PHYSICAL ENVIRONMENT AND SYMBIOTIC NITROGEN FIXATION

VI.* NITROGEN RETENTION WITHIN THE NODULES OF *TRIFOLIUM SUBTERRANEUM* L.

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Summary

The effect of bacterial strain and root temperature on the retention of nitrogen in the root system of *Trifolium subterraneum* plants was re-examined. The root systems of plants nodulated by the moderately effective *Rhizobium trifolii* strain NA30 possessed a higher percentage nitrogen than those nodulated by the fully effective strain TA1, although the number of nodules formed by each strain was similar. The difference was due to a greater weight of nodule tissue on the NA30-nodulated plants, and also to a higher percentage nitrogen in the NA30 nodules; this latter effect was due to a higher concentration of non-protein nitrogen. The overall effect of these differences was to reduce the amount of nitrogen translocated to the shoots of the NA30 plants, in both absolute terms and as a proportion of the total amount of nitrogen fixed. Another difference between the two strains was the rate of nitrogen fixation per unit (dry weight or leghaemoglobin content) of nodule tissue.

At 8°C root temperature, a higher proportion of the nitrogen fixed was retained in the nodule system compared with that for plants grown at 15 and 22°C. At this lower temperature, nodule dry weight and nodule nitrogen constituted a higher proportion of total plant dry weight and total nitrogen than they did at the higher temperatures.

These findings are discussed in relation to their effect on the efficiency of nitrogen fixation by the two strains, and the effect on plant growth. It is proposed that the best *Rhizobium* symbionts are those strains that not only maintain high rates of fixation per unit dry weight of nodule tissue but also release the highest proportion of fixed nitrogen for use in general plant growth.

I. INTRODUCTION

Previous work showed that the percentage nitrogen in the root system of nodulated *Trifolium subterraneum* L. plants was a function of the strain of *Rhizobium trifolii* Dang. and of the root temperature (Gibson 1966*a*). The bacterial strain effect was considered to be associated with differences in the morphology of the roots rather than to differences in the nodules; nodule numbers and sizes were similar on plants nodulated by each of a number of strains of *R. trifolii*. Furthermore, a higher proportion of the total amount of nitrogen fixed appeared to be translocated to the shoots of the plants with the lower percentage nitrogen in the roots. It was also observed that a higher proportion of the fixed nitrogen was translocated to the shoots when the plants were grown at 18°C root temperature than when grown at 8°C root temperature.

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These observations have been re-examined to determine the factors responsible for the apparent retention of nitrogen in the root system. Two *R. trifolii* strains, one moderately effective and the other fully effective, were used to inoculate *T. subterraneum*, and the development of nodule tissue was followed at three root temperatures. The results are discussed in relation to the efficiency of the nodules as it affects the growth of nodulated plants under different root temperature conditions.

II. Materials and Methods

(a) *Biological Material*

The host plant was *T. subterraneum* cv. Tallarook (F. H. Brunning Pty. Ltd., Melbourne). The two strains of *R. trifolii* used were NA30, of moderate effectiveness with this cultivar, and the highly effective strain TA1 (Gibson 1961, 1965).

(b) *Plant Culture*

The plants were cultured with the roots growing on agar slopes within, and the shoots exposed outside, test tubes (Gibson 1963) and in controlled-environment cabinets in which root and shoot temperatures were controlled independently (Gibson 1965). The light intensity was 2000 f.c. The seedlings were inoculated 3 days after sowing and grown at 22°C root temperature with a 22/15°C shoot temperature regime based on a 16-hr daily light period. In one experiment, the plants were distributed to three root temperature treatments (8, 15, and 22°C) 11 days after inoculation, at which time all plants had commenced to fix atmospheric nitrogen; increase in total nitrogen above 0·40 mg (see Fig. 2) was due to symbiotic nitrogen fixation. After transfer, the shoot temperature regime was 18/11°C, based on a 16-hr daily light period. In the other experiment, the plants were grown at 22°C root temperature, 22/15°C shoot temperature, and a 16-hr daily light period.

