Plant and Soil **34**, 663–673 (1971) Ms. 1463

NODULATION OF ROOTED LEAVES IN LEGUMINOUS PLANTS

by T. A. LIE

Laboratory of Microbiology, Agricultural University, Wageningen, The Netherlands

SUMMARY

Root formation was obtained on the petioles of detached leaves of several leguminous plants, particularly on the primary leaves of bean. Root formation is easily obtained in artificial light at a temperature of 22 to 24°C. In the greenhouse it is optimal in early spring and late autumn. During hot summer seasons no roots but callus was formed on the petioles. Root formation was inhibited when the pulvinus was left on the petiole.

Nodulation of the rooted leaves is inhibited by combined nitrogen and high temperatures. The optimum light intensity for rooted leaves is low in comparison with that of intact plants. Far-red light reduces root-nodule formation; its inhibitory effect is partly eliminated by subsequent irradiation with red light.

INTRODUCTION

Experiments with grafted plants [9 11 12 14] and isolated root cultures [10] may lead to the conclusion that nodule formation in leguminous plants depends on a number of specific factors confined to the roots. This would mean that the shoot only acts in a non-specific way, *viz* by supplying carbohydrates derived from photosynthesis. However, it has been found that light may affect nodulation not only via photosynthesis [6 7]. From the results of those experiments it was concluded that the nodulation process, like many other reactions in the plant, is regulated by phytochrome, a pigment system controlled by light [3]. Part of the data presented in the above-mentioned paper [6] were obtained with rooted leaves, i.e. plant systems of which the shoot is reduced to a single leaf. Although some increase in the volume and the dry weight of the leaf may occur, the main growth of this plant system takes place in the roots [8]. Therefore, this system is useful for studying certain aspects of root-nodule formation without complications due to the growth of the shoot. This system was used earlier for the study of boron uptake [13], nitrogen metabolism [1 2 8] and photosynthesis [4 5]. The present paper deals with root and nodule formation of leaves from leguminous plants, using mainly the primary leaves of *Phaseolus vugaris* L.

MATERIALS AND METHODS

Leaves of the following plants were tested for root and nodule formation: *Phaseolus vulgaris* cv. Walcherse witte, cv. Beka, cv. Amerikaanse zonder draad; *Trifolium pratense* cv. Kühn; *Trifolium repens* cv. Mörso; *Medicago sativa* cv. du Puit; *Ornithopus sativus* cv. Portugal; *Glycine max*, commercial; *Pisum sativum* cv. Rondo; *Vicia faba* cv. Niki; Desmodium spec.; *Phaseolus radiatus*, commercial and the non-leguminous plant *Alnus glutinosa*.

The leaves, with a piece of the petiole, were cut at the end of the photoperiod, when the leaves contain a maximum of carbohydrates. Use was made of young, but fully expanded, leaves from plants growing in day light or in fluorescent light (Philips TL 33, daylight). The experiments were carried out mainly with primary bean leaves (cv. Walcherse witte), detached two weeks from sowing. The petiole was reduced to ca. 2.5 cm length and in some cases the lamina was cut to an uniform size, using a borer of 4 cm diameter. Leaves treated with indole-acetic acid (IAA) were placed with the petiole in an aqueous solution of IAA ($10^{-6}M$) overnight. The following morning the petioles were rinsed with water to remove adhering IAA and the leaves were placed in sand moistened with nutrient solution, on agar or directly in the nutrient solution. The solution used is 1/10 strength of that used for pea plants [7].

The leaves were covered with transparent polythene and, when placed in the greenhouse, shaded at first with paper to prevent excessive evaporation. When grown under artificial light, fluorescent lamps (Philips TL 33; 30 000 ergs/cm^2 sec.) were used. Blue light was obtained by filtering blue light (Philips TL 18) through a filter (Röhm and Haas, no. 627) and red light from Philips TL 15 lamps, filtered through a red filter (Röhm and Haas, no. 501). Far-red light was obtained by filtering incandescent light (Philips Comptalux 150 watt) through 10 cm water and then through a red and blue filter.

The roots normally appeared after 5–7 days and then the leaves were transferred to a fresh solution of $\frac{1}{4}$ strength. When the leaves turned yellow, urea or ammonium nitrate was given, either by spraying the lamina (urea, 100 mg N/l) or in the nutrient solution (ammonium nitrate 10 mg N/l).

RESULTS

A. ROOT FORMATION

The formation of roots on leaves is irregular and depends on the plant species, the age of the plants and the environmental conditions.

