PHYSICAL ENVIRONMENT AND SYMBIOTIC NITROGEN FIXATION

IV.* FACTORS AFFECTING THE EARLY STAGES OF NODULATION

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Summary

The influence of root temperature on the initial nodulation of *Trifolium subterraneum* L. was examined, and observations were made on the effect of light period and shoot temperature on this character. The maximum constant root temperature at which nodules would form was 33°C, and the minimum was in the vicinity of 7°C. The most rapid initial nodulation (2–3 days after inoculation) was observed at 30°C, and plants growing at this temperature had the highest rate of nodule appearance. Below 22°C, there was a marked increase in the "time to first visible nodule" and a general decline in the rate at which they appeared. Differences were observed in the time to first visible nodule, and in the rate of nodule appearance, between different cultivars of *T. subterraneum*. There was an indication of a temperature × cultivar interaction for these characters. With the three strains of *Rh. trifolii* used, no differences in their ability to form nodules were observed, although it was known that their subsequent symbiotic behaviour differed under certain root temperature conditions.

Transfer experiments suggested that the infection of root hairs was stimulated by 30°C root temperature, and that infection continued at a rapid rate up to 33°C, but was severely retarded above this temperature. Meristematic development of the nodules, but not of the roots, was severely retarded above 30°C. Although infection was retarded by lower root temperatures (7–17°C), it appeared that these temperatures had a greater effect on nodule meristem initiation than on infection. Furthermore, there was an indication that low temperature retardation of nodulation was not due solely to direct effects on root hair infection or nodule initiation. Leghaemoglobin formation in the nodules was also retarded by lower root temperatures.

Shoot temperatures in the range 18–25°C had little effect on nodule formation. With a root temperature of 12°C, a daily shoot temperature regime of 15/10°C delayed initial nodule formation, but did not affect the rate of nodule appearance, when compared with a regime of 18/14°C. With 4- and 8-hr daily light periods, initial nodulation was retarded; for plants receiving 12, 16, or 20 hr light/day, the time to first visible nodule values were similar.

I. Introduction

In any examination of the effects of the physical environment on the symbiosis between the legumes and their nodule bacteria (*Rhizobium* spp.), it is essential to distinguish between factors affecting nodule formation and factors affecting nitrogen fixation and associated functions in the nodule. The processes involved in nodule formation (infection of the root hairs, stimulation of the host tissue to produce the nodule structure, and bacterial proliferation) are very different from those involved in the functioning of the nodule (nitrogen fixation, transport of material to and from the nodules, and both host and bacterial tissue maintenance).

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Most of the previous work on the effect of the environment on nodulation has been concerned with effects observed after a considerable period of plant growth (e.g. Jones and Tisdale 1921; Mes 1959; Pate 1962; Possingham, Moye, and Anderson 1964 for temperature studies; and Sironval, Bonnier, and Verlinden 1957 for light studies). In an attempt to isolate the two broad phases of the symbiotic association, Barrios, Raggio, and Raggio (1963) used excised root culture to study the time course of nodulation in *Phaseolus vulgaris*. Hely and Williams (1964) determined the time to initial nodule formation in two lines of *Trifolium subterraneum* L. grown under a range of root temperatures, although their later observations on nodule development were confounded with other effects resulting from nodule activity.

In this paper, observations on nodule formation are restricted to the period before nitrogen fixation commences. Within this period, a number of stages in the formation of the nodules have occurred (Bergersen 1957; Nutman 1962), and these may be conveniently grouped as follows:

Inoculation	Infection	Nodule Initiation	Nodule Development
	Infection of root hairs and growth of the infection thread down the root hair and into the root cortex	Activation of tetraploid centre to form nodule meristem; development of meristem and early tissue differentiation	Enlargement and further differentiation of tissue, occupation of cells by bacteria, and appearance of leghaemoglobin
	Time to first visible nodule →		Rate of nodule appearance (No. of nodules) ⇢

The determination of the time to first visible nodule on plants subjected to varying conditions after inoculation enables conclusions to be drawn regarding the effect of these conditions on the stages involved in infection and nodule initiation. Observations on the rate of nodule appearance in the period immediately following the appearance of the first nodule provide information on the number of successful infections completed, and the ability of the plant to permit these infections to develop into nodules.

The principal observations made in this investigation concern the effect of root temperature on infection and nodule initiation; those on nodule development are subsidiary. Plants are exposed to different temperature conditions for varying times after inoculation in order to study the effects of root temperature on the different stages of nodule formation involved. In addition, observations are made on the effect of light period and shoot temperature on nodule formation.

II. Materials and Methods

(a) *Plant Culture*

All plants were cultured with the roots growing on agar slopes within test tubes, and with the shoots exposed (Gibson 1963). The plants were grown in controlled-environment cabinets in which root and shoot temperatures were controlled independently (Gibson 1965), and which provided a light intensity of 2000 f.c.

Unless otherwise stated, the light period was 12 hr/day. For the "higher root temperature" studies, the shoot temperature regime was 25/22°C (light/dark) and for the second experiment in the "lower root temperature" experiments, it was 18/14°C. The shoot temperature conditions were generally in the range expected, under natural conditions, for the root temperatures being used.

For the first 3 days after sowing, the seedlings were held at 22°C root temperature, and with a shoot temperature regime of 22/16°C (16 hr light/day). The plants were then inoculated with 3-day cultures of *Rh. trifolii* strains TA1, NA30, or CC17, as indicated in the text. The bacterial growth on a yeast extract mannitol agar slope was washed into 5 litres seedling nutrient solution, which was added to the tubes at the rate of 18 ml/tube. Each tube received 10^6–10^7 nodule bacteria.

