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Growth of *Trifolium subterraneum* L. Selected for Sparse and Abundant Nodulation as Affected by Root Temperature and *Rhizobium* Strain

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ABSTRACT

Root temperature greatly affected plant growth whether or not plants depended on symbiotic nitrogen fixation. The two plant selections responded differently to the three strains of *Rhizobium* and this response was differentially affected by root temperature.

Plant yield was significantly decreased by each fall of 4 °C in temperature from 19 to 7 °C by amounts that depended both on the host and *Rhizobium* strain. Symbiosis with strain TA1, originally isolated from a cold environment, was most tolerant of a root temperature of 11 °C; TA1 produced as much or more plant material of the abundantly nodulating host in 40 days growth at 7 and 11 °C as did the uninoculated plants given KNO_3.

Root temperature affected the number, rate of formation, and distribution of nodules on the root system. At 7 °C fewer nodules formed than between 11 and 19 °C. At 7 °C nodules did not form on secondary roots by 40 days but at 11 °C the secondary roots nodulated rapidly between 30 and 40 days. Nodule formation at 19 °C was almost completed at 20 days, when secondary root nodules accounted for 60 per cent of the total. Within the range 15 to 19 °C, at which the original selections for sparse and abundant nodulation were made, plants nodulated true to selection, but not at 11 °C. At 7 and 11 °C plants nodulated with TA1 yielded more with increasing number of nodules.

INTRODUCTION

Root temperature greatly influences both nodule formation and nitrogen-fixing efficiency. Its effect on the nodulation and growth of *Trifolium subterraneum* was first investigated by Meyer and Anderson (1959) who found a marked reduction of nitrogen fixation at 30 °C as compared with 20 °C. Sub-optimum temperature delayed nodule formation, decreased nodule number and N_2-fixation, and favoured N retention by the root (Gibson, 1963, 1965, 1966, 1967*a* and *b*). The effects were also dependent on *Rhizobium* strain (Gibson, 1963).

Nutman (1967) found that the growth of subterranean clover and the amount of N_2 fixed was independent of nodule number, but this relationship is not general because Hely, Costin, and Wimbush (1964) showed that the dry weight of *Trifolium* spp. grown with roots kept cold depended on nodule number.

This paper reports on the growth and nitrogen fixation by nodulated subterranean

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clover plants selected for sparse and abundant nodulation when grown at root temperatures at and below the optimum for their symbiosis.

MATERIALS AND METHODS

Plant culture. Two F_5 lines of subterranean clover selected for sparse or abundant nodule-forming characters from crosses between the cultivars Northam 2nd Early and Mount Barker (Nutman, 1967) were grown with *R. trifolii* strains 0403, isolated in England, SU297 ioslated from northern New South Wales, and TA1 isolated from Tasmania, Australia. Seed was graded to a uniform size to eliminate variation in nodule formation from seed size (Nutman, 1967). Seed was surface sterilized, incubated on 1 per cent plain agar plates at 4 °C for 24 h, and then at 25 °C for 36 h.

Seedlings with 10-mm radicles were inoculated with rhizobia and planted one plant per tube on 8 ml N-free agar slopes (Jensen, 1942) in 150×18 mm test-tubes loosely stoppered with cotton wool (Thornton, 1930).

Uninoculated seedlings were also grown for comparison, some with 0·05 per cent KNO_3 providing *c.* 0·6 mg N per tube; these tubes were also watered with 0·05 per cent KNO_3. From the fourth day after planting all inoculated plants were examined daily for nodulation with a ×10 lens.

Environmental conditions for plant growth. Root temperatures of 7, 11, 15, or 19±0·5 °C were maintained with controlled water tanks. Warm-white fluorescent tubes supplemented by incandescent bulbs above each tank gave a light intensity of 19 000 lx at seedling level The air in the plant chamber was maintained at *c.* 20 °C during the day (16 h), and *c.* 15 °C at night by circulating fans.

Dry weight and nitrogen determination. Plants were dried at 90 °C for a minimum period 6 h. Total nitrogen was determined separately on tops and roots by micro-Kjeldahl digestion, distillation, and titration (Bremner, 1965). Nitrogen gained from fixation was defined as the increase in nitrogen as compared with that in the seed.

TABLE 1. *The effect of root temperature and* Rhizobium *strain on the time for initial nodule formation on sparse and abundant selections of* T. subterraneum.