(c) *Harvest Determinations*

Before the temperature treatments commenced, the plants inoculated with each strain were ranked according to size and leaf development. For the first experiment, the plants were then divided into 21 groups of 13; from each group of 13 plants one was harvested and the others transferred in subgroups of four to 8, 15, and 22°C root temperature. One plant from each four (i.e. 21 replicates per strain × temperature treatment) was harvested 4, 9, 14, and 18 days after transfer (15, 20, 25, and 29 days after inoculation). At each harvest, the plants were divided into shoots, roots, and nodules, dried at 80°C in a forced-draught oven, weighed, and the total nitrogen content determined.

For the other experiment, the plants in each strain treatment were ranked 24 days after inoculation, and divided into 12 groups of 4. Two plants from each group were harvested at that time, and the remainder 8 days later. On each occasion, the nodules from one plant in each group were dried, weighed, and both protein and non-protein nitrogen determined; from the nodules on the other plant, the total leghaemoglobin was extracted and determined.

(i) *Total Nitrogen.*—The material was digested in a concentrated H_2SO_4 (1 litre)–K_2SO_4 (100 g)–Se powder (1 g) mixture. For smaller amounts of nitrogen, the sample was distilled into 2% (w/v) H_3BO_3 and titrated with 0·01N HCl. For larger amounts, the nitrogen was determined colorimetrically using a Technicon Auto-Analyser (Williams and Twine 1967).

(ii) *Protein and Non-protein Nitrogen.*—The dried material was ground in 0·5 ml 10% (w/v) sodium tungstate and 4·0 ml 0·1N H_2SO_4. After centrifuging at 7000 r.p.m. for 20 min, the supernatant containing the non-protein nitrogen was decanted and the procedure repeated. The residue contained protein nitrogen. Both the protein and non-protein fractions were digested in the H_2SO_4 mixture, and total nitrogen in each determined by distillation and titration. The determinations were based on four replicate samples of nodules.

(iii) *Leghaemoglobin.*—After harvest, the nodules were stored at −10°C. The nodules (approximately 300 mg fresh weight) were crushed in 2·5 ml 0·1M phosphate buffer, centrifuged at 10,000 r.p.m. for 10 min, and the supernatant decanted. The procedure was repeated with 2·0 ml buffer. The supernatants were combined and the total volume determined. Pyridine haemochromogens were formed from the leghaemoglobin in the extract by mixing 1·0 ml of the combined supernatant fractions with 1·5 ml H_2O and 2·5 ml alkaline pyridine reagent (2·2M pyridine in 0·2M NaOH) (Paul, Theorell, and Åkeson 1953). Protohaem, and hence leghaemoglobin, concentrations were then calculated from the reduced−oxidized pyridine haemochromogen spectrum as described by Porra *et al.* (1967) and assuming a difference coefficient (i.e. extinction coefficient at 417 mμ minus that at 450 mμ) of 144, as found for an authentic sample of protohaem.

III. Experimental Details and Results

(*a*) *Effect of Root Temperature and Bacterial Strain*

To examine the effect of root temperature and bacterial strain on nitrogen retention in the root system, plants nodulated by strains TA1 and NA30 were grown at 8, 15, and 22°C root temperature from 11 days after inoculation and harvested at regular intervals. The shoot and root growth of the TA1-nodulated plants was