Certified commercial seed of *T. subterraneum* cultivars Dwalganup, Mount Barker, Tallarook, and Yarloop was obtained from F. H. Brunning Pty. Ltd., Melbourne. The cultivar Tallarook (fast and abundantly nodulating) was used for the higher root temperature studies, and Mount Barker (slower, and less abundantly nodulating) for the lower root temperature experiments. Both cultivars were examined under the full range of root temperature conditions, whilst Dwalganup and Yarloop were included for comparative purposes at the lower root temperatures.

(*b*) *Observations*

Unless otherwise stated, the roots were observed at daily intervals, such observations commencing 2–5 days after the plants were inoculated, and ceasing when the majority of the plants in any treatment had one nodule containing leghaemoglobin, as shown by the appearance of a pink colour in the nodule tissue. The observations were made against a well-illuminated, back-lit, frosted glass screen. This enabled the observer to identify the nodules at an early stage of development, and to better differentiate between nodules and lateral roots.

For these studies, the occasional nodule forming on the first 0·5 in. of root below the aluminium cap on the tubes was ignored, as the temperature in this section of the tube showed a gradient between the specified root and shoot temperatures. The few nodules forming on the lateral roots towards the end of any observation period were also disregarded. In both instances, such nodules constituted less than 5% of the total number of nodules found in any treatment.

The time to first visible nodule for any treatment was calculated as the mean of the time taken from inoculation to the appearance of the first nodule on each plant in any treatment.

III. Experimental

(*a*) *Temperature Effects on Time to First Visible Nodule*

Within the range 25–36°C, there were marked effects of root temperature on the time to first visible nodule (Table 1). Nodules appeared most rapidly at 30°C. At 33°C they appeared more slowly, and at 36°C none of the plants were nodulated

7 days after inoculation, at which time they were showing extensive leaf necrosis and nodulation was unlikely.

TABLE 1

TIME TO FIRST VISIBLE NODULE, MEAN NODULE NUMBER 7 DAYS AFTER INOCULATION, AND MEAN ROOT LENGTH 3 DAYS AFTER INOCULATION FOR TWO *T. SUBTERRANEUM* CULTIVARS GROWN AT FOUR ROOT TEMPERATURES FROM THE TIME OF INOCULATION

Data pooled for three strains of *Rh. trifolii* (TA1, NA30, CC17). 27 replicates

Root Temp. (°C)	Time to First Visible Nodule (days)		Mean Nodule Number on Day 7		Primary Root Length on Day 3 (cm/plant)	
	Mount Barker	Tallarook	Mount Barker	Tallarook	Mount Barker	Tallarook
25	4·7	4·2	3·5	9·1	4·9	6·5
30	4·1	4·0	11·3	18·7	9·8	10·1
33	5·1	4·7	1·2	6·5	11·0	10·0
36	>7·0	>7·0	0	0	8·4	6·4
Least significant differences ($P = 0·05$)*						
Within temperatures		0·4		2·0		0·7
Between temperatures		0·3		1·5		0·5

* Results from 36°C root temperature treatment excluded when calculating significant difference values for nodulation characters.

TABLE 2

TIME TO FIRST VISIBLE NODULE FOR PLANTS OF FOUR HOST CULTIVARS OF *T. SUBTERRANEUM* INOCULATED WITH *RH. TRIFOLII* STRAIN TA1, AND GROWN UNDER SIX TEMPERATURE REGIMES FROM THE TIME OF INOCULATION

15 replicates

Temperature Regime (°C)		Time to First Visible Nodule (days)			
Roots	Shoots	Mount Barker	Dwalganup	Tallarook	Yarloop
22	22/18	5·7	4·7	4·1	4·9
17	22/18	9·0	6·5	6·4	6·9
17	18/14	8·2	6·6	6·3	6·8
12	18/14	12·8	10·4	10·5	11·1
12	15/10	13·7	11·3	12·7	13·1
7	15/10*	24·2 (53%)	20·2 (71%)	20·2 (88%)	22·8 (56%)

* To obtain mean values for this treatment, unnodulated plants were given a value of 25. The percentage values in parenthesis refer to the proportion of nodulated plants in this treatment 24 days after inoculation.

Below 22°C root temperature, the formation of the first visible nodules was markedly retarded (Table 2). At 7°C, a proportion of the plants had failed to nodulate 24 days after inoculation, and as the plants appeared to be dying, eventual

nodulation was doubtful. In addition to delaying initial nodule formation, lower temperatures extended the time interval during which the plants in any treatment

