

SOME ASPECTS OF ROOT NODULATION IN TROPICAL LEGUMES

N. RAJAGOPALAN AND T. S. SADASIVAN

University Botany Laboratory, Madras-5

AMONG plant tissues, root nodules of legumes have two unique features: they fix atmospheric nitrogen and contain haemoglobin. Despite comprehensive study of this symbiosis and the resultant specific protein synthesis *in vivo*, the problem has not lost its fundamental interest. In this paper, recent work on root nodules carried out here is presented in the light of foregoing knowledge on the subject.

SPECIFICITY OF THE ASSOCIATION BETWEEN RHIZOBIA AND LEGUMES

Depending on collections of legume species that could exchange rhizobia with each other, rhizobia are classified into six cross-inoculation groups, viz., *Rhizobium leguminosarum* (*Pisum*, *Vicia*, *Lens*), *Rh. phaseoli* (*Phaseolus*), *Rh. trifolii* (*Trifolium*), *Rh. meliloti* (*Melilotus*, *Medicago*, *Trigonella*), *Rh. lupini* (*Lupinus*, *Ornithopus*) and *Rh. japonicum* (*Soya*). While temperate rhizobia exhibit this specialization in infective kinships to particular plant hosts, the vast majority of tropical legumes belong to the so-called cow-pea cross-inoculation group in which infective promiscuity appears to be very common. Thus, a *Rhizobium* strain from groundnut which belongs to the cow-pea group was shown in this laboratory¹ to induce effective nodulation in *Arachis hypogaea* L., *Phaseolus mungo* L., *Clitoria ternatea* L., *Centrosema pubescens* Benth., *Vigna catjang* Walp., *Psophocarpus tetragonolobus* DC., *P. palustris* DC., *Cyamopsis tetragonoloba* (L.) Taub., *Cajanus cajan* L. Millsp., *Dolichos lablab* L., *Dolichos biflorus* L., *Gliricidia macrocarpa* H.B. et K. and *Crotalaria juncea* L.—representatives of the tribes Gensiteæ, Galegeæ, Hedysareæ and Phaseoleæ of the subfamily Papilionatæ. While cross-inoculation specificity has been based on the economically important and intensively studied temperate species, study of the remaining 90% of the legume genera which still have to be examined in respect of nodulation² might confirm or contradict the observations of Wilson³ who claimed "more than five hundred reasons for abandoning the cross-inoculation groups".

Allen and Allen⁴ found *Rh. japonicum* from soybean to produce a few moderately effective nodules on *Arachis hypogaea* which belongs to the cow-pea group. In this laboratory (Gopala-

krishnan and Raju, unpublished), inoculating with three effective strains of *Rh. trifolii*, four of *Rh. leguminosarum* and one from lucerne failed to induce nodulation in the same host. With *Rh. leguminosarum* a few diminutive ineffective nodules developed on *Crotalaria. Lathyrus sativus* L. which belongs to the Pea and Vetch group of *Rh. leguminosarum* did not, however, nodulate with an effective strain from *A. hypogaea*. While reasons for these are not clear, studies on infective relationships between rhizobia and legumes have to await further investigation of the "big cow-pea group (*Vigna*) which has always been somewhat of a problem child of *Rhizobium* classification"⁵ Bowen and Kennedy⁶ suggest the possibility of establishing effectiveness sub-groups in tropical legumes.

THE EFFECT OF SOIL TYPE AND SOIL REACTION ON NODULATION

In temperate countries it is well known that extremes of soil reaction could adversely affect nodulation. The importance of this factor does not seem to have been studied widely in the tropics. Inoculation experiments in this laboratory with effective rhizobia was observed to lead to different responses in nodulation of groundnut in red and black soils—two of the major soil types in South India. An effective strain (R4) induced heavy nodulation in *A. hypogaea* in red soils which are acid to neutral (pH 5.5–7.0) more than in black soils which are distinctly alkaline (pH 8.2–10.5) albeit the fact that red soils contain less exchangeable cations than black soils. In sterile cultures pH levels of 4.0, 5.0 and 6.0 were found suitable for plant growth, optimum nitrogen fixation and haemoglobin formation with a most favourable effect at pH 5.0 while fixation deteriorated toward pH 3.0, 7.0 and 8.0 (Fig. 1). This agrees well with the observations of Norris⁷ that there is no justification for assuming that tropical legumes need lime because the soil is acid, for they are capable of living and thriving quite normally in distinctly acid soils.

THE LEGUME-RHIZOSPHERE EFFECT

The role of plant root excretions on the microbial composition of rhizosphere is well defined.^{8,9} Thus, Timonin¹⁰ found 275 millions of bacteria

per gram of rhizosphere soil around wheat roots against 21 millions in control soil. The numbers for lucerne were 287 and 22.3 millions respectively, the ratio of rhizosphere to soil in both plants being 12:9.

