Soil Biol. Biochem. Vol. 22, No. 2, pp. 203-207, 1990
Printed in Great Britain.
0038-0717/90 $3.00 + 0.00

NON-DESTRUCTIVE CHLOROPHYLL ASSAY FOR SCREENING OF STRAINS OF *BRADYRHIZOBIUM JAPONICUM*

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(Accepted 15 August 1989)

Summary-In greenhouse-grown soybeans [*Glycine max* (L.) Merr. cv Clark] supplied with different amounts of combined nitrogen (0, 75, 150, 300 or 600 mg N as $NH_4NO)_3$, the chlorophyll content of leaf discs was significantly correlated with shoot dry weight ($r = 0.90$; $P < 0.001$) and shoot total N ($r = 0.88$; $P < 0.001$). In a parallel experiment with plants inoculated with *Bradyrhizobium japonicum* strains of varying effectiveness, there was a highly significant ($P < 0.001$) correlation between chlorophyll and shoot dry weight ($r = 0.89$); shoot total N ($r = 0.88$): shoot N-fixed ($r = 0.88$); nodule dry weight ($r = 0.85$); and total nitrogenase activity ($r = 0.79$). Effectiveness ranking of strains of *B. japonicum* based on chlorophyll estimates and shoot total N were similar. In field-grown soybean plants, the chlorophyll content of leaf discs was also significantly ($r = 0.92$; $P < 0.001$) correlated with N accumulated in the plant tops. Our data support the use of leaf chlorophyll content as an indirect measure of N status of greenhouse and field-grown soybeans and the effectiveness ranking of strains of rhizobia.

INTRODUCTION

Leguminous plants are able to obtain significant amounts of N through N_2 fixation by entering into a symbiotic relationship with rhizobia (e.g. Nutman, 1976; LaRue and Patterson, 1981). However, establishment of effective symbiosis cannot be left to chance but requires a careful selection of effective strains of rhizobia for inoculation. Burton (1974) and Date (1976) suggested that the strains of rhizobia to be used as inoculum should be first screened under controlled environmental conditions for their effectiveness in terms of N_2 fixation before field trials are conducted. Several direct and indirect methods of measuring the rate and magnitude of N_2 fixation have been employed (e.g. Havelka *et al.*, 1985). Of these methods, plant dry matter, plant colour, plant total N (Wynne *et al.*, 1980) and acetylene reduction (e.g. Hardy and Holsten, 1977; Masterson and Murphy, 1976, 1980) are widely used for screening rhizobia for their effectiveness. The acetylene reduction assay, although it has advantages of sensitivity, speed and economy, has been criticized (Minchin *et al.*, 1983), because it is a single point, indirect, and often destructive measure of N_2 fixation potential. To measure the effectiveness of rhizobial strains under controlled conditions, the most useful criterion is the measurement of total N accumulated in plant tissues. This is usually determined by the Kjeldahl technique which is time consuming and expensive. Thus, although several methods have been developed, a need still exists for rapid, inexpensive and reliable method for ranking rhizobial strains for effectiveness. The leading role of chlorophyll in photosynthesis and interdependence of photosynthesis and N supplied, either exogenously or via *Rhizobium* symbiosis, is now well known (e.g. Bethlenfalvay *et al.*, 1978). Wynne *et al.* (1980) used leaf colour as an indicator of the effectiveness of peanut rhizobia. This suggests, that, when all other nutrients are supplied and growth conditions standardized, a relationship may exist between plant total N and chlorophyll content. However, to our knowledge the potential usefulness of such a relationship for estimating symbiotic N_2 fixation in legumes grown under greenhouse and field conditions has not been reported. Our objectives were to use soybean plants to (a) develop and evaluate a non-destructive, simple, inexpensive quick and reliable assay for chlorophyll determination of greenhouse and field-grown plants; (b) use this assay as an indirect indicator of N content of plants, and (c) use the assay for ranking strains of *Bradyrhizobium japonicum* differing in their N_2-fixing effectiveness.