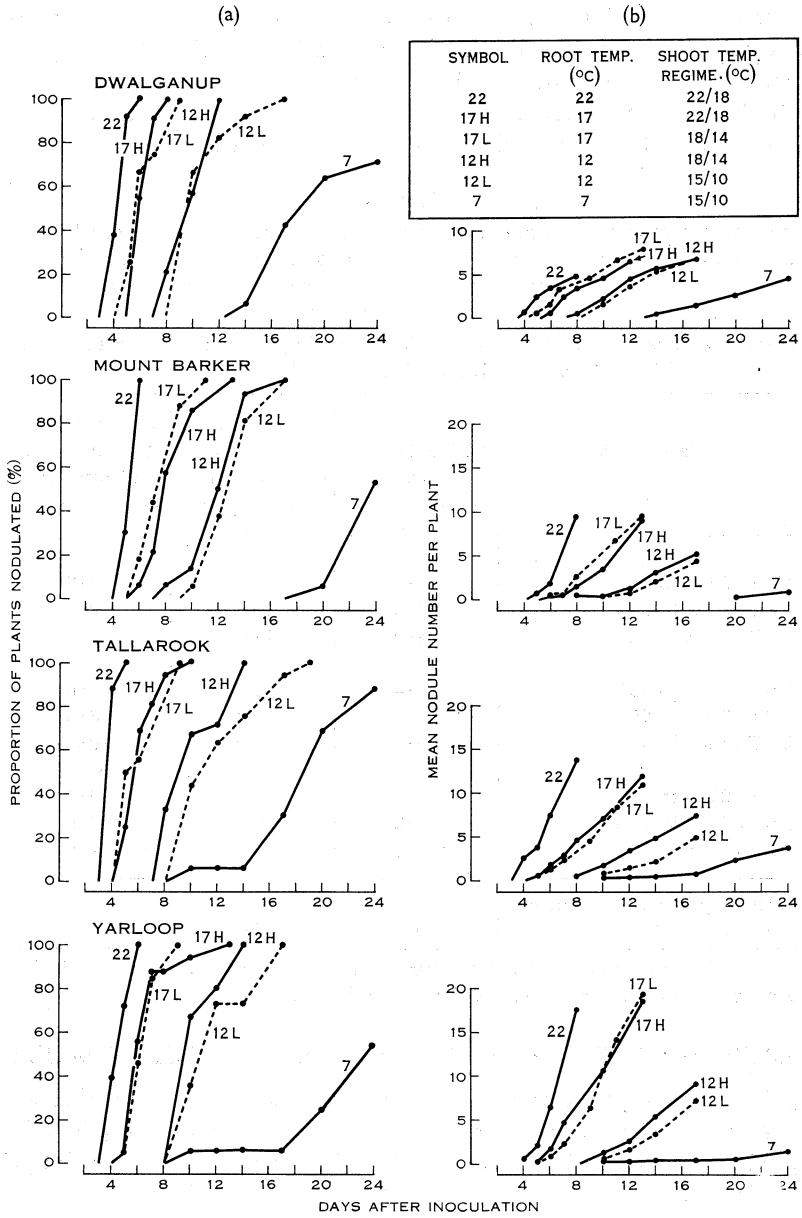

Fig. 1.—(*a*) Progressive proportion of plants nodulated (as a percentage) in six temperature regimes for each of four cultivars of *Trifolium subterraneum* inoculated with *Rh. trifolii*, strain TA1. (*b*) Increase in mean number of nodules per plant for the treatments mentioned in (*a*).

nodulated [Fig. 1(*a*)], the length of the interval and the rate at which the plants nodulated being dependent on the host cultivar.

At all root temperatures examined, except 30°C, the time to first visible nodule for cv. Mount Barker was greater than that for cv. Tallarook, Yarloop, and Dwalganup (Tables 1 and 2), with the difference being greater at the lower temperatures than at 25 and 33°C. After 24 days at 7°C, the lowest proportion of nodulated plants was found in cv. Mount Barker. Although the plants were inoculated with three strains for the higher temperature experiment, and with two in the lower temperature experiment (Table 2), there were no bacterial strain differences between the time to first visible nodule values.

TABLE 3

TIME TO FIRST VISIBLE NODULE, MEAN NUMBER OF NODULES PER PLANT 7 DAYS AFTER INOCULATION, AND PRIMARY ROOT LENGTH PER PLANT 3 DAYS AFTER INOCULATION, FOR PLANTS TRANSFERRED FROM, OR TO, 25°C ROOT TEMPERATURE 36 HR AFTER INOCULATION

Results were pooled for the three symbiotic combinations: Mount Barker–NA30, Mount Barker–TA1, and Tallarook–TA1. 27 replicates

Treatment	Time to First Visible Nodule (days)	Mean No. of Nodules/Plant on Day 7	Primary Root Length/Plant on Day 3 (cm)
25°C control	4·6	4·9	5·1
From 25°C to 30°C	4·6	7·6	6·7
From 30°C to 25°C	3·9	10·4	8·6
30°C control	4·0	13·2	9·9
From 25°C to 33°C	5·4	1·5	7·6
From 33°C to 25°C	4·3	9·9	8·6
33°C control	5·3	1·8	10·8
From 25°C to 36°C	>7·0	0	6·4
From 36°C to 25°C	6·3	2·9	7·8
36°C control	>7·0	0	7·6
Least significant difference ($P = 0·05$)*	0·3	2·0	0·7

* Results from 36°C temperature treatments excluded when calculating least significant difference values for nodulation characters.

(*b*) *Higher Root Temperature Effects on the Stages of Nodule Formation*

By transferring plants from one temperature condition to another at various intervals after inoculation (given in days), and for different times (given in hours), it is possible to draw conclusions about the effect of temperature on infection and nodule initiation.

To examine temperature effects on the stages of nodule formation, plants were held at 30, 33, or 36°C root temperature for 36 hr immediately after inoculation, and then transferred to 25°C. At the same time, another group of plants were transferred from 25 to 30, 33, or 36°C. The plants held at 30°C for 36 hr nodulated at the same time as the 30°C controls, and earlier than the controls at 25°C (Table 3).

Similarly, the plants at 33°C for 36 hr nodulated earlier than the controls at 25°C, even though the controls at 33°C nodulated later than those at 25°C. These results suggest that, 36 hr after inoculation, the infection of the root hairs was further advanced at 30 and 33°C than it was at 25°C. At 36°C, infection was inhibited, as the nodulation of the plants transferred from 36 to 25°C was delayed by approximately 1·5 days.