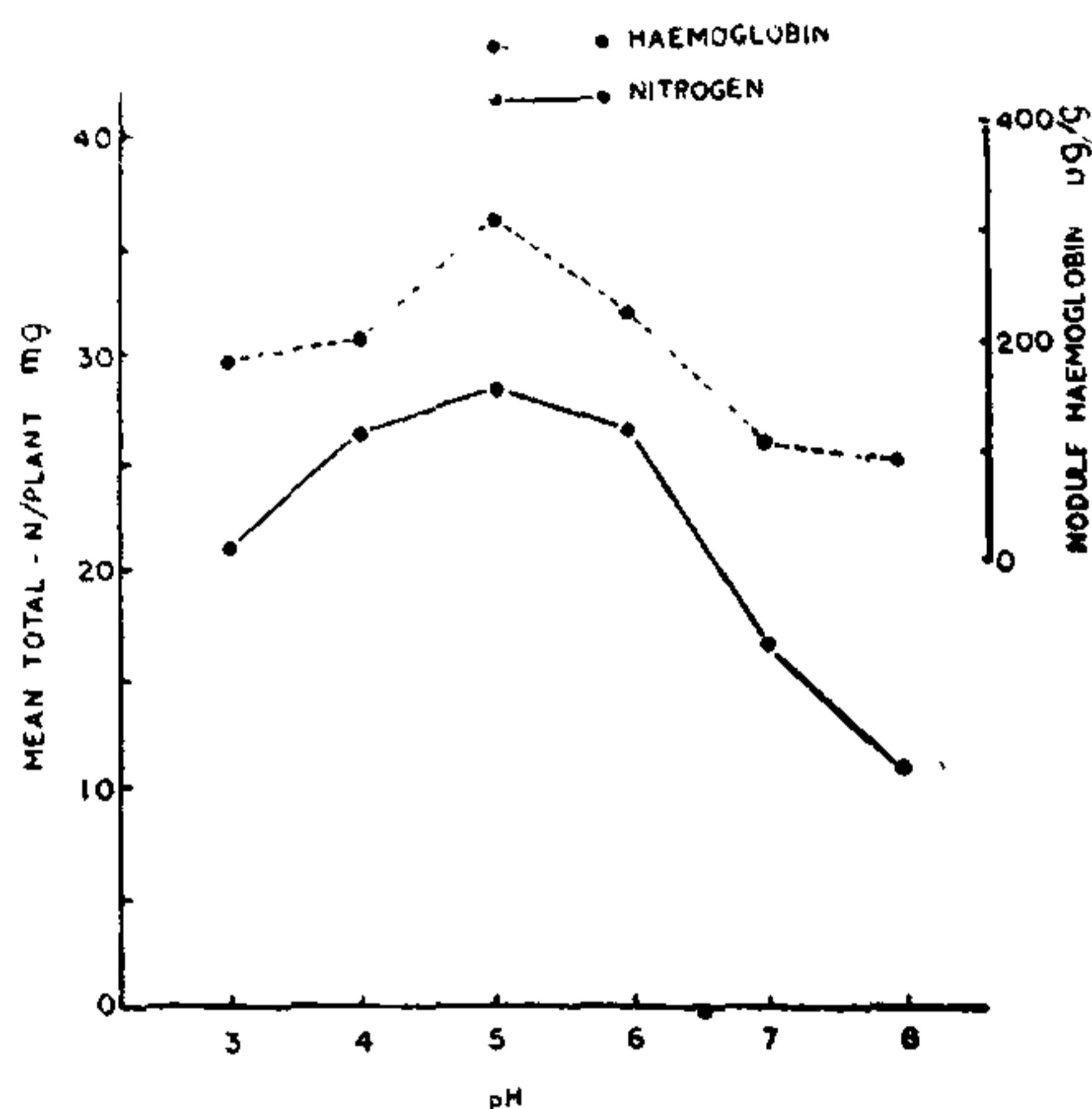


FIG. 1. The influence of soil reaction on nitrogen fixation and haemoglobin content in *Arachis hypogaea*.

However, the legume rhizosphere might stimulate the nitrifying group of bacteria. Thus, at 20 days from germination in *Dolichos lablab* nitrifying group bacteria reach 240 millions per gram of rhizosphere soil from an initial 43 millions at five days. Foliar sprays of sucrose or urea further increased their numbers, while those with thiouracil and gibberellin had an adverse effect.¹¹ Similarly, nitrifiers which comprised 2% in control soil increased to 12.3% in the rhizosphere soil of *Phaseolus mungo*, while applications of farmyard and green manure changed the percentage of nitrifiers in the rhizosphere to 10.0 and 15.0 in the two treatments. Ammonium sulphate further increased their numbers from 5.0% in the control soil to 48.0 and 43.0% in rhizosphere soil of *Phaseolus* and *Vigna*.¹²

There does not seem to be specific evidence in literature in favour of the rhizosphere effect on rhizobia. While Starkey¹³ claimed that rhizobia thrive particularly in contact with legume roots, they have since been reported to occur around the roots of Polygonaceae, Gramineae and Malvaceae.¹⁴ Besides, nodule number in legumes may not bear a relation to either the number of rhizobia in the rhizosphere or to the size of the inoculum¹⁵ although rhizobia may thrive in response to soil amelioration.¹⁶ In

fact, the number of rhizobia present in the rhizosphere of clover and alfalfa (10^6 – 10^9 organisms per ml. of medium at the time of initial nodulation) have been shown to greatly exceed that required for maximum nodulation.¹⁷

The role of aspergilli in the rhizosphere of legumes in acid sands where the microbial production of organic acids helps acid-tolerant lupines and seradellas to thrive and enable lucerne and clover to overcome the influence of humic acid was recently emphasized.¹⁸ The predominance of aspergilli in the rhizosphere of groundnut plants¹⁹ grown in lateritic soils is of interest since organic acids such as oxalic, gluconic and citric acids produced by this fungus might increase the availability of soil phosphates under certain conditions.

THE EFFECT OF PLANT EXUDATES

Among organic substances exuded from plant roots, organic acids, sugars, amino-acids, reducing compounds, growth factors, exo-enzymes and nucleic acid derivatives are well known.