Root temp. °C	Mean time to form initial nodule (days)					
	TA1	SU297	0403	Sparse	Abundant	Mean
7	15·8*	14·7	18·0	14·6†	17·8	16·2
11	11·8	11·3	10·7	11·5	11·1	11·3
15	9·5	8·7	8·6	8·9	9·0	9·0
19	6·9	6·2	7·3	6·5	7·1	6·8
Mean	11·0	10·2	11·2	10·4	11·2	

Factor	S.E. difference between means
Temp.	0·19
Strain	0·17
Plant	0·14
T×S	0·33
T×P	0·27

* Mean for 48 replicate plants. † Mean for 144 replicate plants.

RESULTS

Constant root temperatures

Nodule formation. Table 1 shows the time taken by strains TA1, SU297, and 0403 to form nodules on both host selections at 7, 11, 15, and 19 °C. The mean time for nodule formation increased progressively as root temperature fell within the range of 19 to 7 °C. Strain TA1 took consistently longer to form nodules than SU297 but the time required by 0403, although not different from SU297 within the range of 11 to 19 °C, increased greatly at 7 °C .The time for nodule formation of each host differed only at 7 °C; the sparse line nodulated earlier with each strain and with TA1 the differential was 5 days.

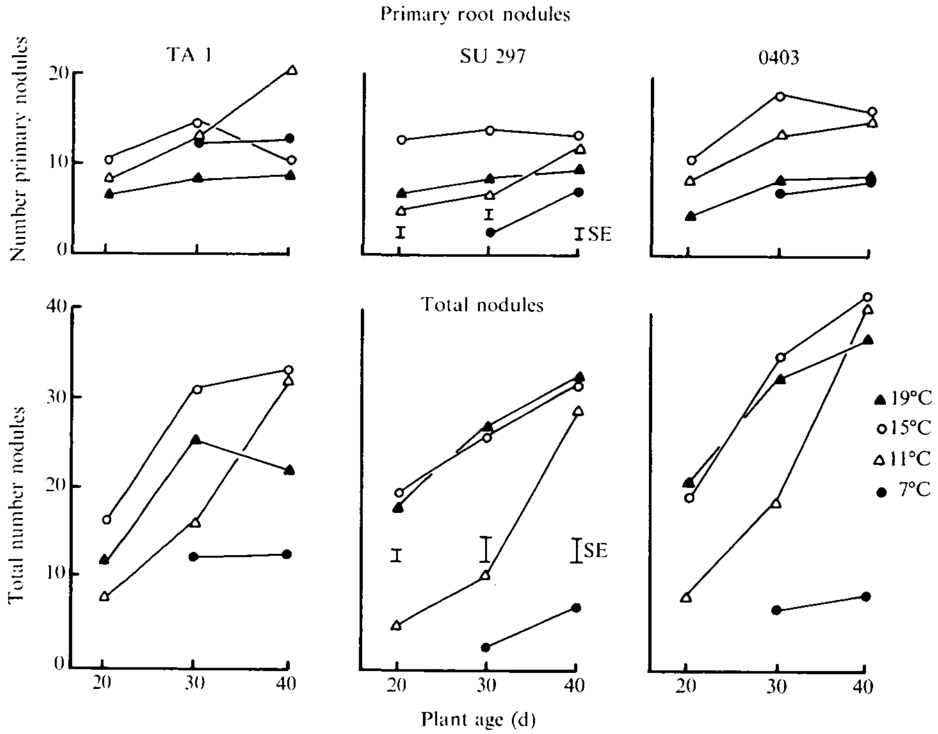

FIG. 1. Effect of root temperature and *Rhizobium* strains TA1, SU297, and 0403 on the number of nodules formed on the primary root and on the whole root system (total no.) of *T. subterraneum*.

Nodule number. Root temperature significantly affected the total number of nodules formed and their distribution on the root system. Nodules formed very slowly at 7 °C. The temperatures for maximum nodule production by each strain depended on plant age (Fig. 1). For example, at 11 °C, nodule formation was slow initially but after 20 days increased rapidly until at 40 days nodules were not significantly fewer than at temperatures that favoured early rapid nodule formation.