TABLE 4

TIME TO FIRST VISIBLE NODULE FOR CULTIVAR TALLAROOK PLANTS INOCULATED WITH STRAIN TA1, AND THEN GROWN FOR 24, 48, OR 72 HR AT ONE ROOT TEMPERATURE BEFORE TRANSFER TO ANOTHER ROOT TEMPERATURE

15 replicates

Transferred to:	Time of Exposure before Transfer (hr)	Time to First Visible Nodule (days)*			
		Transferred from:			
		20°C	25°C	30°C	35°C
20°C	0 (control)	5·7	—	—	—
	24	—	5·1	4·1	5·9
	48	—	5·4	3·9	6·3
	72	—	4·9	3·9	>7·0
25°C	0 (control)	—	4·5	—	—
	24	4·9	—	3·5	5·5
	48	5·6	—	3·3	6·1
	72	5·7	—	3·6	6·1
30°C	0 (control)	—	—	3·7	—
	24	4·4	4·1	—	4·5
	48	5·2	5·0	—	5·6
	72	5·9	4·4	—	6·1

* Least significant difference ($P = 0·05$) = 0·6.

The results of this experiment suggest that the later stages of nodule formation (i.e. nodule initiation) occurred at a similar rate at 25 and 30°C, but were retarded at 33°C. Plants transferred from 25 to 30°C nodulated at the same time as the 25°C controls. Those plants transferred from 25 to 33°C nodulated later than these controls. At 36°C, there appeared to be a complete inhibition of the later stages of nodule formation, as plants transferred from 25 to 36°C failed to nodulate during the course of the experiment.

Two further lines of evidence indicate that the major stimulation of nodule formation caused by a root temperature of 30°C was due to an effect on infection. Plants held at 30°C for 24, 48, and 72 hr after inoculation, and then transferred to 25 or 20°C, all nodulated as rapidly as the 30°C controls (Table 4). This indicated that the stimulation occurred during the first 24 hr after inoculation, and that further exposure to 30°C had little effect on subsequent stages of nodule formation. Corroborative evidence indicating that it was the infection stage that was stimulated by 30°C came from plants which were held at 20 and 25°C before transfer to a higher

temperature (Table 4). In most cases, the delay in time to first visible nodule, relative to that of the higher temperature controls, was proportional to the period at the lower temperature before transfer; where the delay was 48 or 72 hr, the plants did not nodulate significantly earlier than the controls at the lower temperature. The results for plants exposed to 35°C indicate that infection was either severely retarded or completely inhibited, while later stages were also severely retarded [Fig. 2(*e*)]. None of the 35°C controls nodulated in the period of the experiment.

The second line of evidence supporting the conclusion that 30°C stimulated infection came from an experiment in which plants normally growing at 20°C were exposed to 30°C for 24 hr, such exposure commencing immediately, or 1, 2, or 3 days after inoculation. Observations were made 12-hourly. The plants transferred to 30°C immediately after inoculation nodulated as rapidly as the 30°C controls (3·4 and 3·0 days respectively). The plants in the other 24 hr treatments nodulated later (4·0 days) than the 30°C controls, but earlier than the 20°C controls (5·3 days). In the same experiment, groups of plants were exposed to 30°C for 6, 12, or 24 hr immediately after inoculation, and then transferred to 20°C. The time to first visible nodule values were 4·7, 4·0, and 3·4 days respectively. This indicates that the length of exposure to 30°C has a marked effect on the degree of stimulation, and that the stimulatory effect is cumulative over the 24-hr period. In another treatment, plants were exposed to 30°C for 12 hr commencing 1 day after inoculation, and the time to first visible nodule (4·0 days) indicated that the stimulation was the same as that of two other treatments — a 24-hr exposure commencing at the same time, and a 12-hr exposure commencing immediately after inoculation.

The stimulatory effect of 30°C was an effect on the plant–bacteria association, and not on the plant alone. Plants inoculated *after* exposure to 25 or 30°C for 24 hr, and then grown at 20°C, had the same time to first visible nodule value as the 20°C controls; plants inoculated *before* such exposure treatments nodulated significantly earlier than the 20°C controls. The stimulatory effect of 30°C was not an effect on bacterial multiplication in the medium as plants inoculated with 10^3 bacteria/tube nodulated at the same time as plants inoculated with 10^8 bacteria/tube. This effect was observed at 20, 25, and 30°C.

(*c*) *Lower Root Temperature Effects on the Stages of Nodule Formation*

Lower root temperatures markedly retard infection. Plants held at 22°C for 48 hr immediately after inoculation, and transferred to a range of lower temperature conditions (7–17°C), nodulated earlier than control plants at the latter temperatures (Table 5). If it is assumed that the stages of nodule formation in both the transferred and control plants, beyond those occurring in the first 48 hr at 22°C, are similarly affected by the lower temperatures, the difference between the time to first visible nodule values for the two sets of plants indicates the extent of the delay to the early stages by continuous exposure to the lower temperatures. For example, taking the values for Dwalganup at "12–15/10", the time to first visible nodule for the controls was 11·3 days, and for the transferred plants 8·8 days, a difference of 2·5 days. Hence the control plants took 4·5 days (2·5+2·0) to reach the same stage of nodulation as that reached by the 22°C controls in 2 days. If this is regarded

as the infection stage, the infection of the control plants took 1·5 times longer at 17°C, 2·3 times longer at 12°C, and 4·3 times longer at 7°C than the infection of the plants at 22°C. The relative increase in the time for infection was the same for both cultivars, even though there was a considerable difference between the time to first visible nodule for the controls of the two cultivars under each temperature condition.

TABLE 5

TIME TO FIRST VISIBLE NODULE OF *T. SUBTERRANEUM* CULTIVARS DWALGANUP AND MOUNT BARKER TRANSFERRED FROM THE "22–22/18" TEMPERATURE REGIME TO FIVE DIFFERENT TEMPERATURE REGIMES 2 DAYS AFTER INOCULATION WITH STRAIN TA1

15 replicates

Temperature Regime (°C)		Time to First Visible Nodule (days): cv. Dwalganup		Time to First Visible Nodule (days): cv. Mount Barker	
Roots	Shoots	Transferred Plants	Control Plants	Transferred Plants	Control Plants
22	22/18	—	4·7	—	5·7
17	22/18	5·5	6·5	7·3	9·0
17	18/14	5·4	6·6	7·1	8·2
12	18/14	6·6	10·4	9·2	12·8
12	15/10	8·8	11·3	10·6	13·7
7	15/10	13·6	20·2*	17·7*	24·2*

* Some plants in these treatments were not nodulated 24 days after inoculation.