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MATERIALS AND METHODS

Soybean seeds and rhizobia

Seeds of nodulating and non-nodulating isolines of soybean [*Glycine max* (L.) Merr. cv Clark], were obtained from the NifTAL Project, University of Hawaii, Paia. Strains of *B. japonicum* included in the study were: USDA 110; USDA 123; USDA 136b; USDA 138; USDA 31 and USDA 33 (obtained from USDA-ARS culture collection, Beltsville, Md); and SM-5, and ineffective mutant of Nitragin strain 61A76 from W. J. Brill, University of Wisconsin, Madison. Cultures were grown in yeast mannitol broth (Vincent, 1970) for use as inoculum.

Table 1. Effect of various N concentrations on shoot dry weight, shoot total, N_2 contents and chlorophyll measurements of glasshouse-grown soybean [*Glycine max* (L.) Merr cv Clark]

Nitrogen Concentrations (mg N per jar)	Chlorophyll (a + b) (mg 100 cm^{-2})	Shoot dry weight (g per jar*)	Shoot total N (mg per jar*)	Shoot % N
0	0.58d†	1.17e	21.17e	1.76cd
75	1.18c	5.78d	94.65d	1.63d
150	1.36c	8.89c	164.26c	1.86c
300	1.90b	15.10b	317.87b	2.11b
600	2.35a	17.83a	610.02a	3.43a
cv (%)	17.68	10.52	10.67	8.46

*Values are per Leonard jar containing 2 plants.
†Means having the same letter within a column are not significantly different at 5% level of probability as shown by Duncan's Multiple Range Test.

Plant culture

Seeds were sorted for uniform size, surface sterilized using 2% NaOCI for 2 min followed by at least 5 rinses in sterile water and left to imbibe in sterile water for 2-3 h in a refrigerator. After two additional rinses, seeds were germinated at room temperature for 36 h on 1 % (w/v) water agar in Petri dishes. Three seedlings were planted per modified Leonard jar (Vincent, 1970) packed with horticulture-grade vermiculite as rooting medium. An N-free nutrient solution (Broughton and Dilworth, 1971) was used in the Leonard jars. After sowing and inoculation the surface was covered with a layer *(ca* 0.5 cm deep) of sterilized gravel to avoid contamination. Plants were grown in a greenhouse under natural light. Seven days after planting they were thinned to 2 per Leonard jar.

Relationship between chlorophyll and total N

To investigate the relationship between chlorophyll and total N contents of the shoots, soybean plants were grown in the Leonard jars and supplied with five different concentrations of combined nitrogen as NH_4N0_3 applied as liquid stock solution in equal split applications at time zero and 17 days after planting. The five treatments were: 0, 75, 150, 300 or 600 mg N. These were pippeted directly into the reservoir portion of the Leonard jar containing the N-free nutrient solution. There were 8 replicates of each treatment and the experiment was arranged in completely randomized design. At harvest (42 days after planting) leaves were sampled for chlorophyll determination then the plant tops were oven dried at 65°C for 48 h and dry weights recorded. Dried plants were then milled and subjected to Kjeldahl digestion followed by measurement of total N by the method of Mitchell (1972) with a Technicon autoanalyser.

Ranking strains of B. japonicum

To rank the strains of *B. japonicum* using chlorophyll content as indirect indicator of effectiveness, the nodulating cultivar of soybean was inoculated with strains of *B. japonicum* known to differ in their N2 fixation capacity (Singleton *et al.,* 1985). Immediately after sowing each treatment was inoculated with *ca* 10^9 rhizobia per seedling of the appropriate strains of *B. japonicum.* The ineffective SM-5 served as non-fixing control. There were 5 replicates of each of the inoculated treatments and the experiment was arranged in a completely randomized design. At harvest (42 days) plants were sampled for chlorophyll determination, dry weight and shoot total N. For total nitrogenase activity (C_2H_2 reduction) roots of de-topped plants were shaken free of vermiculite and exposed in 10% (v/v) acetylene in air-tight polypropylene bottles for 1 h. Samples were analyzed for C_2H_2 production by gas chromatography. Roots were then washed, nodules removed and oven-dried at 65°C for 48 h for recording dry weight. Shoot N-fixed was derived by computing the differences in total N between plants inoculated with *B. japonicum* strains of varied effectiveness and the non-fixing SM-5-inoculated control plants.