Preston *et al.*²⁰ have shown that the growth regulator α -methoxy phenylacetic acid, when applied to bean hypocotyls moves into the roots, is secreted by the roots into the medium, taken up by the roots of many plants and transmitted to their leaves. Elkan²¹ found nutritionally different groups of microbes and higher numbers of rhizobia in the rhizosphere population of a normally nodulating (NN) soybean when compared to that of a near isogenic non-nodulating (*nn*) soybean strain. Since root excretions of this mutant (*nn*) resulted in highly significant decreases in nodulation of the normal plants (NN) Elkan concluded that non-nodulating soybean strains exude nodulation inhibiting principles. Deleuil²² described that leachates of the roots of perennial species of *Rosmarinus officinalis* and *Erica* prevent the growth of the associated annual legumes in the same soil. Germination and growth of legumes were inhibited when seeds of these were watered with leachates from the toxic Rosmarino-Ericon soil. Root and nodule macerates of the nodulating annual legume species offset this inhibitory effect. Deleuil thus showed that roots and nodules of legumes produce a substance antagonistic to the toxin excreted by the perennial species of the Rosmarino-Ericon association. Again intercropping of cow-peas and *Phaseolus* with grasses cause concern since roots of several Gramineae inhibit the development of bacteria not only in their rhizosphere but in non-rhizosphere as well.²³

Additions of decomposing plant litter generally promote the growth of amino-acid requiring organisms in the rhizosphere. Similarly, application of decaying organic matter may promote nodulation.²⁴ However, Garrett²⁵ points out that toxins from plant litters may predispose plants to greater invasion by root-rot causing organisms. For instance, straw and litter of wheat, barley and rye produce four phenolic inhibitors: *p*-hydroxycinnamic, *p*-hydroxybenzoic, vanillic and ferulic acids, while with rice straw *p*-oxybenzoic and *p*-coumaric, ferulic, caffeic, protocatechuic and salicylic acids are reported.²⁶ It is, therefore, evident that "many specific and aspecific metabolic accelerators or inhibitors are involved in the interactions between root exudates and rhizosphere microfloras".²⁷ The study of soil and rhizosphere factors, therefore, becomes essential if legumes are to play their full role in farm management.

EFFECTIVE AND INEFFECTIVE RHIZOBIA

Rhizobia may be effective or ineffective depending on their capacity to fix nitrogen during symbiosis. Strain effectiveness cannot, however, be correlated to their (i) cultural and physiological characteristics, (ii) antigenic structure, (iii) colonial mutation, (iv) phage sensitivity and (v) sensitivity to antibiotic action.²⁸ Thus, the only criterion, at present, to distinguish between effective and ineffective rhizobia appears to be through careful evaluation of nitrogen fixation in defined host-strain partnerships. Nitrogen fixation depends on both the plant and the rhizobium since the formation of bacteroids—the 'X' and 'Y' forms in which bacteria occur within nodule cells—depends not solely on either bacterium or host but upon a complex interaction between the two entities.²⁹ Further, effectiveness could be influenced by temperature characteristics, genetic plant factors, host metabolism and its nutrition, for rhizobia could even become pathogenic in the absence of boron.³⁰

EFFECTIVE AND INEFFECTIVE ROOT NODULES

Hæmoglobin, shown to be a prerequisite for nitrogen fixation, characterizes effective root nodules. Absorption spectra of the pigment obtained with aqueous extracts of nodules after conversion to solutions of pyridine hæmochromogen show sharpened absorption maxima, characteristic of hæmoglobin as detailed in Figs. 2, 3 and 4 for root nodules of *Cajanus*, *Cyamopsis* and *Vigna*. Virtanen³¹ found a positive correlation between hæmoglobin content

and the intensity of nitrogen fixation. Bacterial strains are known to differ in their effectiveness depending on their association with particular host species. There are strains which are more effective than the other strains in association with certain plants.³² Thus as shown in