Although the early stages of nodule formation were affected by the lower temperatures, the results of a second experiment indicated that the lower temperature effects were stronger on the later stages of development (Table 6). The exposure of plants to 12°C for 24 hr increased the time to first visible nodule in relation to that of the 22°C controls only when such exposure commenced 2 and 3 days after inoculation. Similar effects were evident where the exposure period to 12°C was longer.

(*d*) *Temperature Effects on Nodule Development*

For plants growing at 22°C, leghaemoglobin was observed in the nodules 2–4 days after the nodules were recorded. There was no indication that any post-inoculation treatment affecting the time to first visible nodule also affected the development of leghaemoglobin. Within a treatment, there was an indication that the later nodulating plants developed leghaemoglobin more rapidly than the earlier nodulating plants (2–3 days; cf. 3–4 days). Above 22°C, the time taken for leghaemoglobin to appear was similar to that for plants growing at 22°C, but no development of pigment was observed at 33°C.

The nodules on plants growing at 12°C took 5–8 days for leghaemoglobin to appear after the nodules were first recorded. Again there was no effect of any post-inoculation treatment which affected initial nodulation.

(*e*) *Higher Temperature Effects on Rate of Nodule Appearance*

As well as stimulating initial nodulation, 30°C also promoted the rapid rate of nodule appearance (Table 1; Fig. 2). Exposure of plants to 30°C for 24–48 hr after inoculation also caused a high rate of nodule appearance when the plants were subsequently transferred to a lower root temperature [Table 3; Fig. 2(*d*)]. This indicates that plants growing at the lower root temperatures continuously are capable of forming nodules at a higher rate than that achieved by the controls, but the main limitation to a high rate of appearance is the number of successful infections made. Hence, exposure to 30°C not only stimulates the individual infections, but also promotes more infections than are achieved at lower root temperatures. The longer

TABLE 6

TIME TO FIRST VISIBLE NODULE OF PLANTS NORMALLY GROWING AT 22°C ROOT TEMPERATURE WHEN THEY WERE EXPOSED TO 12°C FOR VARYING TIMES, AT VARYING PERIODS AFTER INOCULATION

Plants were cv. Mount Barker inoculated with strain TA1, and they received 16 hr of light per day. 20 replicates

Period of Exposure at 12°C (hr)	Days after Inoculation before Exposure to 12°C	Time to First Visible Nodule (days)*	Period of Exposure at 12°C (hr)	Days after Inoculation before Exposure to 12°C	Time to First Visible Nodule (days)*
0	0	5·3	48	0	6·2
	1	—		1	6·1
	2	—		2	7·0
	3	—		3	6·9
24	0	5·6	72	0	6·4
	1	5·6		1	6·8
	2	6·0		2	7·4
	3	6·4		3	7·0

* Least significant difference ($P = 0{\cdot}05$) = 0·07.

the exposure to a higher root temperature, the greater is the number of nodules appearing during the period of observation [Figs. 2(*b*), 2(*d*)]. Likewise, the longer the plants are held at a lower temperature before transfer to a higher temperature, the fewer the number of nodules [Figs. 2(*a*), 2(*c*)].

Plants transferred from 33 to 25°C 36 hr after inoculation developed a similar number of nodules by day 7 to that developed by plants transferred from 30°C at the same time. This indicates that as many successful infections were made at 33°C as had been made at 30°C (Table 3). However, for plants transferred from 35°C at various times after inoculation, the results suggest that the infection rate had fallen to a level comparable with that achieved at 20°C [Figs. 2(*f*), 2(*c*)]. The stimulatory effect of 30°C on the number of infections is well illustrated in Figure 3(*b*), which shows a sharp increase in the rate of nodule appearance approximately $2\frac{1}{2}$ days after any 24-hr exposure to 30°C commenced. The cumulative effect of exposure to 30°C is shown in Figure 3(*a*).

(*f*) *Lower Temperature Effects on Rate of Nodule Appearance*

Below 22°C, there was a decline in the rate of nodule appearance [Fig. 1(*b*)], with the cultivars examined varying in their response to the lower root temperatures. At 22°C, Yarloop achieved the highest rate of nodule appearance (4–5 nodules/day)