At 15 and 19 °C nodulation by strain TA1 was almost complete at 30 days, but with SU297 and 0403 nodulation on secondary roots continued at these temperatures and by 40 days 0403 had formed significantly more nodules than the other two strains.

Nodules were restricted to the primary root at 7 °C and in the range 11 to 15 °C the proportion of nodules on the secondary root increased with plant age except on sparse plants inoculated with TA1 at 11 °C which continued to form nodules on primary roots until 40 days. For strains SU297 and 0403, most nodules formed on secondary roots at 11 °C; the proportion of primary nodules fell from 100 per cent at 20 days to 48 per cent after 40 days, whereas at 19 °C the proportion of primary nodules remained fairly constant at about 30 per cent.

TABLE 2. *The effect of root temperature on the mean* number of nodules formed on hosts selected for sparse and abundant nodule formation*

Root temp. °C	Sparse						Abundant					
	20 days		30 days		40 days		20 days		30 days		40 days	
	Prim.	Total	Prim.	Total	Prim.	Total	Prim.	Total	Prim.	Total	Prim.	Total
7	..	..	5·6	5·6	7·6	7·6	..	..	9·3	9·3	11·4	11·4
11	5·8	5·8	8·5	15·9	15·3	36·5	8·9	8·9	14·7	15·0	17·1	31·5
15	7·6	17·3	15·0	34·5	13·8	31·8	15·6	19·5	16·5	28·3	13·4	39·5
19	6·9	18·0	8·4	26·5	8·1	26·4	5·2	16·7	9·9	30·3	10·7	35·1
Mean	6·8	13·7	9·4	20·6	11·2	25·6	9·9	15·0	12·6	20·7	13·1	29·4
S.E. mean	0·47	0·86	0·47	1·04	0·58	1·15	0·47	0·86	0·47	1·04	0·58	1·15

S.E. host × temp.

	20 days	30 days	40 days
Primary	0·81	0·95	2·31
Total	1·49	2·08	3·53

*8 Replicates.

TABLE 3. *Influence of root temperature,* Rhizobium *strain and host on yield (mg/plant) of* T. subterraneum *at 40 days*

Root temp. °C	Sparse line TA1	SU297	0403	Host mean	Abundant line TA1	SU297	0403	Host mean	Temp. mean	Bacterial strain TA1	SU297	0403	Uninoculated −N	+N
7	15·0	24·0	18·6	19·2	25·2	17·8	14·0	19·0	19·1	20·1	20·9	16·3	22·3	25·1
11	29·7	21·3	24·2	25·1	45·1	21·2	18·6	28·3	26·7	37·4	21·2	21·4	27·4	35·3
15	35·7	41·1	19·2	32·0	31·3	27·5	28·2	29·0	30·5	33·5	34·3	23·7	24·6	47·4
19	39·2	40·5	28·1	35·9	43·5	40·6	39·0	41·0	38·5	41·4	40·5	33·5	27·5	..
Mean	29·9	31·7	22·5	28·5	36·3	26·8	24·9	29·3	28·7	33·1	29·2	23·7	25·5	35·9

Factor	S.E. difference between means		
Temp.	0·95	T×S	1·64
Strain	0·82	T×H	1·34
Host	0·67	S×H	1·16
		T×S×H	3·3

The number of both primary and secondary root nodules were significantly influenced by the selection of plants for sparse and abundant nodule formation (Table 2). The total number of nodules was greater on the abundant than on the sparse selection at 40 days when grown at 15 and 19 °C but fewer at 11 °C.

Plant dry weight. Analyses of plant dry weight for eight replicates showed that root temperature influenced growth at all times of harvest, with the effects of strain increasing and of plant selection decreasing with time.

Table 3 shows the influence of root temperature, bacterial strain, and host selection on yield at 40 days. Except for plants inoculated with TA1 at 11 °C, only plants grown at 15 and 19 °C yielded more than the uninoculated N-free controls. During the 20- to 40-day period the increase in mean plant dry weight at 11 °C was only 67 per cent of that at 19 °C.