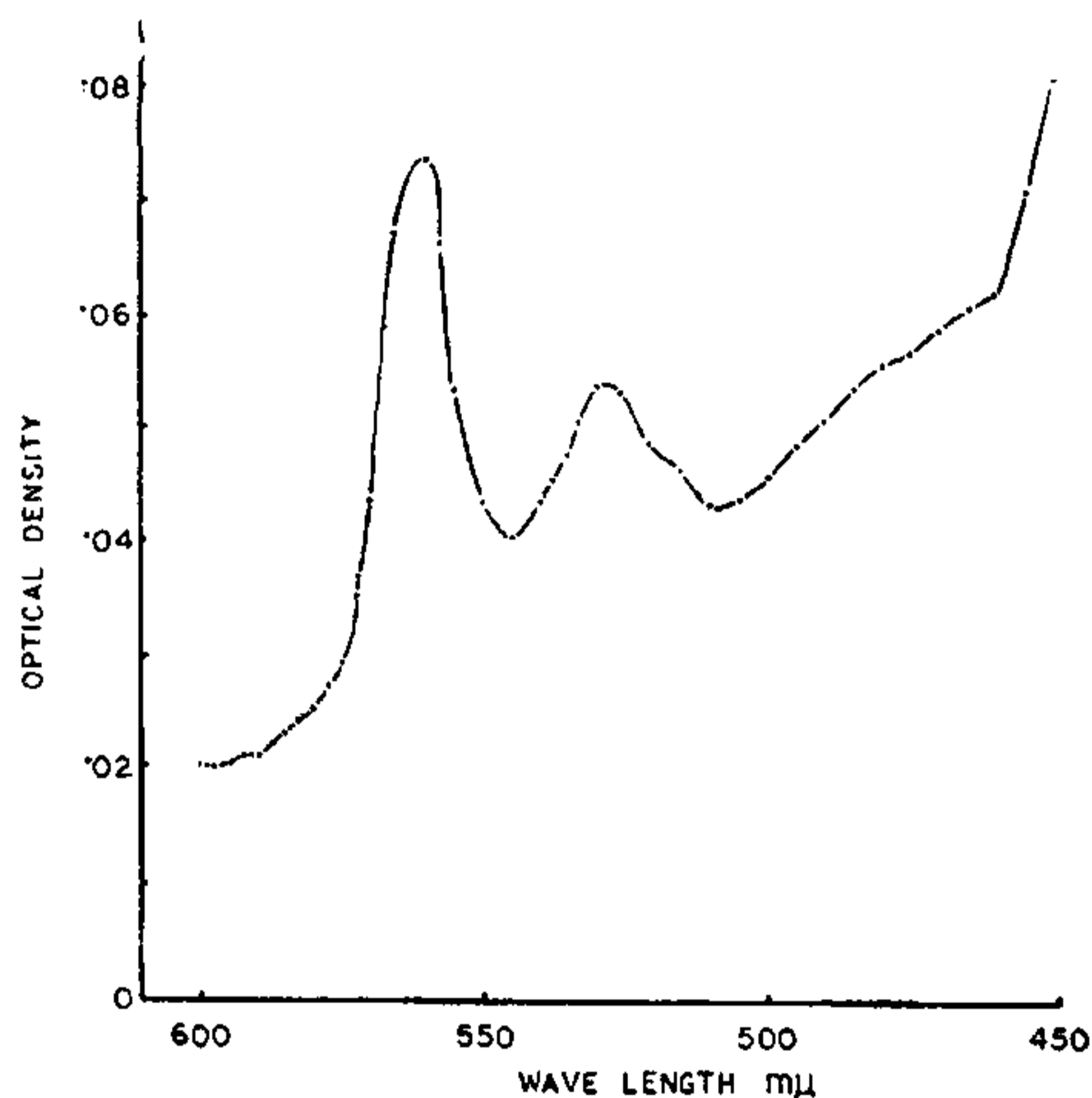


FIG. 2. Absorption spectrum of pyridine hæmochromogen from the root nodules of *Cajanus cajan*.

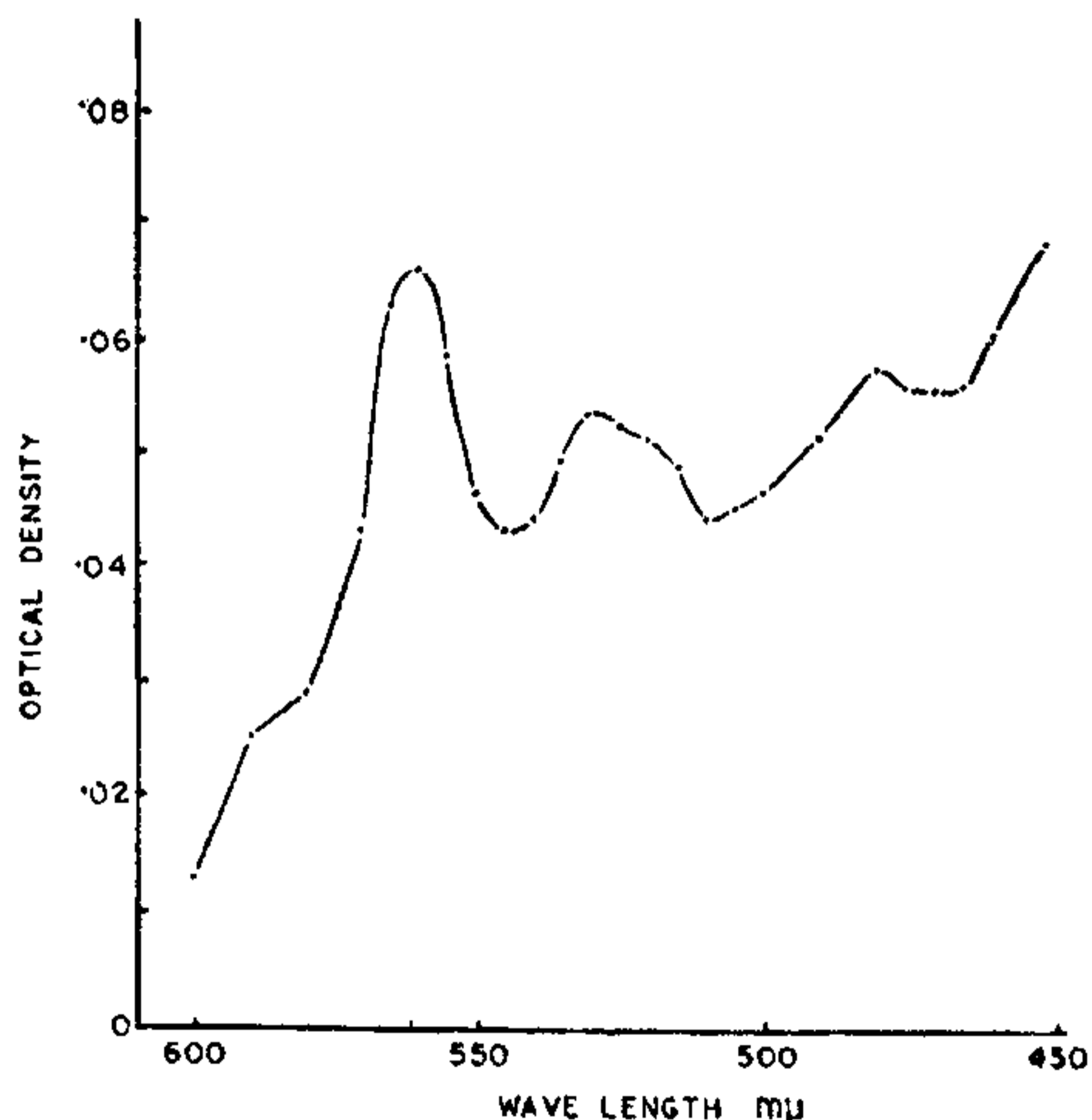


FIG. 3. Absorption spectrum of pyridine hæmochromogen from the root nodules of *Cyamopsis tetragonoloba*.