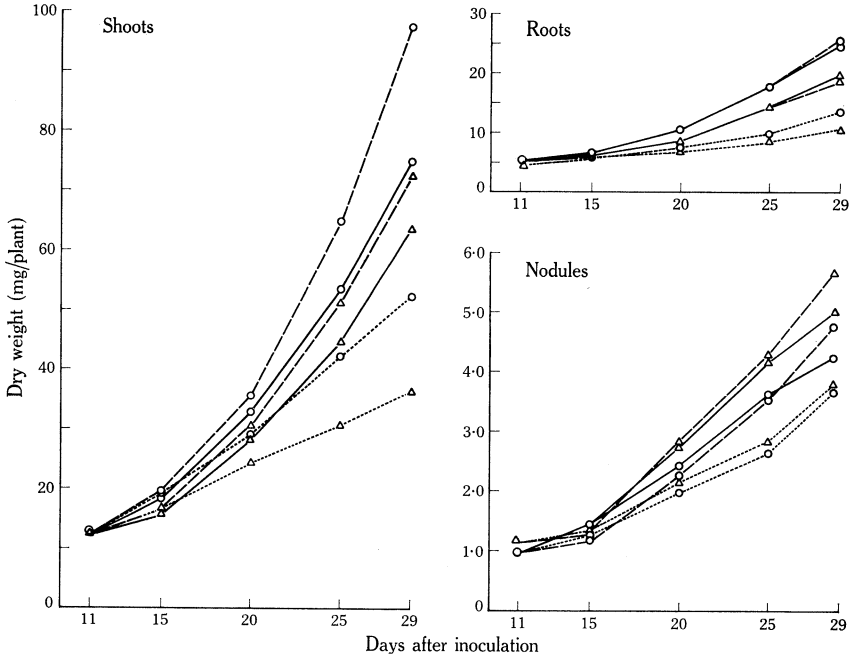

Fig. 1.—Dry weight increase in the shoots, roots, and nodules of plants nodulated by *R. trifolii* strain TA1 (○) or NA30 (△) and grown at 8 (- - -), 15 (——), or 22°C (— —) root temperature (21 replicates).

greater than that for the NA30-nodulated plants at all three root temperatures (Fig. 1) although the values for the plants inoculated by each strain were similar at the commencement of treatment. By contrast, total nodule weight for the NA30-nodulated plants exceeded that for the TA1-nodulated plants from 15 days after

inoculation, especially at 15 and 22°C. Whereas shoot growth was greater at 22 than at 15°C, both root growth and the increase in nodule weight was the same within a strain treatment at these temperatures. There was a slower increase in nodule weight at 8 than at 15°C, but during the 18 days of temperature treatment this amounted to a threefold increase, as compared with a twofold increase in root growth.