At 11 °C, TA1 was more effective on the abundant host than either SU297 or 0403 which produced less than 50 per cent of the dry weight produced by TA1. Strain response was influenced by the host plant; TA1 was more effective on the abundant than the sparse line, whereas SU297 reversed this response. The large

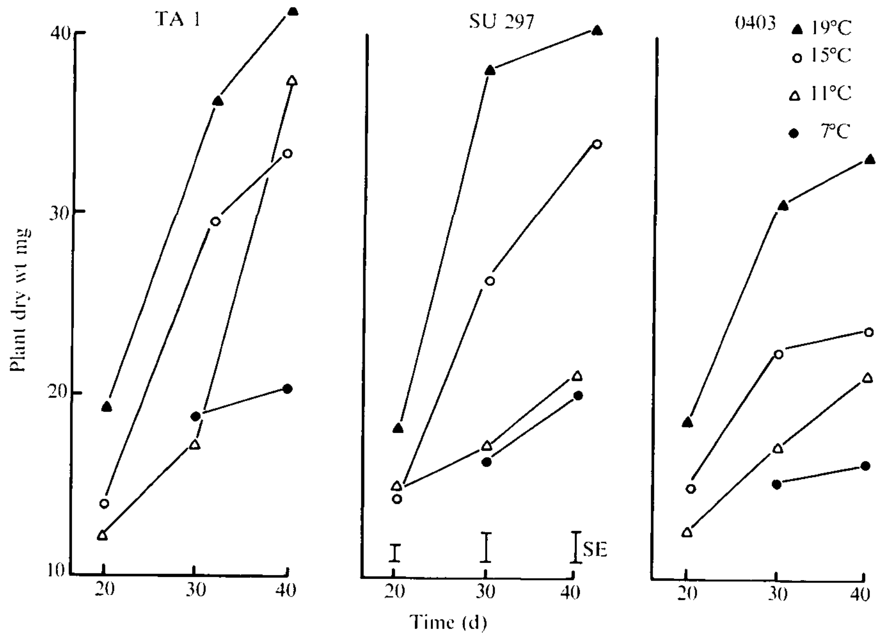

FIG. 2. Dry weights of *T. subterraneum* plants inoculated with *Rhizobium* strains TA1, SU297, and 0403 and grown at root temperatures of 7, 11, 15 and 19 °C for 20, 30, and 40 days.

yield of 45·1 mg by the abundant line, inoculated with TA1 and grown at 11 °C was outstanding. Strain 0403 only effectively nodulated the sparse line at 19 °C but the abundant line at both 15 and 19 °C.

Fig. 2 shows the effect of root temperature on the effectiveness of each strain. Except for TA1 at 11 °C, where once fixation started plant growth was rapidly stimulated, the relative effect of temperature was the same for each strain, i.e. plant yield diminished as root temperature decreased.

Table 4 shows the relationship between plant yield at 7 and 11 °C and nodule number for two *Rhizobium* strains. Yield was related to the number of nodules formed by TA1 but not by SU297.

Plant top/root dry-weight ratio was affected by temperature at 30 days and by temperature and strain at 40 days. At 30 days the top/root ratio fell with increasing root temperature but at 40 days top growth relative to root growth was largest at the two extremes of root temperature, regardless of bacterial strain.

Nitrogen content. Five replicate plants were analysed for N content. Table 5 shows the effect of root temperature, bacterial strain, and host selection on nitrogen gained by 40 days and Fig. 3 shows the increase in nitrogen at each harvest for each strain.

Root temperature determined the time fixation began but the amount fixed and the rate of fixation was affected by the interaction between temperature and strain. The accumulation of nitrogen was not linear. At 19 °C there was a rapid early fixation by all strains but this did not continue beyond 30 days. At this temperature strain 0403 fixed less nitrogen than TA1 and SU297.

TABLE 4. *The relationship between mean nodule number,* Rhizobium *strain and the mean dry weight of* T. subterraneum *grown at root temperatures of* 7 *and* 11 *°C*

Root temp. °C	Strain TA1				Strain SU297			
	Sparse		Abundant		Sparse		Abundant	
	Nodule no.	Dry weight mg	Nodule no.	Dry weight mg	Nodule no.	Dry weight mg	Nodule no.	Dry weight mg
7	11·3	15·0	14·5	25·2	3·3	24·0	11·3	17·8
11	24·3	29·7	40·0	45·1	37·4	21·9	20·5	21·2

TABLE 5. *The influence of root temperature,* Rhizobium *strain and host selection on the N content of plants less seed N (in mg) at* 40 *days*