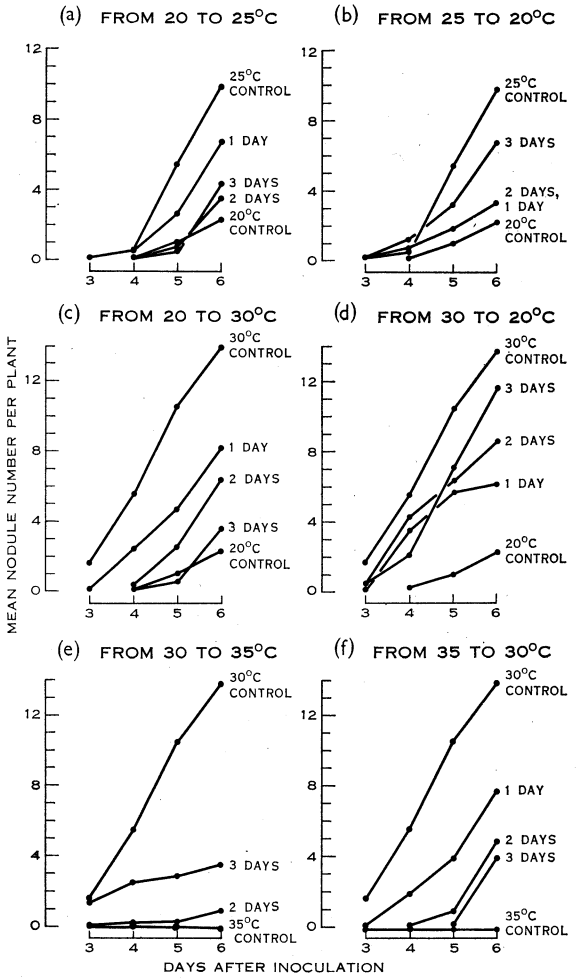

Fig. 2.—Increase in mean nodule number per plant for plants grown at 20, 25, 30, and 35°C root temperatures, either at the one temperature throughout the period of observation (controls), or transferred from one temperature to another after the periods indicated. Cultivar Tallarook plants inoculated with strain TA1 on day "0".

and Mount Barker and Dwalganup the lowest rate (1–2 nodules/day). At 7°C, the rate of nodule appearance for Yarloop, Tallarook, and Mount Barker was greatly reduced from that observed at 22°C, with the values being similar for each cultivar. The rate for Dwalganup was little reduced from that achieved at 22°C. At any root temperature, the rate of nodule appearance was similar for plants inoculated with strain TA1 or strain NA30.

The rate of nodule appearance on plants transferred from 22 to 17, 12, and 7°C root temperatures 2 days after inoculation was not greater than that of the control plants grown continuously at these latter temperatures (Fig. 4), although displaced in time due to retarding effects on the formation of individual nodules. In most instances the rate of nodule appearance on the lower temperature controls was slightly higher than that on the transferred plants, so that the final numbers of nodules for the two treatments were not very different. These effects were evident on both cultivars examined, even though they differed in the rate of nodule formation, especially at the lower temperatures. This could indicate that a number of otherwise successful infections on the transferred plants were unable to proceed to nodule formation due to temperature affecting some factor other than infection or nodule initiation.

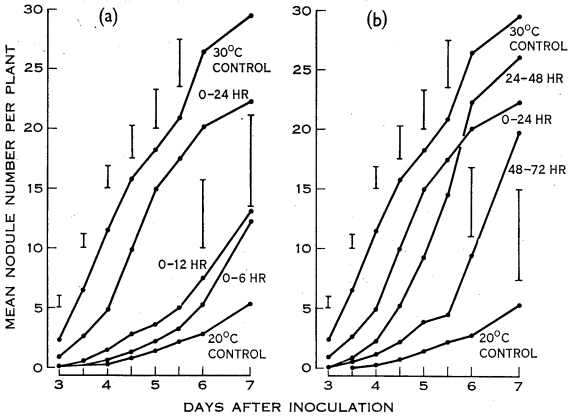

Fig. 3.—Increase in mean nodule number per plant for plants normally growing at 20 or 30°C root temperature, or transferred from 20 to 30°C for different times (hr) at different intervals after inoculation. Cultivar Tallarook, inoculated with strain TA1. $P = 0 \cdot 05$ confidence limits are shown on the figure.

Support for this suggestion was obtained in another experiment. The infection of plants normally growing at 22°C was progressively retarded by exposing groups of these plants to 12°C for 24, 48, and 72 hr. On return to 22°C, the rates of nodule appearance for all treatments were similar to each other, and to that of the 22°C controls (Fig. 5). In the second part of the experiment, plants normally growing at 12°C were exposed to 22°C for 24, 48, and 72 hr. On return to 12°C, there were large differences in the number of nodules formed by the ninth day after inoculation (Fig. 5). However, these differences between treatments were reduced by the 12th day, due to higher rates of nodule appearance on the 12°C controls, and on the plants given a short exposure to 22°C. The plants exposed to 22°C for longer periods were likely to have more potentially successful infections than the other plants at 12°C, and these results would suggest that some factor other than infection was retarding the rate of nodule appearance.

(g) *Root Temperature Effects on Root Development*

The extension of the primary root was stimulated by root temperatures above 25°C (Table 1), even where nodule formation was retarded (33°C) or inhibited (36°C). There was little difference between the lengths of the primary roots of Mount Barker grown at 12 and 22°C, but they were relatively short (5 cm 6 days after