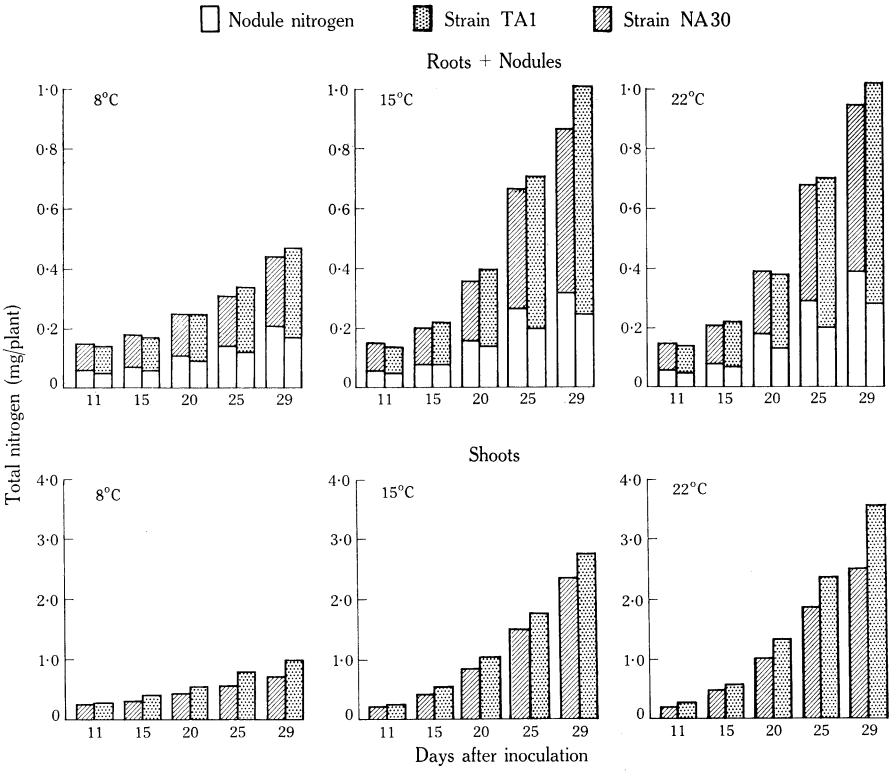

Fig. 2.—Total nitrogen in the shoots and root systems (roots+nodules) of plants nodulated by *R. trifolii* strain TA1 or NA30 and grown at 8, 15, or 22°C root temperature (21 replicates).

The total nitrogen content of the shoots, roots, and nodules (Fig. 2) confirmed, and in some cases accentuated, the differences between the strains and the temperature treatments evident in the dry weight data (Fig. 1). The difference in shoot nitrogen between the TA1 and NA30 plants increased with time at all three root temperatures (Fig. 2). The values for "root+nodule" nitrogen were similar for the two strains at any root temperature despite the large differences in shoot nitrogen, but there were marked differences in distribution. For strain NA30, nodule nitrogen constituted 37–48% of the root+nodule nitrogen, with the proportion being greater at the lower temperature and remaining constant with time; with strain TA1, nodule nitrogen constituted 25–36% of the total root+nodule nitrogen, and, apart from that at 8°C, the proportion decreased with time.

The effect of rhizobial strain on the percentage of nitrogen in the various plant parts is clearly evident in Figure 3. For the roots+nodules, the values for NA30 were consistently higher than those for TA1, except at 15°C where the difference was slight. Examination of the two components of the root system showed that the values for the TA1-nodulated roots were slightly, but consistently, higher than those for the NA30-nodulated roots. More importantly, however, the percentage nitrogen values for the NA30 nodules were markedly higher than those for the TA1 nodules. The values for TA1 nodules tended to remain constant (15 and 22°C) or fall (8°C) whereas those for the NA30 nodules rose (15 and 22°C) or remained constant (8°C).

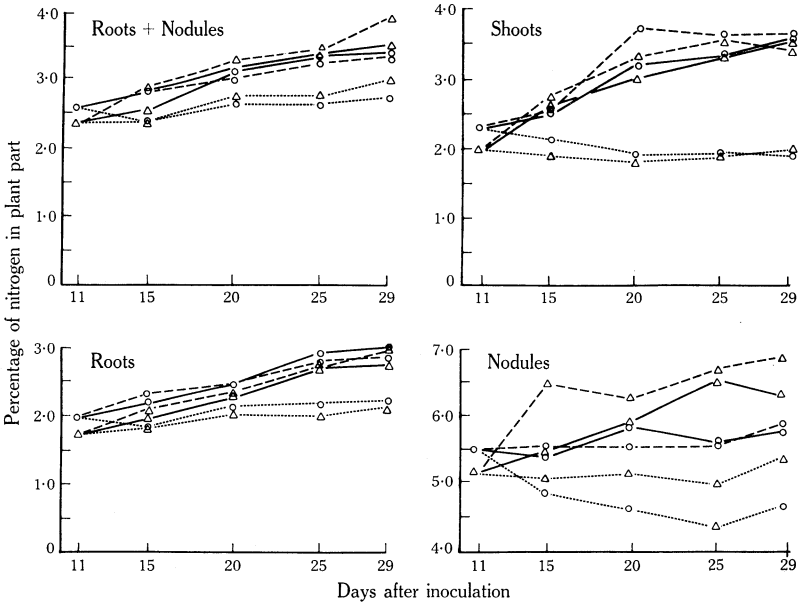

Fig. 3.—Percentage of nitrogen in shoots, roots, nodules, and roots+nodules of plants nodulated by *R. trifolii* strain TA1 (○) or NA30 (△) and grown at 8 (- - -), 15 (——), or 22°C (— —) root temperature (21 replicates).