Table I an effective cow-pea strain (R 4) isolated from *Arachis hypogaea* which produced 245 µg. hæmoglobin/g. fresh nodules in *A. hypogaea*, produced as much as 600 µg./g. pigment in association with *Crotalaria juncea*. In comparison,

strains S1, S5, S6 and S7 isolated from *Crotalaria* were only moderately effective (Rajagopalan and Raju, unpublished).

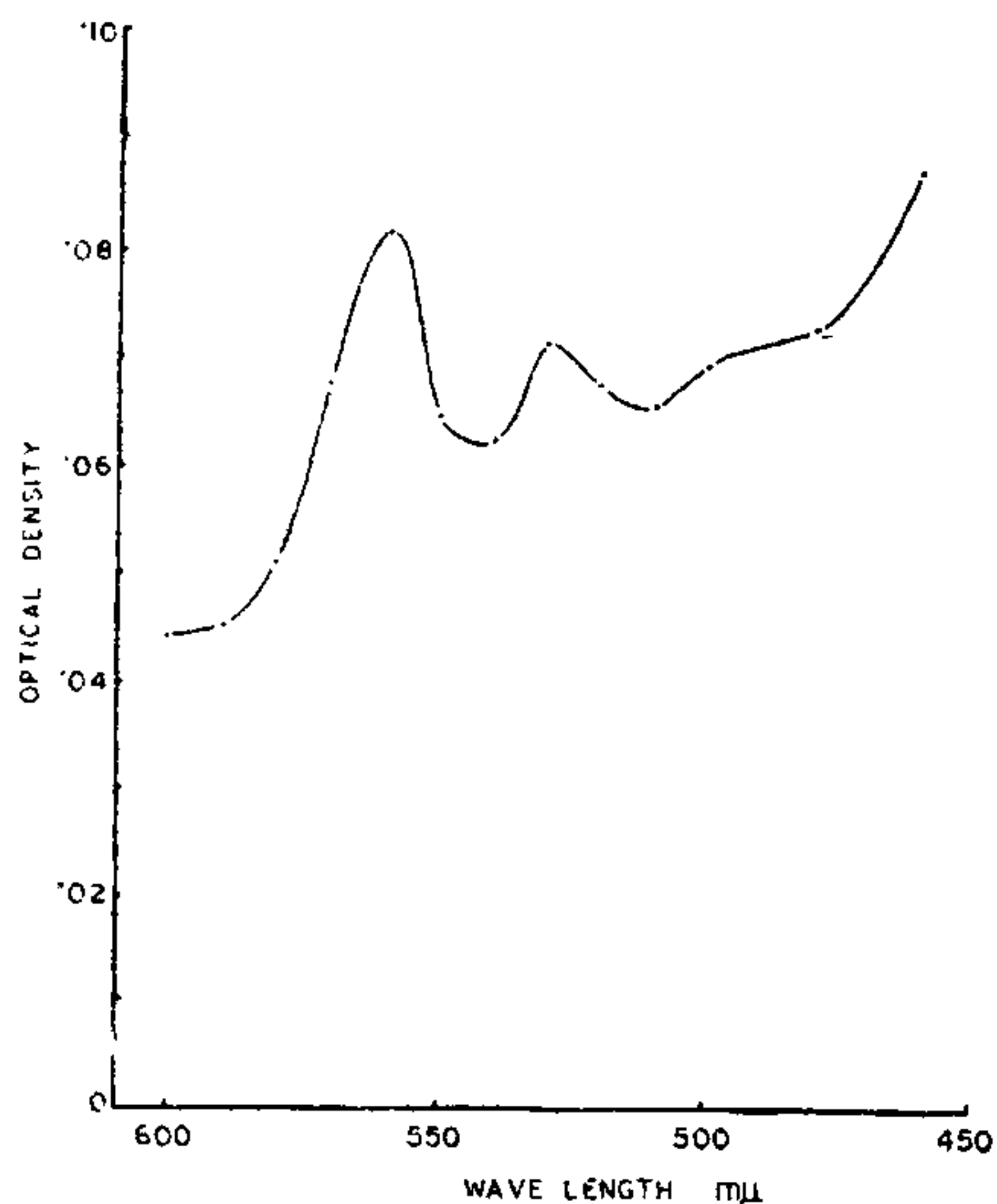


FIG. 4. Absorption spectrum of pyridine haemochromogen from the root nodules of *Vigna catjang*.

TABLE I

Haemoglobin content of nodules produced by various cow-pea rhizobia strains

<i>Arachis hypogaea</i>		<i>Crotalaria juncea</i>	
Strains	Haemoglobin $\mu\text{g./g.}$	Strains	Haemoglobin $\mu\text{g./g.}$
R 1	245	S 1	250
R 2	200	S 5	275
R 3	145	S 6	250
R 4	245	S 7	250
R 5	190	R 4	600

Nodules formed by ineffective strains do not contain haemoglobin. Effective nodules in which bacteroid formation accompanies the appearance of the pigment lose their nitrogen fixing ability when it turns green,³³ although as stated by Thornton³⁴ caution should be exercised in such study, for disintegration of bacteroid tissue equally characterises ineffective nodules, old nodules and those kept in the dark.

