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HAEMOGLOBIN DETERMINATION AND ITS VALUE AS AN EARLY INDICATION OF PEANUT RHIZOBIUM EFFICIENCY *

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SUMMARY

A modified procedure based on the cyanmethaemoglobin method for haemoglobin (Hb) determination in peanut nodules is described. It gave reliable results when tested on peanuts at all stages of vegetative growth, including when the green pigment was present in the nodules.

It was shown that early (32–34 days after planting) determination of Hb in nodules of peanuts grown in a greenhouse under bacteriologically controlled conditions can be taken as a measure of the nitrogen-fixing ability of peanut Rhizobia and thus much of the time and greenhouse facilities usually required for strain screening may be conserved.

Early determination of nitrogen concentration in leaflets of peanuts proved to be also a useful measure of Rhizobium efficiency, but less sensitive than the early determination of nodule haemoglobin.

Virtanen *et al.*[9 10] studied the relation between haemoglobin (Hb) content of leguminous root nodules and the intensity of nitrogen fixation by the associated root nodule bacteria. They employed the prosthetic group of haemoglobin, haematin, determined as pyridine haemochromogen, for evaluation of haemoglobin concentration in nodules of peas, horse bean, and soyabean. Most other investigators[1 5 7 8] who confirmed Virtanen's findings also employed this method for the evaluation of haemoglobin in nodules of various legumes from temperate and hot regions, like vetch, lupin, clover, alfalfa, soyabean, cowpea, and peanut.

A serious disadvantage of the pyridine haemochromogen method seems to be its lack of reliability[2 5 9] when used for Hb evaluation in

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nodules which contain, in addition to Hb, the green pigment cholеglobin which results from Hb decomposition. Such decomposition occurs under the influence of plant aging or unfavorable growth conditions, or as a result of relatively ineffective bacterial strains.

Graham and Parker [3] used a modified cyanmethaemoglobin (CMHb) method for Hb determination in nodules of lupin, but did not give details of their procedure.

Wilson and Reisenauer [11] listed several disadvantages of the pyridine haemochromogen method and described a modified CMHb method, which they found useful for Hb determination in soyabean nodules. This method, as described by the authors, gave erroneous results in our experiments with peanuts. It was therefore necessary to modify further the procedure in order to make it suitable for the determination of Hb in peanut nodules.

A further aim of the present investigation was to ascertain whether, under controlled greenhouse conditions, early determination of the Hb content of peanut nodules, or of the nitrogen concentration in the leaves of the plant, might be useful in predicting the relative efficiency of Rhizobium strains or isolates. This would eventually permit the speedy elimination of isolates of little value from further trials. Usually, the screening of strains is performed under bacteriologically controlled conditions in the greenhouse where legumes inoculated by isolates under examination are compared with controls consisting of uninoculated plants and of plants inoculated by a standard Rhizobium strain. Such comparisons become possible only after the plants have been growing for the relatively long time needed to permit differences to be observed between the various treatments with respect to the dry weight of the tops and their nitrogen content. The time in the case of peanuts is 2 to 3 months. A less time-consuming method for screening of strains would produce savings also in greenhouse space.

MATERIALS AND METHODS

Haemoglobin determinations

Hb determinations were performed on peanut nodules originating from plants grown under field and greenhouse conditions for various growth periods.

Absorption spectra measurements revealed whether the Hb derivative

(methaemoglobin) or other non-related pigments prevailed in nodule extracts prepared by various procedures.

The reliability of the Hb determinations was checked by reproducibility tests, at various stages of peanut growth.

The possibility of preserving the peanut nodules before carrying out the Hb determination was also considered.

Strain screening

Two separate experiments were conducted under bacterial- and temperature-controlled conditions in a greenhouse. One experiment lasted from June 16 to August 24 (69 days) and the other from December 6 to February 8 (64 days). The first experiment included six Rhizobium isolates of unknown performance chosen at random, and the second one included five other previously untested isolates. In both experiments, peanuts inoculated by the isolates were compared with controls consisting of peanuts inoculated by a standard effective strain (205A), and non-inoculated and nitrogen-non-fertilized peanuts.