In addition to an increase in total shoot nitrogen with an increase in root temperature (Fig. 2), there was an increase in the nitrogen distribution index (i.e. in the proportion of the total nitrogen increase for the plants that was found in the shoots) (Table 1). The lower distribution index at 8°C was associated with the retention of a higher proportion of the fixed nitrogen in the nodules rather than in the roots. It is noteworthy that, at all temperatures, the NA30 nodules retained a higher proportion of the total amount of nitrogen fixed than did the TA1 nodules.

There were marked differences between the strains, and at the different root temperatures, in the rates of nitrogen fixation per unit of nodule tissue (Table 2). The values for strain TA1 were approximately 50% higher than those for NA30 when based on nodule dry weight (except at 15°C, when it was only 35%), and somewhat greater when based on total nodule nitrogen. The rates were relatively constant with time within a strain×temperature treatment.

(*b*) *Fractionation of Nodule Nitrogen*

To determine whether there were differences in the protein and non-protein nitrogen fractions of the nodules formed by strains TA1 and NA30, plants nodulated by each strain were grown at 22°C root temperature and harvested 24 and 32 days

TABLE 1

PERCENTAGE OF TOTAL NITROGEN INCREASE DISTRIBUTED TO THE SHOOTS, ROOTS, AND NODULES OF *T. SUBTERRANEUM* PLANTS NODULATED BY *R. TRIFOLII* STRAINS TA1 AND NA30 AND GROWN AT THREE ROOT TEMPERATURES FOR 18 DAYS

Results are means from 21 replicates

Root Temperature (°C)	Bacterial Strain	% Total Nitrogen Increase Distributed to:		
		Shoots	Roots	Nodules
8	NA30	62	19	19
	TA1	69	20	11
15	NA30	74	17	9
	TA1	74	20	6
22	NA30	74	15	11
	TA1	78	16	6

TABLE 2

NITROGEN FIXATION RATES FOR *T. SUBTERRANEUM* PLANTS NODULATED BY *R. TRIFOLII* STRAINS TA1 AND NA30 AND GROWN AT THREE ROOT TEMPERATURES

Nitrogen fixation rates are expressed either as μg nitrogen/mg nodule dry weight/day, or as μg nitrogen/100 μg nodule nitrogen/day, and are means from 21 replicates

Root Temp. (°C)	Fixation Rate Basis	Rates for Following Periods (days) after Inoculation with: Strain NA30					Strain TA1				
		11–15	15–20	20–25	25–29	Mean	11–15	15–20	20–25	25–29	Mean
8	Dry weight	19	23	16	20	20	33	28	30	27	30
	Nitrogen	36	46	32	39	38	64	58	67	58	62
15	Dry weight	42	62	55	55	54	74	71	68	79	73
	Nitrogen	78	107	88	86	90	136	124	118	137	129
22	Dry weight	59	70	63	47	60	89	110	91	90	95
	Nitrogen	101	111	96	69	94	160	198	163	157	170

after inoculation. There was a greater dry weight of nodules on the NA30 plants than on the TA1 plants at each harvest (Table 3). The number of nodules present at the second harvest was similar for both strains (NA30: 33 per plant; TA1: 35 per plant). The NA30 nodules contained more nitrogen than the TA1 nodules, and the percentage nitrogen values were higher for the former strain. Fractionation of the nodule nitrogen showed that total protein nitrogen was higher for NA30 than for

TA1, although, when expressed as a percentage of nodule dry weight, the values for the two strains were similar. The major difference occurred in the non-protein nitrogen values; for strain NA30 these values were more than twice those for strain TA1. With NA30, non-protein nitrogen constituted 39% of the nodule nitrogen, whereas with TA1 it was 25%.