1. *Effect of the plant*

No roots were obtained on leaves of peas and broad beans. The number of rooted leaves was rather low in clovers, lucerne, serradella, soybean and alder. In general, root formation was more successful in sand than in water culture. The best results were obtained with *Phaseolus vulgaris*. Although the trifoliate leaves (Plate 1A) rooted readily, the two opposite, primary leaves (Plate 1B, C, D) are more suitable for experiments, due to the more uniform size. The leaves were usually detached two weeks from sowing when the laminae were fully expanded. Young leaves rooted readily but were more susceptible to water dammage. At the other hand, older leaves formed callus at the base of the petiole instead of roots.

The small primary leaves of *Phaseolus radiatus* (Plate 2G) were found to be suitable for the sterile culture of rooted leaves. In this case, aseptically grown seedlings in Petri dishes were used for the supply of sterile leaves. Very often callus was formed, not only on the petiole, but also on the veins of the lamina (Plate 2I).

The effect of the pulvinus on root formation was also observed. In a number of primary bean leaves the petioles were cut just above the pulvinus, connecting the petiole with the stem, whereas in the opposite leaves the pulvinus was left on the petiole. In general, the leaves were only rooted on the petioles without the pulvinus.

2. *Environmental conditions*

Under greenhouse conditions, with supplementary light and heating during the winter period, root formation was easely obtained in early spring, late autumn and winter, when the temperature was 20–24°C and the light intensity rather low. During the summer months the leaves were rather stiff and brittle, and no roots but only callus was formed at the base of the petiole.

Good results were obtained throughout the year, when the bean plants were raised and the rooting performed under fluorescent light.

TABLE 1

The effect of IAA ($10^{-6}M$) on root formation* of detached primary bean leaves in blue light (17 000 ergs/cm² sec.) or in red light (8000 ergs/cm² sec.). The laminae of the leaves are reduced to an uniform size (4 cm diameter, see Plate 2 H)

	Blue light		Red light	
	−IAA	+IAA	−IAA	+IAA
Number of roots per leaf	1.4	8.4	9.4	19.4
Mean length of the root (mm)	4.9	4.0	10.2	10.5
Total length of the roots per leaf (mm)	6.9	33.6	95.9	200.6

* Mean values of 12–25 leaves.

Under conditions of equal dry matter production, it was found that more roots were formed in red than in blue light. In the latter case, roots appeared only at the base of the petiole whereas in red light roots protruded in four rows throughout a large zone above the cut end. Treatment with IAA promoted root formation both in blue and in red light, but proportionally more in the former (Table 1). It is of interest to note, that leaves treated with IAA, when placed in blue light, produce roots in a large zone above the base (Plate 2H).

Under humid conditions, roots may also be formed on the veins on the lower or even on the upper side of the lamina. These roots may, eventually become nodulated (Plate 1B).

B. NODULE FORMATION

Nodulation was obtained on rooted leaves from *Phaseolus vulgaris*, *Phaseolus radiatus*, clovers, lucerne, serradella and soybean. If due precautions were taken, the leaves could be maintained for a long period in a nitrogen-free medium. In one case, nodulated bean leaves were kept for 13 months in the absence of combined nitrogen. The root system was more 100 cm long and the lamina was dark green. In another experiment nodulated bean leaves had an average dry weight of roots of 736 mg.

The supply of nitrogen from the nodules to the leaves is shown in Tables 2 and 4. The non-nodulated leaves were yellow, whereas the nodulated ones were dark green. Using serradella leaves, nitrogen fixation was also detected using the acetylene-reduction technique.

TABLE 2

Nitrogen fixation* by nodulated primary bean leaves

Treatment	Nodules		Dry weight (mg)			Nitrogen (mg)		
	Number	Fresh wt.	Leaves	Roots	Total	Leaves	Roots	Total
Uninoculated	0	0	97	52	149	2.0	1.0	3.0
Inoculated	16	33	102	57	159	2.9	1.2	4.1

* Mean values per leaf of 6–9 replicates at harvest, 36 days after inoculation.

1. *Pretreatment with IAA*

As described in part A, root formation was strongly stimulated by pretreatment with IAA. To study whether nodulation might be also affected by IAA, a comparison was made between leaves kept overnight either in water or in IAA. The leaves were allowed to form roots in white light for one week and were then transferred to cabinets with white, blue or red light. The light intensities of blue and red light were adjusted in such a way, that approximately the same number of light quanta was obtained in each light cabinet. Due to technical reasons the leaves in the white cabinet received more light quanta than those in the other cabinets.

