alter the physiology of the root in such a way as to inhibit subsequent infection and nodule development.
- (2) An existing nodule appears to exercise a localized and cumulative influence tending to restrict further nodulation, lateral development, and primary root extension of the seedling.
- (3) The restrictive influences exerted by a nodule increase as the nodule ages. Stimulation of nodule numbers or infection rates by delayed inoculation can then be interpreted as follows: the later inoculation is delayed the larger the area of root-hair-invested root that is presented to the bacteria at inoculation, and hence the more nodules that can develop initially on the various segments of infectable root before the earlier-formed of these nodules impose restrictions on further infection.
- (4) In addition to the interpretation presented in (3) the clumping effect produced by excessive delay in inoculation implies that resistance of the host plant to infection is lowered as the root system expands in nitrogen-deficient media in the absence of nodule bacteria. This enables an extraordinary number of foci to be utilized on root portions susceptible to invasion at inoculation. The infection thread-nodule initiation sequences of roots are now being studied to determine the efficiency of bacterial utilization of the infection potential of the root with different delay treatments.

- (5) The restrictive influence of a maturing nodule is dependent on its size and growth activity. In the strain pair studied here, E and I nodules appeared to exercise quantitatively similar effects for the first 2 or 3 weeks of their growth. Thus the three bacterial series show almost identical nodulation responses on primary roots and similar restrictive influences on primary root growth and lateral initiation (Figs. 2, 3, and 4). Differences in potency of inhibitory influence developed when nodules were 2–3 weeks old, coincident with the appearance of size, colour, and meristem differences in the two nodule types. The greater restrictive influence of the mature E primary root nodule is reflected in the fact that nodulation of lateral roots following inoculation at germination or with moderate delays is consistently less extensive in the E series than in the E + I or I series (see Fig. 4). Nutman (1949) described much greater differences between nodule production responses to delayed inoculation using an effective and ineffective strain on red clover. The ineffective association he used might be designated as abundantly nodulating, where the small nodules produced would exert only a meagre and transient restrictive influence on further nodulation of the root.

Two hypotheses may be advanced as alternative interpretations of the mechanism whereby existing nodules might affect host plant development and nodulation. The first, a *nutritional* hypothesis assumes that certain nutrient substances are utilized in both root and nodule development and that an existing nodule (or lateral) intercepts and sequesters these substances in their passage down the root, thereby restricting development distal to the developing nodule (or lateral). It should be noted that this hypothesis is compatible with anatomical objections raised by Allen and Allen (1958) against Nutman's (1949) hypothesis of identical meristematic foci on the root being used in lateral and nodule formation. In this connection it is pictured that nutrient factors delivered to the infected region would be shared in either the fulfilment of pericyclic stimulation for lateral production or in the successful exploitation of cortical initials by bacteria in nodule formation.

The second hypothesis assumes that nodules, *per se*, produce inhibitors whose cumulative effect is to arrest primary root growth and attendant lateral or nodule formation. This *inhibitor* hypothesis is essentially that introduced by Nutman (1949, 1958) as a possible mechanism for the regulation of nodule and lateral root numbers on the host legume. As stated above the hypothesis need not be coupled with Nutman's hypothesis of an origin of laterals and nodules from identical foci in the root. Pate (1958c) has shown that legume nodules contain large amounts of several growth substances. A backward diffusion of inhibitory concentrations of such substances from the nodule might be held responsible for the nodule–root growth interactions described here and in Nutman's studies on red clover.

Until further information is available on the biochemistry and physiology of exchange reactions between nodule and root either hypothesis remains acceptable. In future experiments it is hoped to approach the problem by combining delayed inoculation with timed applications of nutrient substances to top or root to test whether the arrested root growth and nodulation associated with existing nodules



can be wholly or partially alleviated by the supply of extra, possibly limiting, nutrients to the seedling. As a sideline to these studies it may be possible to specify an ideal inoculation-fertilizer treatment promoting maximum symbiosis over the life cycle of the association.