Peanut plants were grown according to Leonard's technique [6] in vessels which contained 3½ kg of sand poor in nitrogen.

The Hb content of the nodules, and the nitrogen and dry matter contents of the leaflets (without the rachis of the compound leaves) and the plant tops were determined at various stages of growth on three plants taken from each treatment group.

The Hb content of the nodules and the nitrogen percentage in the leaflets at early stages of growth were correlated with the usual 'late' criteria of Rhizobium effectivity, *i.e.* dry matter in plant tops and leaflets, percentage of nitrogen in tops and leaflets, and the total nitrogen content of tops and leaflets at the conclusion of the experiment.

RESULTS AND DISCUSSION

Hb determination

The absorption spectra of extracts of peanut nodules prepared according to Wilson and Reisenauer's procedure did not reveal the presence of Cn-methaemoglobin. Even extracts of young nodules (free from the green pigment) did not show the characteristic absorption peak at 540 mμ (Fig. 1). However, we found the following preparation procedure suitable. Peanut nodules were carefully detached from the plant and placed in a dish containing distilled water, completely freed from any adjacent rootlets and then dried with blotting paper. Representative 0.5-g samples were immediately crushed using a pestle in a flat bottom tube containing 3 ml of Drabkin's solution [11], for precisely one minute. Immediately thereafter, the supernatant was decanted into a centrifuge tube. The

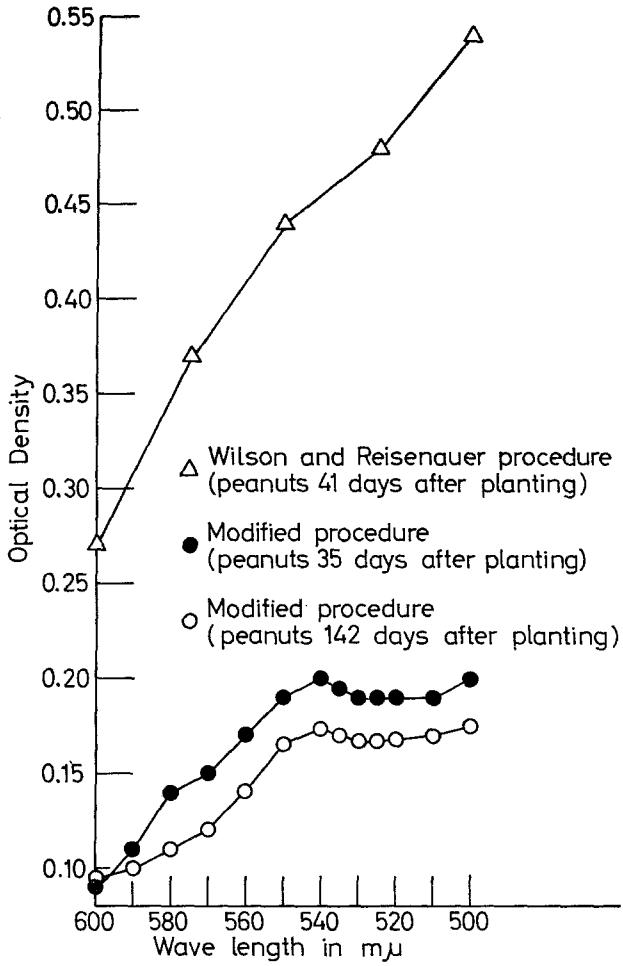

Fig. 1. Absorption spectra of peanut nodule extracts prepared by different procedures.

remaining sediment was twice washed quickly with 1 ml and once with 0.5 ml of Drabkin's solution, and each supernatant was added to the centrifuge tube. The combined extract was centrifuged in a Servall-type SS-1A centrifuge at 500 rpm for 15 minutes. The supernatant was then filtered through a sterilizing Seitz filter, which was afterwards washed by filtering thrice 1 ml and once 0.5 ml of Drabkin's solution. The combined filtrate was transferred to a 10-ml

volumetric flask, brought to 10 ml with Drabkin's solution, mixed and centrifuged at 20,000 rpm for 30 minutes. The absorbance of the clear supernatant was read at 540 mμ against Drabkin's solution in a Coleman Universal Spectophotometer model 14. Standard curves were prepared using Acuglobin, the CN-methaemoglobin solution of Ortho Pharmaceutical Corporation, Raritan, New Jersey, U.S.A.