The results are given in Plate 1C, 1D and Table 3. In agreement

TABLE 3

The effect of pre-treatment with IAA ($10^{-6}M$) on nodulation* of primary bean leaves in blue (17 000 ergs/cm^2 sec.), red (12 000 ergs/cm^2 sec.) and white light (16 000 ergs/cm^2 sec.)

Light quality	IAA	Nodule number	Nodule fresh wt. (mg)
Blue	–	4.5	13
Blue	+	10.0	34
Red	–	8.9	25
Red	+	9.0	28
White	–	10.0	32
White	+	17.0	53

* Mean values per leaf of 16–22 replicates.

TABLE 4

The effect of combined nitrogen on nodulation* and nitrogen content* of primary bean leaves

Ammonium nitrate (mg N/l)	Rhizobium	Nodules		Nitrogen (mg)		
		Number	Fresh wt. (mg)	Leaves	Roots	Total
0	–	0	0	3.4	1.6	5.0
0	+	15.2	49.3	4.9	1.8	6.7
25	+	7.0	16.4	5.2	2.4	7.6
50	+	2.4	1.6	5.0	3.6	8.6
100	+	0	0	6.8	4.0	10.8

* Mean values per leaf of 5 replicates.

with our previous observations it was found that the IAA-treated leaves produced considerably more roots than the untreated ones under all light conditions. Nodulation was also increased in blue and white light, but not, or only slightly, in red light. It is not possible to decide from this experiment, whether the increase in nodulation was due to an increase of root growth or to residual IAA taken up by the leaves at the start of the experiment.

2. *Combined nitrogen*

To study the effect of combined nitrogen on nodulation, rooted bean leaves were transferred from nitrogen-free medium to fresh culture solutions, containing different amounts of ammonium nitrate. The results are given in Table 4. The uninoculated leaves, without combined nitrogen, suffered from nitrogen starvation. The best nodulation was obtained in the absence of combined nitrogen. The inhibitory effect of ammonium nitrate was already significant at 25 ppm N and total inhibition was observed at 100 ppm N.

3. *Root temperature*

The effect of root temperature on nodulation was studied by keeping the jars in waterbaths of different temperatures. The roots were kept constant at the desired temperature but the leaf temperature varied according to the temperature of the greenhouse (20–24°C). The number of root nodules was counted 11 days after inocu-

TABLE 5

The effect of temperature on nodulation* and growth* of primary bean leaves

Temperature (°C)	11 days	21 days (harvest)							
	Nodule number	Nodule number	Nodule fr. wt. (mg)	Dry weight (mg)			Nitrogen (mg)		
				Leaves	Roots	Total	Leaves	Roots	Total
20	2	20	44	63	88	151	2.1	0.5	2.6
25	12	25	81	63	69	132	2.5	0.6	3.1
28	11	17	27	71	60	131	2.5	0.5	3.0
30	0	0	0	66	45	111	1.9	0.5	2.4

* Mean values per leaf of 4 replicates.

lation and at harvest time (21 days). The optimum temperature for nodulation of rooted bean leaves was found to be 25°C, similar to that of intact bean plants [6]. At 20°C the nodules appeared slowly but at the end nodulation was satisfactory. In contrast, at 28°C nodules were formed rapidly, but at harvest time both nodule number and nodule weight were rather low. At 30°C nodulation was completely inhibited, except for one small nodule present on one of the leaves. The nodules at 20 and 25°C were large and red, whereas those formed at 28°C were small and white. The production of roots was highest at the lowest temperatures and strongly reduced at higher temperatures (Table 5).

4. *Light intensity*

In agreement with the results of Mothes and Englebrecht [8] it was observed that rooted leaves are extremely sensitive to high light intensities. At light intensities, in the greenhouse and in light cabinets, where nodulation and growth of intact plants were satisfactory, rooted leaves turned yellow and abscission followed. In fact, to cultivate them for long periods, the leaves must be shaded and protected from direct sun light.

The effect of light intensity was studied in a light cabinet with white light at four different intensities. To standardize the leaf surface, the laminae were reduced to the same size, using a borer of 4 cm diameter (Plate 2H).