While the maxima of root nodule haemoglobin are normally reached just prior to the time of flowering, this may be a function of the age of the plant and condition of its growth. Thus, in *Arachis hypogaea* varieties TMV 2 and TMV 3 grown in unsterile soil under conditions of

TABLE II

The concentration of haemoglobin in the root nodules of *Arachis hypogaea* varieties

Variety	Root nodule haemoglobin $\mu\text{g./g.}$ fresh nodules					
	Days from sowing					
	20	25	35	48	60	70
TMV 2 ..	130	230	260	295	235	225
TMV 3 ..	117	244	295	340	300	220
TMV 5 ..	111	215	285	320	340	180
HG 1 ..	140	159	284	315	330	275

nodulation by endemic rhizobia, the maximal haemoglobin content of nodules are observed between 45 and 50 days; in varieties TMV 5 and HG 1 these maxima are reached at 60 days from sowing.¹ In these varieties, flowering starts from about 22 days after sowing.

The transformation of the red to the green pigment, which is known to result in the onset of conditions leading to cessation of N_2 fixation, does not seem to occur until 53 days in *Arachis hypogaea* (Fig. 5). As seen in the figure,

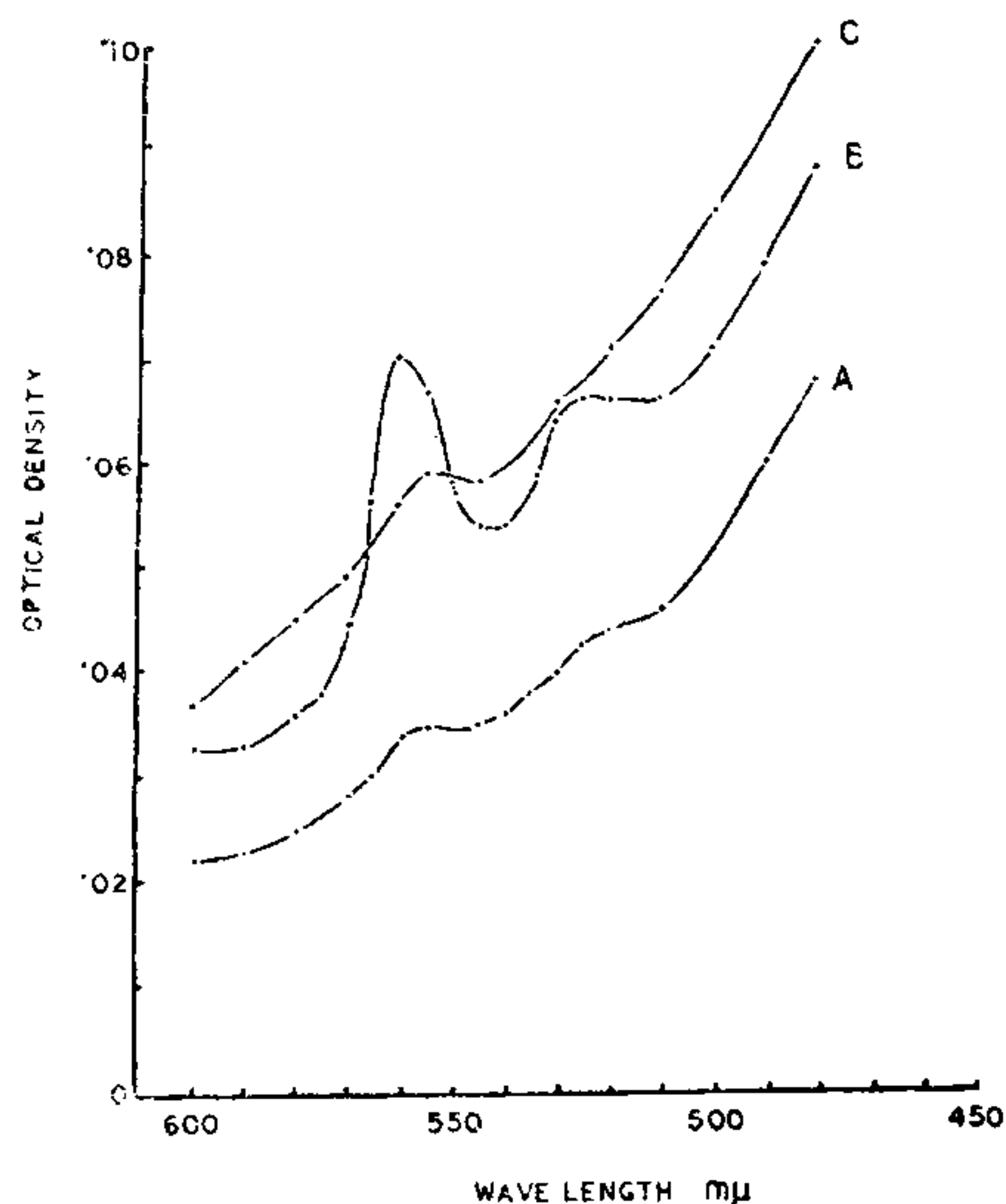


FIG. 5. Absorption spectra of pyridine extractions of root nodules of *Arachis hypogaea*. Curve A: White nodules, 22 days old. Curve B: Red nodules, 53 days old. Curve C: Green nodules, 75 days old.

within 75 days from sowing the absorption maxima of the pyridine haemochromogen from *A. hypogaea* nodules fade rapidly with the change of the red into the green pigment, a time at which the fertilized flowers rapidly put

forth their gynophores for subterranean seed-setting. Further, in this species in which the flowering is extended over a considerable period,³⁵ the maxima in haemoglobin content are found much later during the flowering period.