Root temp. °C	Sparse line			Sparse × temp.	Abundant line			Abundant × temp.	Temp. mean	Bacterial strain			Per cent N in shoot
	TA1	SU297	0403		TA1	SU297	0403			TA1	SU297	0403	
7	0·00	0·18	0·11	0·10	0·27	0·00	0·05	0·11	0·11	0·13	0·09	0·08	65
11	0·80	0·27	0·15	0·40	1·71	0·30	0·15	0·72	0·56	1·26	0·29	0·15	63
15	1·04	1·23	0·13	0·77	0·89	0·71	0·71	0·77	0·77	0·97	0·97	0·42	68
19	0·96	1·18	0·68	0·94	1·11	0·90	0·84	0·95	0·94	1·04	1·04	0·76	80
Mean	0·70	0·77	0·27	0·55	1·00	0·48	0·44	0·64	0·60	0·85	0·60	0·38	

Factor	S.E. difference between means	Factor	S.E. difference between means
Temperature	0·05	T × S	0·09
Strain	0·05	T × H	0·08
Host	0·04	S × H	0·07
		T × S × H	0·18

The symbiosis with TA1 was more efficient than with SU297 at 11 °C but not at 15 or 19 °C. TA1 fixed more nitrogen in association with the abundant selection whereas SU297 fixed more with the sparse.

The significant interaction between temperature, strain, and host was caused by the large accumulation of N in the abundant selection nodulated by TA1 at 11 °C (1·71 mg N), and the response of both hosts nodulated with 0403 at 11, 15, and 19 °C. The results agree well with plant dry weight (Table 3) and show that 0403 fixes N_2 effectively at 15 and 19 °C with the abundant selection and only at 19 °C with the sparse selection.

Table 6 shows the distribution of nitrogen in the plant. When dependent on seed N the sparse selection incorporated more of its nitrogen in the top than did the abundant selection. The two extremes of temperature favoured nitrogen retention by the top. Supplying 0·05 per cent KNO_3 accentuated this difference

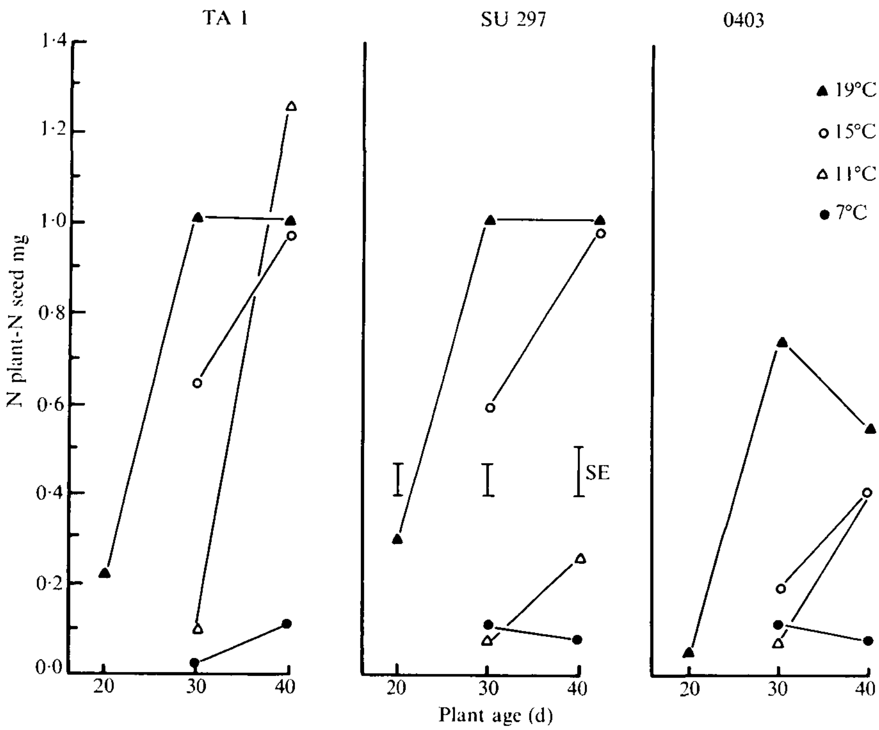

FIG. 3. Nitrogen contents of *T. subterraneum* plants inoculated with *Rhizobium* strain TA1, SU297, and 0403 and grown at root temperatures of 7, 11, 15 and 19 °C for 20, 30, and 40 days.