TABLE 6

The effect of light intensity on nodulation* and growth* of rooted primary leaves** of *Phaseolus vulgaris*

Light intensity (10^3 ergs/cm^2 sec.)	Nodules		Dry weight (mg)		
	Number	Fresh wt. (mg)	Lamina	Petiole	Roots
5	1.3	4	50	16	23
10	7.6	23	59	27	40
15	11.7	40	55	32	51
20	11.5	41	54	35	52

* Mean values per leaf of 16–20 replicates.
** Lamina reduced to an uniform size (4 cm ∅, see Plate 2 H).

From Table 6 it will be seen that both nodule numbers and nodule weights increased when the light intensity was increased to 15000 ergs/cm^2 sec. With higher light intensities no further increase and sometimes even a decrease of nodulation was observed. This was due to a rapid senescence of the leaves at high light intensities. The leaves at 5000 ergs/cm^2 sec. were green, whereas those at 15 and 20 000 ergs/cm^2 sec. had yellow spots. It is of interest to note that the petioles of the leaves increased in thickness and in weight at high light intensities since elongation did not take place.

5. *Red and far-red light*

The rooted leaf is particularly useful for studies where complications due to shoot growth must be eliminated. Light of different wave lengths may affect shoot elongation in different ways. Therefore, to study the effect of red and far-red light on nodule formation, rooted leaves are more suitable than intact plants, since in them shoot elongation is absent. Rooted bean leaves were inoculated and grown in white light (8 hours photoperiod, 20 000 ergs/cm^2 sec.). At the end of the photoperiod, the leaves were irradiated with red or far-red light or both for 10 minutes each and then kept in complete darkness until the next photoperiod. The leaves were so treated during the 5 days following inoculation.

For unknown reasons not all the leaves were nodulated during the first two countings, but at harvest the majority of the leaves were,