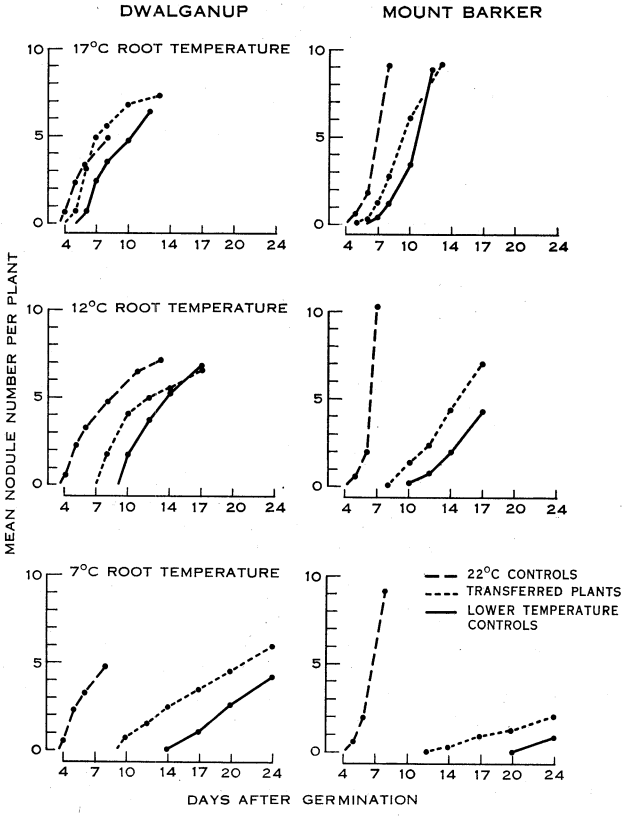

Fig. 4.—Progressive increase in mean nodule number per plant for cultivars Mount Barker and Dwalganup growing at 22, 17, 12, and 7°C root temperatures continuously, or transferred from 22°C to the other temperatures 2 days after inoculation. The shoot temperature regimes were 22/18°C for the 22 and 17°C root temperature treatments, and 15/10°C for the 12 and 7°C root temperature treatments. The light period was 16 hr/day. The plants were inoculated with strain TA1. 20 replicates.

germination) and they grew slowly (1–2 cm in the following 4 days). Observations on the roots of other cultivars indicated that the primary root extension of Tallarook and Yarloop was severely retarded at 12 and 7°C.

With Mount Barker, lateral root initiation was retarded at 12°C root temperature relative to that at 22°C. With exposure to 12°C for different periods, marked differences were found between the treatments 4 days after inoculation, but these differences were not evident 4 days later. Similarly, the exposure to 22°C of

plants normally growing at 12°C root temperature rapidly stimulated lateral root formation. There was an indication that the stimulation was greater with the shorter exposures when they were commenced 2–3 days after inoculation.

(*h*) *Shoot Temperature Effects on Time to First Visible Nodule*

In a number of experiments for which the data is not presented, shoot temperatures in the range 18–25°C (with appropriate root temperatures) had no effect on the time to first visible nodule, or on the rate of nodule appearance. However, with a root temperature of 12°C, a difference in the shoot temperature regime of 3–4°C (18/14°C; cf. 15/10°C) increased the time to first visible nodule by 1–2 days for all cultivars (Table 2). There was no corresponding effect on the rate of nodule appearance [Fig. 1(*b*)].

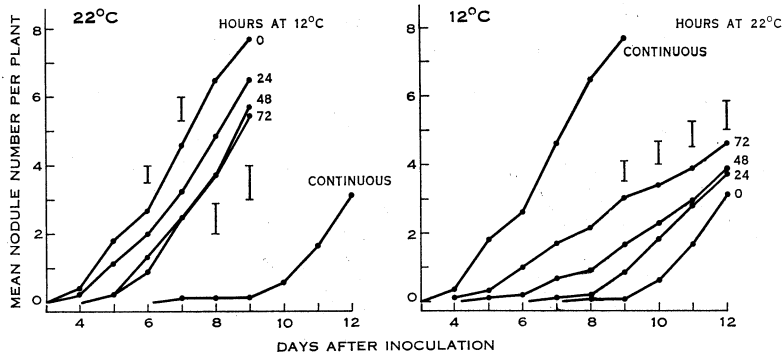

Fig. 5.—Progressive increase in nodule number per plant for cv. Mount Barker inoculated with strain TA1, and growing at 22 and 12°C root temperature. The controls were held at these temperatures continuously through the experiment. The values for the other treatments are the mean of four subtreatments, varying in the time after inoculation (0, 1, 2, and 3 days) before exposure to the alternate temperature for the periods indicated. The shoot temperature regime was 18/14°C. 20 replicates per subtreatment.

(*i*) *Effects of Diurnal Root Temperature Fluctuation on Nodule Formation*

The transfer of plants between all pairs of four root temperatures (20, 25, 30, and 35°C) showed a strong stimulatory effect of higher root temperatures on time to first visible nodule and the number of nodules formed 6 days after inoculation. The exposure to the higher root temperature during the light period was similar in its effect to such exposure during the dark period. None of the plants in any treatment involving 35°C root temperature formed nodules during the course of the experiment.

(*j*) *Light Period Effects on Nodule Formation*

There was a strong effect of the length of the daily light period on the time to first visible nodule. At 30°C root temperature, the mean values for the 4-, 8-, 12-, 16-, and 20-hr light treatments were 5·4, 4·0, 3·7, 3·2, and 3·3 days respectively. At 25°C, the values were slightly higher, but the pattern of the results was similar except that only 9 of the 15 plants in the 4-hr treatment had nodulated 7 days after inoculation.

IV. Discussion

The earliest-formed nodules were recorded 2 days after inoculation, but this was confined to four observations in more than 2000. Each of the four plants showing this early nodule formation were inoculated 4 days after germination. At 30°C, and to a lesser extent at 25°C, a number of plants were nodulated 3 days after inoculation, and from their size it would seem that the majority of the nodules formed during the latter part of the third day. The upper limit to nodule formation was 33°C (Tables 1 and 3) although there was some evidence that either, but not both, infection and nodule initiation could proceed slowly up to 35°C. The lower temperature limit to nodule formation was in the vicinity of 7°C, at which temperature many plants took 20 days or longer to nodulate, or failed to nodulate at all [Fig. 1(*a*)]. With each successive fall of 5°C below 22°C, there was a disproportionate delay in initial nodule formation (Table 2), and a marked fall in the rate of nodule appearance [Fig. 1(*b*)]. The observed effects of lower temperatures on the time to first visible nodule are in general accord with those of Hely and Williams (1964), and extend the available information to other cultivars of *T. subterraneum*. The rate of nodule appearance data seems to differ from the observations on *Medicago tribuloides* and *Vicia atropurpurea* (Pate 1962) that extensive and, for some bacterial strains, maximum numbers of nodules could be found on plants growing at a constant shoot and root temperature of 6°C.