TABLE 6. *The distribution of N in 40 day old plants expressed as a ratio of N in tops: N in roots as affected by root temperature, host selection,* Rhizobium *strain and* KNO_3

Root temp. °C	Uninoculated $-KNO_3$			Uninoculated $+KNO_3$			Inoculated TA1		
	Sp.	Ab.	Temp. mean	Sp.	Ab.	Temp. mean	Sp.	Ab.	Temp. mean
7	1·98	1·85	1·91	4·74	3·36	4·05	1·33	1·33	1·33
11	1·72	1·31	1·52	4·58	3·88	4·23	2·50	1·46	1·98
15	1·45	1·05	1·25	6·76	3·41	5·08	2·71	2·17	2·44
19	2·44	1·46	1·95	6·77*	6·21*	6·49*	5·73	4·32	5·03
Mean	1·90	1·42		5·36†	3·55†		3·05	2·59	
S.E. difference between means	0·096		0·135	0·65†		0·80†	1·17		0·587

* Plants harvested at 30 days.
† Mean and S.E. applies to temperature range 7–15 °C only

between hosts. For nodulated plants nitrogen translocation to the tops was favoured by increase in temperature, and particularly at 19 °C.

Transfer from a sub-optimum to an optimum root temperature

Plants of the sparse selection inoculated with strain TA1 were grown at a root temperature of 7 °C for 9, 16, 23, or 30 days before being transferred to a root temperature of 19 °C.

Number of nodules. Table 7 shows that the duration of exposure to 7 °C affected the rate nodules formed but not the number finally formed. Plants rapidly formed nodules immediately after transfer, e.g. plants transferred from 7 °C at 16 days formed more nodules during the next 4 days than plants grown continuously at 7 °C for 20 days. When exposed to 7 °C for longer than 16 days subsequent nodulation was slower.

TABLE 7. *Effect of length of exposure to a root temperature of* 7 °*C before transfer to* 19 °*C on the number of nodules formed by* T. subterraneum

Length of exposure to 7 °C in days	Number nodules at harvest		
	20 days	30 days	40 days
0	10·0	19·0	23·0
9	16·7	18·7	24·0
16	11·7	13·7	20·7
20	4·3	. .	. .
23	. .	14·7	17·3
30	. .	10·7	16·3
40	. .	. .	13·0
S.E. difference between means	1·4	2·8	2·5

TABLE 8. *Effect of length of exposure to a root temperature of* 7 °*C before transfer to* 19 °*C on the dry weight and nitrogen content of plant tops*

Time exposed 70 °C, days	20 days		30 days		40 days	
	D.W. top mg	N content mg	D.W. top mg	N content mg	D.W. top mg	N content mg
0	15·1	0·41	31·6	1·14	43·3	1·72
9	10·8	0·29	23·6	0·97	34·5	1·34
16	11·1	0·18	11·2	0·61	31·0	1·08
20	8·8	0·21	. .	. .	. .	. .
23	. .	. .	5·7	0·42	31·1	1·09
30	. .	. .	8·7	0·44	15·0	0·40
40	. .	. .	. .	. .	9·7	0·10
S.E. difference between means	1·4	0·06	1·3	0·09	2·9	0·11

Dry weight and nitrogen content of tops

Table 8 shows the effect of duration of exposure to a root temperature of 7 °C on the dry weight and nitrogen content of plant tops at 20, 30, and 40 days. Plants exposed to 7 °C for 9 days before transfer to 19 °C showed no recovery at 20 days and despite rapid growth between 20 and 30 days, neither their dry weight nor their nitrogen content were as large as for plants grown continuously at 19 °C. Plants exposed to 7 °C for more than 9 days showed little or no recovery by 30 days but those exposed to 7 °C for 16 or 23 days grew rapidly during the period 30 to 40 days and at 40 days contained the same amount of N.

Root development

Table 9 shows the effect of exposure to 7 °C on the growth of roots and the ratio of dry weight top/root. Root development of plants exposed for 9 days before transfer recovered after 11 days at 19 °C; the root weight of such plants was approximately three times that of the plants remaining at 7 °C. The weight of roots at 40 days was unaffected by exposure to 7 °C for as long as 23 days.

Because roots responded to transfer faster than tops the transfer decreased the top/root dry weight ratio.