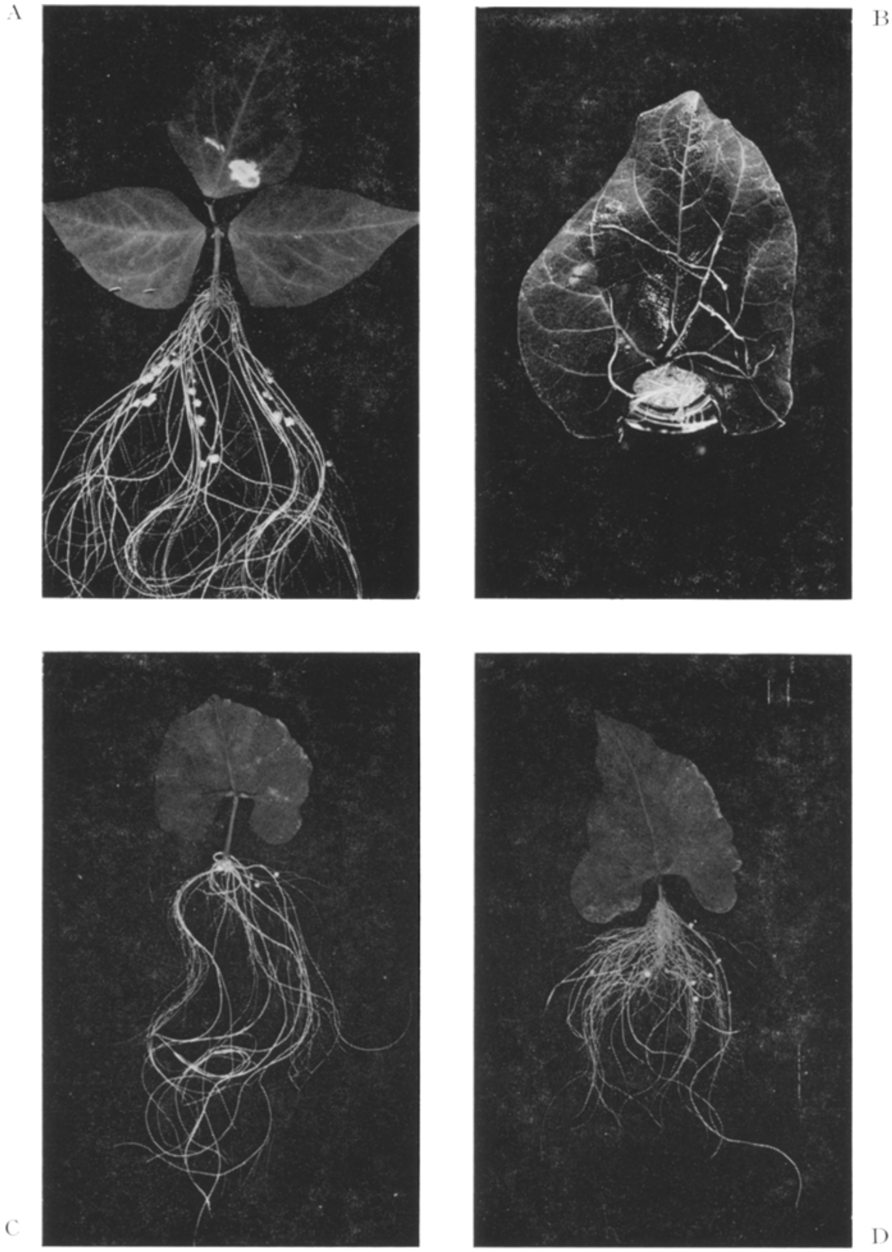

Plate 1. Root and nodule formation in *Phaseolus vulgaris* leaves. **A**. Trifoliate leaf; **B**. Primary leaf with nodulated roots coming from the veins, on the upper side of the lamina; **C** and **D**. Primary leaves growing in blue light, untreated (**C**) or treated with IAA (**D**).

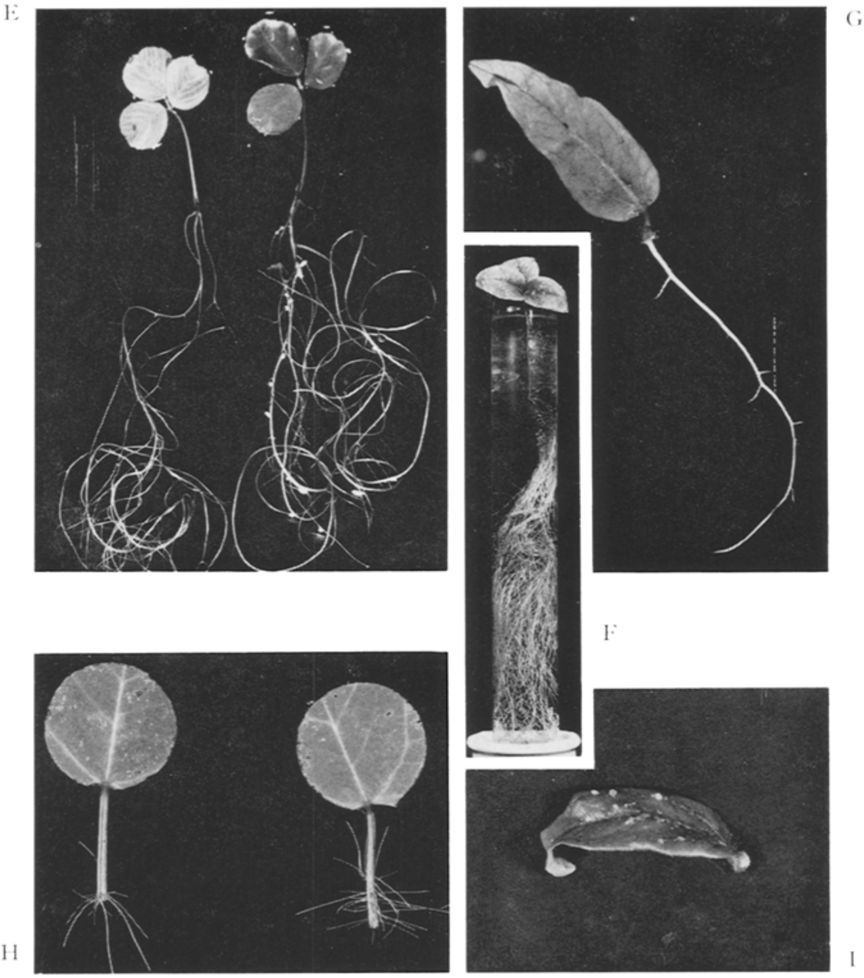

Plate 2. **E**. Lucerne leaves with or without nodules; **F**. Red clover leaf; **G**. *Phaseolus radiatus* leaf; **H**. Primary leaves of *Phaseolus vulgaris* growing in blue light; lamina reduced to an uniform size (4 cm diameter). Left, untreated and right, treated with IAA; **I**. Callus formation on the veins and the petiole of *Phaseolus radiatus* leaf.