Nodule extracts prepared in this manner gave, irrespective of the plant's age, an absorption spectrum with the characteristic Cn-methaemoglobin peak at 540 mμ (Fig. 1).

TABLE 1

Reproducibility of the modified CN-methaemoglobin determination procedure

Age of peanut plant (days after planting)	Hb/g nodules fresh weight (means) (γ)	Standard deviation	Coefficient of variance
17	1361	41.70	3.06
20	1982	58.37	2.94
28	3652	58.94	1.61
35	3690	29.46	0.80
58	3838	49.66	1.29
82	4968	52.73	1.06
97	4182	72.30	1.73
110	3723	24.75	0.66

The coefficient of variance in reproducibility tests (Table 1) ranged from a minimum of less than 1% to a maximum of about 3%, and was in most cases considerably less than the maximum. Considering the inherent variability of such biological material as root nodules, the reproducibility of the haemoglobin extraction tests prepared by this modified method seems to be satisfactory.

Preservation of the root nodules for longer periods of time pending Hb determination presented problems (Table 2). Detached root nodules kept for 24 hours at temperatures just above freezing yielded slightly less Hb than when analysed without delay, the differences in some cases being significant. Storage for 48 hours or freezing of nodules lowered the amount of haemoglobin extracted significantly.

TABLE 2

The effect of storage of nodules on their haemoglobin content

	Experiment 1 Storage at 4°C* or at −14°C** Hb (γ)	Experiment 2 Storage at 4°C Hb (γ)	Experiment 3 Storage at 4°C* (or in a portable ice-box at about the same temp.**) Hb (γ)	Experiment 4 Storage at 4°C* (or in a portable ice-box at about the same temp.**) Hb (γ)
Shortly before storage	3838 a	3723 a	3673 a	3612 a
After 24 hours	3655*a	3641 a	3542* b	3396* b
	2854**b	—	3577**b	3433**b
After 48 hours	2762* b	3075 b	—	—
S.E.	60.9	29.0	16.2	21.0

Means in a given column followed by the same letter do not differ significantly; those with different letters are significantly different at $P = 0.05$ according to the sequential multiple range test [4].

Strain screening

The results of the two greenhouse experiments suggest that under the stated experimental conditions, Hb concentration in nodules and nitrogen percentage in leaflets at a relatively early period of growth predetermine also to a large measure the levels of these components during the later stages of vegetative growth.

In the first experiment (Figs. 2, 3), three distinct strain groups can be distinguished. One group consists of strains 242A and 249A and is characterized by consistently low levels of Hb and nitrogen during the growth period.

The second group is composed of strains 225A and 238A, with intermediate Hb and nitrogen levels, and the third, of strains 205A, 259A, and 263A, displaying high Hb and nitrogen production.

In the second experiment (Figs. 4, 5) again two separate groups of strains are apparent: strains 265A and 267A from one group, having lower concentrations of Hb and of nitrogen, while the other group is composed of strains 205A, 264A, 268A and 269A, with higher concentrations of Hb in the nodules and of nitrogen in the leaflets.