HAEMOGLOBIN CONTENT OF ROOT NODULES

Thornton³⁶ and Chen and Thornton³⁷ showed in a study of effective and ineffective temperate nodules that the amount of nitrogen fixed was a function of the central nodule tissue and it is now known that the central tissue of effective nodules contains haemoglobin along with the bacterial component. The haemoglobin content of nodules was used as an index of effectiveness in nitrogen fixation and a variation in nodule haemoglobin content from 105 μ g. to 1,130 μ g./g. fresh nodules has been described for peas, bean, alfalfa, vetch and clover. In tropical legumes wide variations are known in the effectiveness of nodules. Of the papilionaceous genera examined in this laboratory, species of *Arachis*, *Phaseolus*, *Clitoria*, *Centrosema*, *Vigna*, *Psophocarpus*, *Cyamopsis*, *Cajanus*, *Dolichos*, *Gliricidia* and *Crotalaria* possessed effective nitrogen fixing root nodules. On inoculation with an effective *Rhizobium* the nodule weight per plant ranged from 1.5 to 3.0 g. in *Arachis* to 20.0-25.0 g. in species of *Psophocarpus*. Their haemoglobin content varied from 240 μ g. to 835 μ g./g. fresh nodule tissue. On the basis of their haemoglobin content (1.707-66.205 μ g. per nodule) these papilionaceous genera possessed effective root nodules.

The haemoglobin content in species of *Dolichos* (780 μ g./g. fresh nodules), *Gliricidia* (835 μ g./g.), *Psophocarpus* (433 μ g./g.) and *Centrosema* (395 μ g./g.) indicate very effective symbioses as these were far in excess of that required for effectiveness.

FREE AMINO-ACIDS AND AMIDES IN ROOT NODULES

Since free amino-acids constitute the primary products in the assimilation of nitrogen in root nodules, study of their relative occurrence in different legumes may serve to define degrees of effectiveness.³⁸ An examination of the protein-free extracts of root nodules in this laboratory has shown that variations occur in free amino-acid and amide composition in different genera growing in symbiotic association with an effective strain. This may be construed in terms of host metabolism influencing symbiotic efficiency. In general, asparagine and glutamine amides preponderated in the soluble nitrogen of many nodules as shown in Table III.

TABLE III

Variation in the amide-nitrogen of root nodules of legumes expressed as percentage of the total soluble nitrogen

Legume	Amide-N
<i>Phaseolus mungo</i>	.. 9.9
<i>Vigna catjang</i>	.. 21.0
<i>Clitoria ternatea</i>	.. 21.5
<i>Dolichos biflorus</i>	.. 20.0
<i>Dolichos lablab</i>	.. 34.3
<i>Psophocarpus tetragonoloba</i>	.. 34.5
<i>Arachis hypogaea</i>	.. 44.0
<i>Gliricidia macrocarpa</i>	.. 56.2
<i>Centrosema pubescens</i>	.. 76.4
<i>Cyamopsis tetragonoloba</i>	.. 88.8

While in *Cyamopsis* asparagine dominated the soluble nitrogen fraction (75%), in *Centrosema* both glutamine and asparagine were present in large quantities. Glutamine was present in all legume nodules in varying amounts; on the other hand, asparagine was not detected in some of them. The amides which constituted 44-88% of the soluble nitrogen of nodules in *Arachis*, *Gliricidia*, *Centrosema* and *Cyamopsis* are suggestive of high fixation levels in these species.

Next to the amides, glycine, alanine, leucine, α -aminobutyric and γ -aminobutyric acid were prominent in many nodules. Aspartic and glutamic acid (γ -methylene glutamic acid in *Arachis*) and arginine, ornithine and cystine occurred in progressively lesser quantities. Of the hydroxy acids, serine occurred in quantities comparable to those of glutamic acid in *Phaseolus*. Pipecolic acid was characteristic of this plant. In the nodules of *Trifolium repens* γ -aminobutyric acid was reported³⁹ but in genera examined here, only small quantities of this amino-acid were detected. β -alanine, a decarboxylation product in Leguminosæ, occurred in large quantities in *Dolichos* and *Vigna*, with less amounts in *Cyamopsis*. Arginine occurred in larger quantity in nodules of *Arachis* than in others, while phenylalanine, tryptophane and tyrosine were not observed in most, with the probable exception of *Dolichos*.

The preponderance of amides in the soluble nitrogen of nodules in the tropical species mentioned here indicate effectiveness of their symbiosis which their high haemoglobin content further serves to emphasize.

THE PRACTICAL ASPECTS OF LEGUME NODULATION TO TROPICAL AGRICULTURE

The quantity of nitrogen fixed by a legume per acre depends on the nodule number, their size, longevity, the bacterial strain, condition of