TABLE 3

NODULE DRY WEIGHT, TOTAL, PROTEIN, AND NON-PROTEIN NODULE NITROGEN, AND TOTAL LEGHAEMOGLOBIN FOR *T. SUBTERRANEUM* PLANTS NODULATED BY *R. TRIFOLII* STRAINS NA30 AND TA1 AND GROWN AT 22°C ROOT TEMPERATURE

Results are means from 12 replicates

Measurement	Unit of Measurement	Days after Inoculation with Strain NA30:		Days after Inoculation with Strain TA1:	
		24	32	24	32
Nodule dry weight	mg/plant	4·5	7·1	3·7	5·0
Nodule nitrogen	mg/plant	0·30	0·52	0·20	0·33
As % of dry weight		6·6	7·3	5·3	6·6
Nodule protein nitrogen	mg/plant	0·19	0·31	0·15	0·25
As % of dry weight		4·1	4·4	4·0	5·0
Nodule non-protein nitrogen	mg/plant	0·11	0·21	0·05	0·08
As % of dry weight		2·5	2·9	1·3	1·6
Total leghaemoglobin	n-moles/plant	0·63	0·86	0·64	0·90
Per milligram nodule dry weight	n-moles/mg	0·14	0·12	0·17	0·18
Total plant nitrogen	mg/plant	2·36	4·59	2·55	5·23
Total nitrogen fixed	mg/plant	2·23		2·68	
% transferred to shoots		58·2		65·5	
% transferred to roots		32·0		29·5	
% transferred to nodules		9·8		5·0	
Mean nodule dry weight	mg/plant	5·8		4·4	
Total nitrogen fixed	*	48		77	
Mean nodule leghaemoglobin content	n-moles/plant	0·75		0·77	
Total nitrogen fixed	†	373		435	

* As μg/mg nodule dry weight/day. † As μg/n-moles leghaemoglobin present/day.

At each harvest leghaemoglobin was determined (as pyridine haemochromogen) in all the nodules of a similar group of plants to those used for nodule-nitrogen fractionation. Total leghaemoglobin per plant was the same for the two strains at each harvest. The increase between harvests (39%) was proportionately less than either the increase in nodule dry weight (48%) or total nodule nitrogen (65%).

IV. DISCUSSION

(*a*) *Effect of Bacterial Strain*

While the present results confirmed that there were differences in the percentage nitrogen values for the root systems of plants nodulated by different strains of *R. trifolii*, they also showed that the effect was associated with the nodules and not, as

previously considered (Gibson 1966*a*), with morphological differences in the roots. The difference was due to two factors. Firstly, the NA30 plants produced a greater dry weight of nodule tissue than the TA1 plants (Fig. 1), in both absolute terms and, more importantly, as a proportion of the entire root system. Secondly, the NA30 nodules possessed a higher percentage nitrogen than the TA1 nodules (Fig. 3). Similar observations were made in a pot experiment using strain TA1 and a strain of intermediate effectiveness, CC10 (Simpson and Gibson, unpublished data); again the higher percentage nitrogen values were associated with the nodules, and root systems, of the plants nodulated by the less effective strain.

Because NA30 nodules contain a lower proportion of infected central tissue than TA1 nodules (65% cf. 95–100%—Bergersen 1961, and unpublished data), it was expected that the percentage nitrogen levels in the nodules formed by strain NA30 would be lower than those formed by TA1. That the reverse was found was due to the high non-protein nitrogen values for NA30 nodules (Table 3); percentage protein nitrogen values were similar for both strains. The nature of this non-protein nitrogen, and the reason for its retention within the nodules, remain to be determined. The difference between the percentage nitrogen values for NA30 and TA1 nodules increased with time (Fig. 3). This divergence occurred from an early stage in the observations, indicating that the higher nitrogen levels were not necessarily a result of nodule breakdown occurring as the nodules aged.