Analysis of the data (Table 3) indicates the existence of a close correlation between Hb concentration in nodules or nitrogen per-

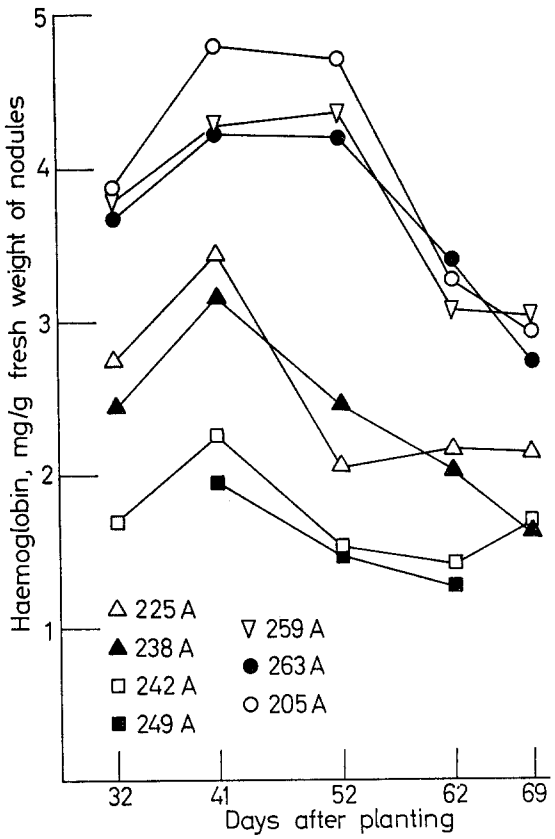

Fig. 2. Experiment 1. Haemoglobin concentration in fresh nodules of peanuts inoculated with various strains of Rhizobium.

centage in leaflets determined at the relatively early stages of peanut vegetative growth (32 or 34 days after planting) and the various parameters normally used for assessing Rhizobium efficiency, obtainable at a much later stage of greenhouse experiments. In our first experiment (16.VI.66), the correlation was significant between Hb or nitrogen concentration at the beginning of growth and all six usual 'late' efficiency parameters, established 69 days after peanut planting (Table 3, exp. 1). At the conclusion of this experiment a significant differentiation was found in plants inoculated with different isolates with respect to five of the six 'late' criteria for Rhizobium efficiency (Table 4).

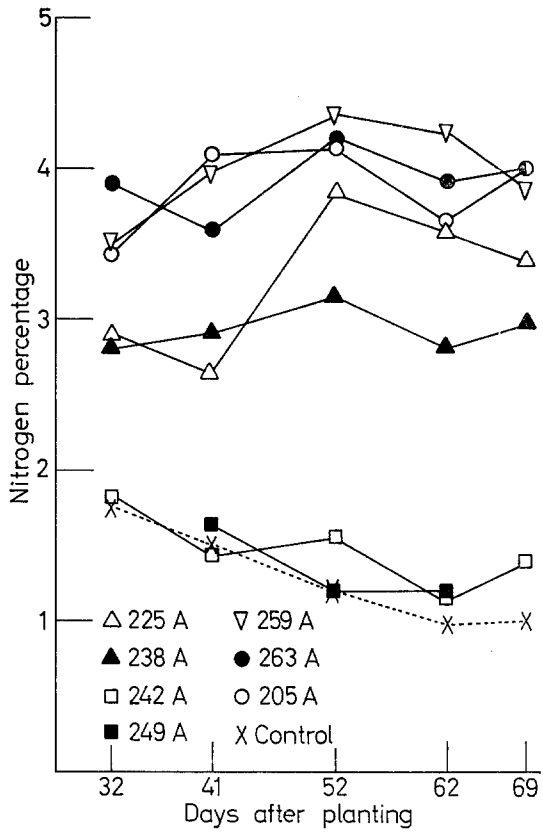

Fig. 3. Experiment 1. Nitrogen percentage in oven-dried leaflets of peanuts inoculated with various strains of Rhizobium.

There was no such pronounced differentiation apparent at the end of the second experiment (6.XII.66, Table 4), probably because of the short days of December and January, which slowed down the rate of plant growth. Thus, in the second experiment, the correlation (Table 3, exp. 2) between the 'early' and 'late' criteria of strain efficiency was not so general as in the first.