TABLE 9. *Effect of length of exposure to a root temperature of 7 °C before transfer to 19 °C on root wet weight and top:root ratio*

Length of exposure to 7 °C (d)	Root wet weight mg			Ratio top dry weight: root dry weight*		
	20 days	30 days	40 days	20 days	30 days	40 days
0	42·1	167·1	119·9	5·0	2·6	6·1
9	47·2	171·9	146·9	3·2	1·9	3·3
16	28·4	63·6	126·6	5·5	2·4	3·4
20	17·3	..	..	7·1	..	..
23	..	29·0	150·9	..	2·7	2·9
30	..	27·8	65·8	..	4·3	3·2
40	..	..	27·2	..	..	5·3
S.E. diff. between means	3·8	15·3	19·9	0·65	0·64	0·87

* Estimated from root wet weight by applying a pre-determined factor for moisture loss of wet weight = 14 × dry weight

DISCUSSION

Root temperature greatly affects the growth of subterranean clover whether dependent on symbiotic nitrogen or given combined nitrogen. The amount of yield reduction at low temperatures depended on plant host and *Rhizobium* strain. The abundant host nodulated by TA1 grew equally well at 7 °C as when supplied with KNO_3 and better at 11 °C, but with other strains it grew less well.

Both continuous exposure to cold root temperatures and exposure for a short period before transfer to a favourable temperature delayed the onset of N_2-fixation. When exposed to 7 °C for only 9 days before transfer, growth was still affected after 31 days at 19 °C.

Strain TA1, which was originally isolated from a colder environment than strain SU297, nodulated plants earlier and promoted more growth at 7 and 11 °C although the two strains produced the same number of nodules. SU297 formed a larger proportion of nodules on the secondary root system and this was related to less efficiency of fixation per nodule. During the period from nodulation to 30 days, bacterial strain did not affect plant growth at 7 and 11 °C so that plant establishment when seeds are sown in cold soil may depend on other factors such as seed size and soil nitrogen.

The sparse selection formed fewer nodules than the abundant except at 11 °C where it formed more. The actual difference at 19 °C in nodule number between the selections (34 per cent) was much smaller than in Nutman's experiments (300 per cent, Nutman, 1967), presumably because of differences in conditions under which the plants were grown. Selection for increased nodule number increased plant yield for some but not all combinations of temperature and strains. There was a clear correlation between yield and nodule number at 7 and 11 °C with strain TA1 but not with strain SU297. Strain TA1 was the strain observed by Hely, Costin, and Wimbush (1964) to promote growth in proportion to nodule number in cold field soils.

Plants grown in cotton wool-stoppered test-tubes are physically restricted affecting leaf expansion and nodulation (Nutman, 1945), so that differences between treatments may be smaller than when growth is unrestricted.

Root temperature influenced the relative development of plant roots and tops. Generally, cold roots favoured top growth, particularly in the early stages probably because of the disproportionate contribution of cotyledon nitrogen. Only the sparse plant nodulated by TA1 confirmed Gibson's (1966) results in which increasing root temperature increased shoot growth relative to root.

Root temperature affected the distribution of nitrogen in plants and cold favoured nitrogen retention by the root. This agrees with Gibson (1966) except that the percentage of N in the root in our plants was independent of bacterial strain. The range of root temperature we used was much smaller than in Gibson's studies, in which the plants were also nodulated and fixing N_2 before being transferred to different temperatures. In our experiments plant roots were grown throughout at the temperatures stated, and this affected the N_2-fixing pattern at 7 and 11 °C by delaying the onset of fixation and resulted in large differences in N_2 fixed at different temperatures during the limited 40 days growth period.

The results emphasize the need to select both *Rhizobium* strains and host lines to suit particular environmental conditions. Strains TA1 and SU297 are highly effective under optimum conditions but TA1 fixed 4 times as much nitrogen as SU297 at 11 °C. The selection for abundant nodule formation did not improve nitrogen fixation under optimum conditions but did improve fixation at 7 °C. This was not related to nodule number *per se*, but depended on the ability of this host to promote bacteroid differentiation at 7 °C (Roughley, 1969). Thus each association of host and *Rhizobium* needs testing under different conditions of root temperature as the efficiency of the symbiosis is not necessarily related to the ability to form nodules.

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