(*b*) *Effect of Root Temperature*

At 8°C root temperature, a higher proportion of the fixed nitrogen was retained in the root system, and particularly in the nodules (Table 1), with a consequent reduction in the proportion translocated to the shoots. This may have been due to a reduced ability of the plants to translocate the products of nitrogen fixation from the roots at lower temperatures. Alternatively it may have been related to the development of a proportionately higher amount of nodule tissue; for example, nodule tissue constituted 8·3 and 5·3% of total plant dry weight gain at 8°C in NA30 and TA1 plants respectively, whereas at 15 and 22°C nodule tissue constituted 5·6 and 3·7% of the total dry weight gain respectively. This result suggests that the effect of lower temperatures in reducing the nitrogen fixation rate per unit of nodule tissue was compensated for by an increase in the amount of such tissue. A similar observation has been made for plants growing at 12 and 22°C temperature (Davidson and Gibson, unpublished data).

The lower percentage nitrogen levels in the nodules at 8°C root temperature (Fig. 3) may have been due to a slower development of bacteroid tissue, relative to host tissue, in the nodules, and also to an accumulation of starch. Both of these possibilities were observed in nodules on *Vigna sinensis* Endl. ex Hassk. grown at 21°C, a suboptimal temperature for this species (Dart and Mercer 1965).

(*c*) *General Considerations*

By definition, the most effective symbiotic combinations are those that fix the greatest amount of atmospheric nitrogen. However, for a true assessment of any symbiotic combination, the efficiency of nitrogen fixation should be considered

(Gibson 1966*b*). That is, high rates of nitrogen fixation per unit of nodule tissue should be maintained, in addition to which a high proportion of the fixed nitrogen should be available for plant growth.

In considering effectiveness, the NA30 plants fixed 76–86% of the amount of nitrogen fixed by the TA1 plants (Fig. 2 and Table 3). In considering efficiency, nitrogen fixation per unit of nodule tissue was lower for NA30 than for TA1 (Tables 2 and 3), in addition to which the nitrogen distribution index for NA30 plants was less than that for TA1 plants (Table 1). The lower efficiency of the NA30 plants may be traced to three causes. Firstly, there was a lower fixation rate per unit of nodule tissue. Secondly, there was a greater production of nodule tissue, and this in turn utilized nitrogen that would otherwise be used for host-plant growth. Thirdly, non-protein nitrogen accumulated in the NA30 nodules.

Relative to strain TA1, nitrogen fixation by strain NA30 was most efficient at 15°C root temperature. At 15°C, the lowest proportion of fixed nitrogen was retained in the NA30 nodules, and the relative growth rate (0·087 mg/mg/day) was nearer to that of the TA1 plants (0·098 mg/mg/day) than at 8 or 22°C. For strain TA1, the highest efficiency was achieved at 22°C, as indicated by the high rates of fixation (Fig. 2) and the high nitrogen distribution index (Table 1).

A continuing problem in legume nodulation studies is the search for a reliable basis on which to express rates of nitrogen fixation in order that such values relate to active nodule tissue. Virtanen (1955) considers that leghaemoglobin concentration in nodules is a sound index of nitrogen-fixing activity, although Bergersen (1961) suggests that it is more appropriately an index of the concentration of bacteroid tissue. These conclusions are not necessarily incompatible, and a logical extension from them is that the total leghaemoglobin in the nodule system of a plant is a guide to the total nitrogen-fixing activity of that plant. However, in this study, total leghaemoglobin in the NA30 and TA1 nodules was the same but the rates of fixation per unit of leghaemoglobin were different (Table 3). This suggests that there is a basic difference in the nitrogen-fixing ability of the two strains, and that total leghaemoglobin per plant may not be a reliable index of the activity of the bacteroid tissue. Similarly, the use of leghaemoglobin concentration as an index of the nitrogen-fixing activity may also be misleading as the difference in concentration (0·13 and 0·18 n-moles/mg nodule dry weight for NA30 and TA1 respectively) was greater than the relative difference in the amount of nitrogen fixed (2·23 and 2·68 mg nitrogen per plant). Such results indicate the need for a closer assessment of the relationship between leghaemoglobin and nitrogen-fixing activity in different symbiotic combinations.