There was a significant correlation between Hb concentration (at 34 days after planting) and four of the six 'late' criteria for strain efficiency. However, the nitrogen percentage in leaflets (at day 34) correlated significantly only with two of the six usual 'late' criteria (Table 3, exp. 2). Thus, when differentiation between strains is slight, early determination of the Hb concentration is a more sensitive

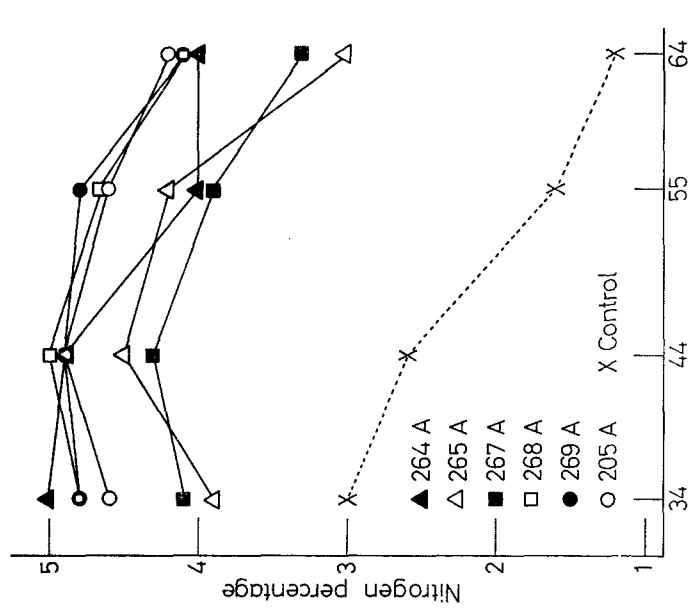

Fig. 5. Experiment 2. Nitrogen percentage in oven-dried leaflets of peanuts inoculated with various strains of Rhizobium.

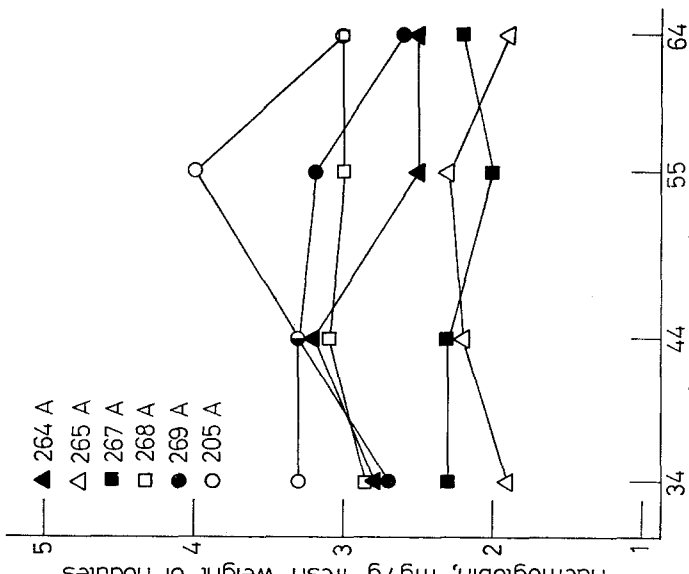

Fig. 4. Experiment 2. Haemoglobin concentration in fresh nodules of peanuts inoculated with various strains of Rhizobium.

TABLE 3

Regression coefficients and correlation between haemoglobin concentration in nodules or nitrogen percentage in leaflets at early stages of growth and the various 'late' parameters of Rhizobium efficiency