The results of the transfer experiments show that a root temperature of 30°C stimulates the development of individual infections of root hairs, and the number of successful infections that are made. This is not an effect on the plant alone as the stimulation is found only after post-inoculation exposure to 30°C, a result which confirms the finding of Barrios, Raggio, and Raggio (1963) with excised roots of *Phaseolus vulgaris*. Nor is it likely to be due to stimulation of bacterial multiplication in the medium. Fahraeus (1957) observed infected root hairs on *Trifolium repens* as early as 2 days after inoculation, although nodules were not observed until 6–8 days later. As nodulation occurred more rapidly in the experiments reported here, it is logical to assume that infection was initiated and the infection thread had commenced to grow down the root hairs within 24 hr of inoculation. Microscopic observation of the root hairs of plants growing at 20 and 30°C failed to reveal any infection threads until nodules had appeared, indicating the difficulty of using this technique for precise studies of temperature effects on the infection of this species. However, extensive root hair curling was observed on the 30°C plants 12 hr after inoculation, whereas such curling did not appear until 12 hr later on plants at 20°C. While the curling of root hairs and infection may not be related causally (Nutman 1959; Munns 1967), the presence of curled hairs indicates that some activity associated with infection is proceeding.

The stimulatory effect of 30°C on individual infections is cumulative, with a 24-hr exposure to 30°C promoting earlier initial nodulation than a 12- or 6-hr exposure. Furthermore, a 12-hr exposure 1 day after inoculation is as stimulatory as one given immediately after inoculation. These observations suggest that both the initiation of infection, and the growth of the infection thread, is stimulated by the higher root temperature. Apart from affecting individual infections, it is evident that the higher temperatures also increase the number of successful infections made.

Higher temperatures also stimulate the post-infection stage of nodule formation (nodule initiation and development) but the effect is not as great as that on the infection stage.

While infection occurs rapidly, and abundantly, at 33°C (Table 3), it is severely retarded above this temperature. The second stage of nodule formation is progressively retarded with increases in temperature above 30°C. These results differ from those of Barrios, Raggio, and Raggio (1963) who considered that the root hair infection of excised roots of *P. vulgaris* was retarded above 25°C, whereas other stages of nodule development were little affected by temperatures up to 30°C. The differences may be due to the use of different species, or to the use of different techniques of root culture.

TABLE 7

ESTIMATED TIME FOR THE COMPLETION OF THE VARIOUS MAIN STAGES IN THE FORMATION OF NODULES UNDER DIFFERENT ROOT TEMPERATURE CONDITIONS

Root Temp. (°C)	Time (days) for Completion of Following Stages:		
	Infection	Nodule Initiation	Nodule Development
33	1–1·5	3·5–4	—
30	1–1·5	1·5–2·5	2–3
22	2–2·5	2·5–3	2–4
17	3–4	3·5–4·5	3–6
12	4·5–6	5–7	5–8
7	>9	>10	Very slow

Root temperatures below 22°C retard the formation of individual nodules through an effect on the infection stage (Table 5) and a greater effect on the second stage (nodule initiation) (Table 6). A more difficult problem to solve is the effect of lower root temperatures on the number of potentially successful infections made and the number of nodule meristems which can develop. The results for plants normally growing at 22°C, and exposed to 12°C for varying periods (Fig. 5) suggest that the number of potentially successful infections made is not greatly affected by the lower root temperature, although the rate of development of individual infections is retarded. At 12°C, the overall rate of nodule formation is lower for plants exposed to 22°C for longer periods than it is for the 12°C controls (0·5 nodules/day; cf. 1·0 nodules/day). This suggests that the lower temperatures are not reducing the number of nodule meristems which are activated and develop into nodules. The fact that nodule numbers tend to equalize for the various treatments (Figs. 4 and 5) suggests that some overriding factor is controlling nodule formation. One possibility is that the activity of previously formed nodule meristems, which continue to be active during the formation of later nodules, retards the formation of new nodules

(Nutman 1952, 1953). For this explanation to be valid, it must be assumed that temperature exerts an influence on this effect, as plants growing at higher root temperatures are capable of considerably higher rates of nodule formation than those found at lower temperatures. An alternative explanation is that the supply of essential growth materials, such as carbohydrates and amino acids or both, is restricted at the lower temperatures so that the number of nodules formed is limited. Although Raggio, Raggio, and Torrey (1965) did not examine the effect of temperature on the nodule formation on excised roots of *P. vulgaris* provided with different levels of sucrose and nitrate, their results indicate that higher levels of sucrose, in the presence of adequate nitrate, stimulate nodule formation. Lower root temperatures also retard the development of lateral roots and it is possible that the factor limiting nodule meristem activation and development is also affecting lateral root development.

Although it was not possible to observe the stages of nodule formation microscopically, an examination of the results suggests that certain arbitrary decisions may be made regarding the time taken for the stages to be achieved under various root temperature conditions. These are summarized in Table 7. A range for each stage under each root temperature is given in order that cultivar differences, and effects of lower shoot temperatures, are taken into consideration.

In the current experiments, there were no differences between strains of *Rh. trifolii* for time to first visible nodule, or rate of nodule appearance. This is not meant to imply that strains do not differ in the speed with which they form nodules, as other information (Gibson, unpublished data) shows that such differences do exist. The interesting feature of the results in these experiments is that two of the strains (NA30 and CC17) fail to fix sufficient atmospheric nitrogen for normal plant growth when plants nodulated by them are exposed to 30°C root temperature (Gibson 1965), although at lower root temperatures, the symbiosis formed is classed as effective. Despite this difference in nitrogen-fixing ability, in comparison with that of strain TA1, there is no effect of the higher temperatures on their ability to form nodules.

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