#### V. ACKNOWLEDGMENTS

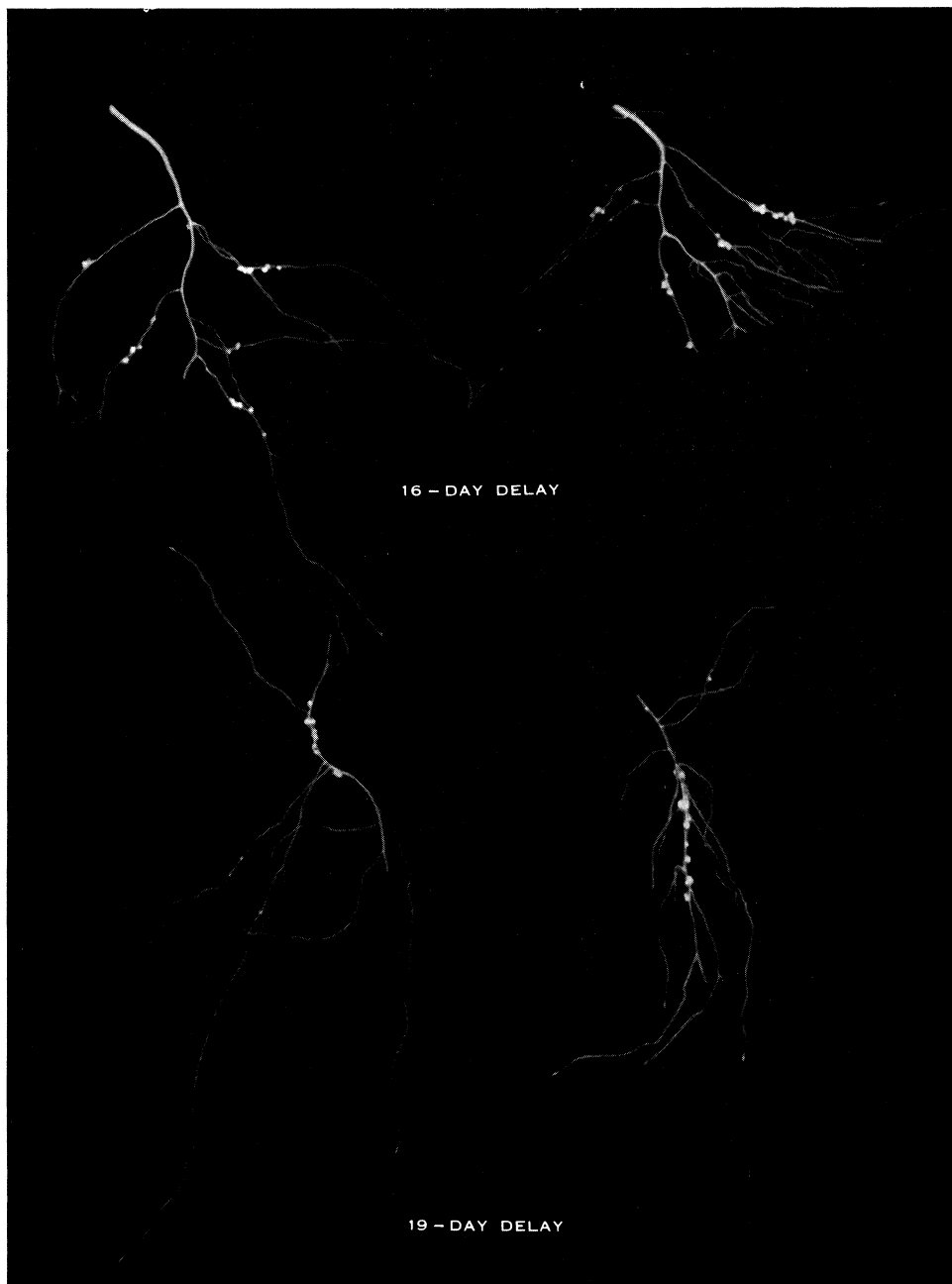
We are indebted to Professor J. M. Vincent, Faculty of Agriculture, University of Sydney, for his useful criticisms and interest in this work and for his permission to use glass-house space in the Agriculture School. We would also like to thank Mrs. L. M. Waters for her supply of the bacterial strains used in this study, and Mr. J. B. Bloomfield for his advice on the use of serological techniques.

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## NODULATION STUDIES IN LEGUMES. III



Portions of root systems of 39-day-old barrel medic (*M. tribuloides*) seedlings showing the characteristic clumping of nodules on distal root segments following long delays in inoculation. Upper two photographs: infected laterals after a 16-day delay in inoculation; lower two photographs: infected zone of primary roots after a 19-day delay in inoculation. Invading *Rhizobium* SU277-1.  $\times 1$ .