There was a decline in leghaemoglobin concentration in the NA30 nodules over an 8-day period during which the concentration was maintained in the TA1 nodules (Table 3). Nutman (1967) showed that bacteroid tissue in nodules formed by a completely ineffective strain persisted for a shorter period than that formed by an effective strain. It is interesting to speculate that the present results indicate a shorter period of nitrogen-fixing activity by the NA30 bacteroids.

There is a need for a suitable index of bacteroid tissue in assessing the effectiveness of nitrogen fixation by the acetylene-reduction technique (Stewart, Fitzgerald, and Burris 1967; Hardy *et al.* 1968). Total nodule nitrogen could be misleading, as

such values would include variable amounts of non-protein nitrogen, while protein nitrogen would include a component due to protein in host cells, inactive or decaying bacteroids, and vegetative rhizobia. It is suggested that nodule dry weight may be the best interim base for such studies; nitrogen fixation (or its equivalent) by all nodules on a plant would give an indication of the total amount fixed at that time, while the rate of fixation per unit nodule dry weight would indicate the efficiency of the symbiosis.

V. Acknowledgments

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VI. References

Bergersen, F. J. (1961).—Haemoglobin content of legume root nodules. *Biochim. biophys. Acta* **50**, 576–8.

Dart, P. J., and Mercer, F. V. (1965).—The effect of growth temperature, level of ammonium nitrate, and light intensity on the growth and nodulation of cowpea (*Vigna sinensis* Endl. ex Hassk.). *Aust. J. agric. Res.* **16**, 321–45.

Gibson, A. H. (1961).—Root temperature and symbiotic nitrogen fixation. *Nature, Lond.* **191**, 1080–1.

Gibson, A. H. (1963).—Physical environment and symbiotic nitrogen fixation. I. The effect of root temperature on recently nodulated *Trifolium subterraneum* L. plants. *Aust. J. biol. Sci.* **16**, 28–42.

Gibson, A. H. (1965).—Physical environment and symbiotic nitrogen fixation. II. Root temperature effects on the relative nitrogen assimilation rate. *Aust. J. biol. Sci.* **18**, 295–310.

Gibson, A. H. (1966*a*).—Physical environment and symbiotic nitrogen fixation. III. Root temperature effects on shoot and root development and nitrogen distribution in *Trifolium subterraneum*. *Aust. J. biol. Sci.* **19**, 219–32.

Gibson, A. H. (1966*b*).—The carbohydrate requirements for symbiotic nitrogen fixation: a "whole-plant" growth analysis approach. *Aust. J. biol. Sci.* **19**, 499–515.

Hardy, R. W. F., Holsten, R. D., Jackson, E. K., and Burns, R. C. (1968).—The acetylene–ethylene assay for N_2 fixation: laboratory and field evaluation. *Pl. Physiol., Lancaster* **43**, 1185–207.

Nutman, P. S. (1967).—Varietal differences in the nodulation of subterranean clover. *Aust. J. agric. Res.* **18**, 381–425.

Paul, K. G., Theorell, H., and Åkeson, A. (1953).—The molar light absorption of pyridine ferroprotoporphyrin. *Acta chem. Scand.* **7**, 1284–7.

Porra, R. J., Vitols, K. S., Labbe, R. F., and Newton, N. A. (1967).—Studies on ferrochelatase. The effects of thiols and other factors on the determination of activity. *Biochem. J.* **104**, 321–7.

Stewart, W. D. P., Fitzgerald, G. P., and Burris, R. H. (1967).—*In situ* studies on N_2 fixation using the acetylene reduction technique. *Proc. natn. Acad. Sci. U.S.A.* **58**, 2071–8.

Virtanen, A. I. (1955).—"Biological Nitrogen Fixation." Proc. 3rd Int. Congr. Biochem., Brussels. (Ed. C. Liebecq.) pp. 425–33.

Williams, C. H., and Twine, J. R. (1967).—Determinations of nitrogen, sulphur, phosphorus, potassium, sodium, calcium, and magnesium in plant material by automatic analysis. Tech. Pap. Div. Pl. Ind. CSIRO, Aust. No. 24.