TABLE 7

The effect of supplementary red (8000 ergs/cm² sec.) and/or far-red light (50 000 ergs/cm² sec.) on nodulation* of primary bean leaves, grown in white light (20 000 ergs/cm² sec., photoperiod 8 hours)

Light Treatment	Number of nodules at †† days after inoculation			Dry weight (mg) of		
	8	11	15	Lamina	Petiole	Roots
C	1.8	2.2	6.7	118	23	65
R	2.0	2.5	6.7	114	28	64
R–I	0	1.3	6.1	108	32	58
R–I–R	1.0	3.3	8.2	120	35	68
R–I–R–I	0	1.7	5.0	108	28	52

* Mean values per leaf of 7–9 replicates. C, control, untreated; R, red light during 10 minutes; I, far-red light during 10 minutes.

and in Table 7 mean values are given. In agreement with previous experiments [7] a reduction of nodule number was observed after irradiation with far-red light. When red light was applied afterwards, the inhibitory effect was partly eliminated. When a number of light treatments were given, the results were determined by the last treatment. The slight effects of the light treatments on the dry weights of the leaves and roots may be due to the effect of light on nodulation and consequently nitrogen supply of the leaves.

DISCUSSION

The use of rooted leaves for the study of root-nodule formation has the advantage that the effect of certain factors can be studied without complications due to differences in shoot growth. This is demonstrated in the study of the effect of light on nodule formation. When intact plants are used, differences in shoot elongation due to light of different wave lengths may be obtained, and a comparison of nodulation of these plants is rather difficult. Furthermore, by reducing the lamina to a standard size, the amount of incident light per leaf can be standardized.

The results on the effect of different factors on nodulation of rooted leaves are in close agreement with those obtained with intact plants. The optimum light intensity for rooted leaves, however, is far lower than that of intact plants. Since artificial light of high intensity is difficult to realize under laboratory conditions, the use of rooted leaves is suitable for the study of light intensities on nitrogen fixation and other processes.

Rooted leaves can be maintained for a very long period, provided that the root system is not limited in its growth. The laminae of nodulated leaves are dark green, sometimes even blueish green, due to the activity of root nodules. A study of this system may be of interest to plant physiologists.

For practical purposes, rooted leaves may be used in addition to stem cuttings for the study of nodulation of leguminous trees or shrubs. The use of genetically identical systems, without sacrifying the whole plant is important in plant species with a high variation in nodulation pattern.

ACKNOWLEDGEMENT

Thanks are due to professor E. G. Mulder for critical perusal of the manuscript.

Received February 21, 1970. Revised September 1970

REFERENCES

1 Chibnall, A. C., Protein metabolism in rooted runner bean leaves. New Phytologist **53**, 31–37 (1954).
2 Gregory, F. C. and Samantarai, B., Factors concerned in the rooting responses of isolated leaves. J. Exp. Botany **1**, 159–193 (1950).
3 Hillman, W. S., The physiology of phytochrome. Ann. Rev. Plant Physiol. **18**, 301–324 (1967).
4 Humphries, E. C., Dependence of net assimilation rate on root growth of isolated leaves. Ann. Botany N.S. **27**, 175–184 (1963).
5 Humphries, E. C., The effect of root temperatures on dry matter and carbohydrate changes on rooted leaves of Phaseolus spp. Ann. Botany **31**, 59–69 (1967).
6 Lie, T. A., Nodulation of leguminous plants as affected by root secretions and red light. Thesis, Wageningen (1964).
7 Lie, T. A., Non-photosynthetic effects of red and far-red light on root-nodule formation by leguminous plants. Plant and Soil **30**, 391–404 (1969).
8 Mothes, K. and Englebrecht, L., Über den Stickstoffumsatz in Blattstecklingen. Flora (Jena) **143**, 428–472 (1956).
9 Nutman, P. S., Nuclear and cytoplasmic inheritance of resistance to infection by nodule bacteria in red clover. Heredity **3**, 263–291 (1949).

10 Raggio, M., Raggio, N. and Torrey, J. G., The nodulation of isolated leguminous roots. Am. J. Botany **44**, 325–334 (1957).
11 Richmon, T. E., Legume inoculation as influenced by stock and scion. Botan. Gaz. **82**, 438–442 (1926).
12 Tanner, J. W. and Anderson, I. C., Investigations on non-nodulating and nodulating soybean strains. Can. J. Plant Sci. **43**, 542–546 (1963).
13 Scholz, G., Über die Translokation des Bors in Tabak-Blattstecklingen mit geteilten Wurzelsystem. Flora (Jena) **148**, 295–305 (1959).
14 Wagenbreth, D., Leguminosenpfropfungen und Wirtsspezifität der Knölchenbakterien. Flora (Jena) **144**, 84–97 (1956).