Y: Parameters of Rhizobium eff. at the end of the experiment, after 69 days (for exp. 1) and after 64 days (for exp. 2)	X: mg Hb/g fresh weight of nodules 32 days (for exp. 1) and 34 days (for exp. 2) after planting		X: % N in leaflets 32 days (for exp. 1) and 34 days (for exp. 2) after planting	
	Regression coefficient (b_{xy})	Correlation coefficient (r)	Regression coefficient (b_{xy})	Correlation coefficient (r)
Experiment 1 – planting date 16.VI.1966				
Amount of total N in tops (mg)	0.101 **	0.97 **	0.1251 **	0.98 **
Amount of total N in leaflets (mg)	0.0736 **	0.98 **	0.0884 **	0.96 **
% N in tops	0.6750 **	0.82 *	0.8368 **	0.94 **
% N in leaflets	1.0600 **	0.94 **	1.3265 **	0.96 **
Dry weight of tops (g)	2.67 **	0.94 **	3.3296 **	0.97 **
Dry weight of leaflets (g)	1.12 **	0.95 **	1.3203 **	0.92 **
Experiment 2 – planting date 6.XII.1966				
Amount of total N in tops (mg)	0.0431 **	0.92 **	—	0.77 n.s.
Amount of total N in leaflets (mg)	0.0367 *	0.91 *	—	0.80 n.s.
% N in tops	0.6220 **	0.99 **	0.5698 *	0.83 *
% N in leaflets	0.8650 *	0.89 *	0.9888 **	0.93 **
Dry weight of tops (g)	—	0.56 n.s.	—	0.42 n.s.
Dry weight of leaflets (g)	—	0.46 n.s.	—	0.51 n.s.

* = significant at $P = 0.05$
** = significant at $P = 0.01$
n.s. = non significant

measure of strain efficiency than early determination of nitrogen concentration in the leaflets.

In view of the above result its seems justifiable to consider the early determination of Hb concentration in peanut nodules a useful measure of Rhizobium efficiency, thus permitting savings in time and greenhouse space when large-scale peanut strain screening is undertaken.

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TABLE 4

Differentiation between isolates in respect to the 'late' Rhizobium efficiency parameters (criteria) at the end of the experiment

Treatments	Late criteria (parameters) of Rhizobium efficiency					
	Dry weight of tops/ plant (g)	Dry weight of leaflets/ plant (g)	% of N in tops	% of N in leaflets	Amount of N in tops/ plant (mg)	Amount of N in leaflets/ plant (mg)
Exp. 1 (16.VI.1966)						
Inoculated with Rhizobium:						
no. 205A (control)	10.61 ab	4.72 a	2.45 a	3.99 a	260 a	190 a
no. 225A	7.55 ab	3.31 a	2.26 a	3.40 ab	170 ab	110 bc
no. 238A	7.17 ab	2.74 a	1.71 b	2.98 b	125 bc	80 cd
no. 242A	4.91 b	2.43 a	0.82 c	1.40 c	40 c	30 d
no. 259A	9.42 ab	4.15 a	2.37 a	3.85 a	225 ab	160 ab
no. 263A	11.71 a	4.93 a	2.47 a	4.02 a	285 a	200 a
Non-inoc. control	5.65 ab	2.63 a	0.71 c	1.01 c	40 c	20 d
L.S.D.	3.74	1.39	0.22	0.45	81	50
S.E.	1.08	0.40	0.065	0.130	23.4	16.1
Exp. 2 (6.XII.1966)						
no. 205A (control)	4.92 a	2.20 a	2.75 a	4.22 a	140 a	100 a
no. 264A	5.51 a	2.57 a	2.54 b	4.02 a	140 a	110 a
no. 265A	3.97 a	1.75 a	1.88 d	3.10 b	80 a	60 a
no. 267A	4.96 a	2.44 a	2.22 c	3.32 b	110 a	80 a
no. 268A	4.79 a	2.35 a	2.50 b	4.08 a	120 a	90 a
no. 269A	4.30 a	2.05 a	2.46 b	4.06 a	110 a	80 a
Non-inoc. control	2.53 a	1.37 a	0.99 c	1.18 c	30 b	20 b
L.S.D.	2.25	1.20	0.19	0.48	48	40
S.E.	0.650	0.348	0.054	0.140	14.1	11.6

Means in a given column followed by the same letter do not differ significantly; those with different letters are significantly different at $P = 0.05$ according to the sequential multiple range test [4].

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