plant growth and crop management. While fixation-benefit depends on the nodule volume per acre of crop, very few field observations have so far been made in the tropics. Crops in Britain have been shown to have higher nodule weight per plant than tropical crops which may possibly be due to higher soil moisture and lower temperature. Thus, Russell⁴⁰ considered that in species of *Vicia*, *Phaseolus*, *Arachis*, *Glycine* and *Vigna* which may have between 100 and 1,000 nodules, the individual nodule weight may vary between 1 and 40 mg. In many cultivated legumes grown in association with an effective cow-pea strain in this laboratory, this weight ranged from 4.5–225.0 mg. per nodule which showed that some Indian crop plants have, on an average, greater weight per nodule than was previously known. Their haemoglobin contents (1.707 to 66.205 µg. per nodule and 240 to 835 µg./g. fresh nodules) clearly indicate effective symbiosis. However, far too little is known about tropical crops to make any generalization. The statement that legumes carry nodules on their roots and that they benefit the soil is only doubtfully true for many large-seeded cultivated crops such as pigeon-pea, bean, groundnut, sunnhemp, green-, black- and horse-gram. This is so because if they carry nodules, which is not often so under good farming conditions, most nitrogen fixed is removed from the land in the seed crop while nearly all the rest goes with vines or straw at harvest. Russell⁴⁰ points out that in the tropics "many legumes grown for the ostensible purpose of raising the soil nitrogen level are not even nodulated for most of their growing season". Whether this lack of nodulation applies only to genera or entire subfamilies remains unknown. Undoubtedly the problem of root nodule nitrogen fixation requires reinvestigation in the tropics.

We thank Drs. L. Saraswathi-Devi and C. E. Sulochana for critically reading the manuscript.

1. Rajagopalan, N., "Studies on nodule bacteria", *Doctoral Thesis*, Univ. Madras, 1963.
2. Allen, O. N. and Baldwin, I. L., *Soil Sci.*, 1954, **78**, 415.
3. Wilson, P. W., *Ibid.*, 1944, **58**, 61.
4. Allen, O. N. and Allen, E. K., *Bot. Gaz.*, 1940, **102**, 121.
5. Jensen, H. L., *Proc. 5th Easter School*, Nottingham Univ., Butterworth Sci. Publ., London, 1958, p. 75.
6. Bowen, G. D. and Kennedy, M. M., *Qd J. agric. Sci.*, 1951, **18**, 161.
7. Norris, D. O., *Proc. 5th Easter School*, Nottingham Univ., Butterworth Sci. Publ., London, 1958, p. 164.
8. Sadasivan, T. S. and Subramanian, C. V., In *Plant Pathology*, Academic Press, New York, 1960, p. 272.
9. Lechhead, A. G., *Annu. Rev. Microbiol.*, 1952, **6**, 185.
10. Timonin, M. I., *Canad. J. Res.*, 1940, **18**, 446.
11. Lakshmi-Kumari, M., "Rhizosphere microfloras and host parasite relationships," *Doctoral Thesis*, Univ. Madras, 1961.
12. Bhuvaneshwari, K., "Studies on the rhizosphere microfloras of crop plants," *Ibid.*, 1958.
13. Starkey, R. L., *Soil Sci.*, 1929, **27**, 319.
14. Manil, P., *Proc. 5th Easter School*, Nottingham Univ., Butterworth Sci. Publ., London, 1958, p. 232.
15. Bhadhuri, S. N., *Ann. Bot., Lond., N.S.*, 1951, **15**, 209.
16. Rovira, A. D., *Aust. J. agric. Res.*, 1961, **12**, 77.
17. Purchase, H. F. and Nutman, P. S., *Ann. Bot., Lond., N.S.*, 1957, **21**, 439.
18. Swaby, R. J. and Sherber, J., *Proc. 5th Easter School*, Nottingham Univ., Butterworth Sci. Publ., London, 1958, p. 289.
19. Rao, A. S., *Plant and Soil*, 1962, **8**, 260.
20. Preston, W. M., Mitchell, J. W. and Reeve, W., *Science*, 1954, **119**, 437.
21. Elkan, G. H., *Canad. J. Microbiol.*, 1962, **8**, 79.
22. Leleuil, G., *C.R. Acad. Sci., (Paris)*, 1954, **238**, 2185.
23. Metz, H., *Arch. Microbiol.*, 1955, **23**, 297.
24. Borner, H., *Beitr. Biol. Ph.*, 1937, **33**, 33.
25. Garrett, S. D., *Biology of Root Infecting Fungi*, Cambridge Univ. Press, 1956.
26. Evenari, M., *Encycl. Pl. Physiol.*, 1961, **16**, 691.
27. Sadasivan, T. S., *Proc. Summer School Bot.*, Ministry Sci. Res. Cult. Aff., Govt. of India, New Delhi, 1962, p. 363.
28. Allen, E. K. and Allen, O. N., *Encycl. Pl. Physiol.*, 1958, **8**, 48.
29. Bergersen, F. J. and Nutman, P. S., *Heredity*, 1957, **11**, 175.
30. Hallsworth, F. G., *Proc. 5th Easter School*, Nottingham Univ., Butterworth Sci. Publ., London, 1958, p. 183.
31. Virtanen, A. I., *Proc. intern. Congr. Biochem., 3rd Congr. (Brussels, 1955)*, p. 425.
32. —, Erkama, J. and Linkola, H., *Acta chem. Scand.*, 1947, **1**, 861.
33. — and Miettinen, J. K., *Ibid.*, 1949, **3**, 17.
34. Thornton, H. G., *Sci. Progr.*, 1954, **42**, 185.
35. Smith, B. W., *Amer. J. Bot.* 1954, **41**, 607.
36. Thornton, H. G., *Trans. 3rd Comm. intern. Soc. Soil Sci.*, 1939, p. 2).
37. Chen, H. K. and Thornton, H. G., *Proc. roy. Soc.* 1949, **129 B**, 203.
38. Steward, F. C. and Thompson, J. F., *Annu. Rev. Pl. Physiol.*, 1950, **1**, 233.
39. Butler, O. W. and Bathurst, N. O., *Aust. J. biol. Sci.*, 1958, **11**, 529.
40. Russell E. J., *Soil Conditions and Plant Growth*, 9th Edn., Longmans, Green & Co., 1961, p. 327.