Chlorophyll measurements

At harvest, using a standard single-hole paper punch (H2 MG 4038, McGill Co., Chicago, 111.) leaf discs (6.35 mm dia) were cut from leaflets taken at random but excluding the youngest and oldest trifoliates leaves. Six leaf discs, the minimum required for analysis (one per leaflet) were removed from the uninoculated controls and from plants inoculated with USDA 33, because of their small size in the greenhouse studies; in all other cases 12 discs were removed. Leaf discs were transferred immediately to screw-capped glass vials containing 5 ml (6 discs) or 10 ml (12 discs) of 80% aqueous acetone (analytical grade). Chlorophyll was extracted by keeping the vials in the dark at 4°C for 12 h. Chlorophyll (a + b) concentration was determined spectrophotometrically (Arnon, 1949; Mackinney, 1941; Bruinsma, 1961). To prevent the decomposition of pigments all operations were performed under subdued light. All glassware had been washed with concentrated sodium phosphate.

Statistical analysis

Each experiment was analysed separately by analysis of variance. Simple coefficients of linear correlation among growth and nodulation characteristics including chlorophyll measurements were computed using individual observations as entries.

RESULTS AND DISCUSSION

Increasing amounts of exogenously-supplied mineral N resulted in highly significant ($P < 0.001$) increases in shoot dry weight, shoot total N and chlorophyll content (Table 1). Shoot dry weight, shoot total N and chlorophyll measurements showed

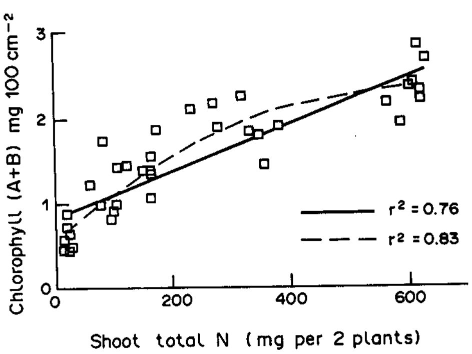

Fig. 1. Relationship between chlorophyll and shoot total N in 42-day-old glasshouse-grown soybean plants grown with 5 different concentrations of N_2.

highly significant correlation with one another as follows: shoot dry weight vs shoot total N, r = 0.93 (P < 0.001); shoot dry weight vs chlorophyll, r = 0.90 (P < 0.001) and shoot total N vs chlorophyll, r = 0.88 (P < 0.001). The relationship between total shoot N and chlorophyll is illustrated in Fig. 1 where data were fitted to a linear function and a regression line was used to describe the relationship although it was probably curvilinear. These results suggest that the response of soybean to mineral N application could be expressed by any of the three measurements made. An R^2 value of 0.76 in Fig. 1, further indicates that ca 76% of variation in chlorophyll is due to varying amounts of N accumulated in

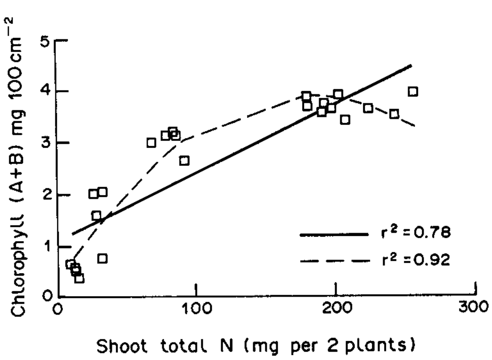

Fig. 2. Relationship between chlorophyll and shoot total N in glasshouse-grown soybean plants inoculated with strains of *B. japonicum* of a range of symbiotic effectiveness.

shoots, and that the chlorophyll measurements could be used as an indirect indicator of N status of soybean plants fertilized with mineral N. In ranking the rhizobia, variability in the effectiveness of the strains of *B. japonicum* was clearly reflected in highly significant effects in shoot dry weight, shoot total N, percent shoot N, shoot N fixed, total nitrogenase activity (TNA), specific nitrogenase activity (SNA) and chlorophyll measurements (Table 2).

The effectiveness of the strains of *B. japonicum* was measured using the established methods for N_2-fixation, as well as the total chlorophyll estimates as an indirect indicator of N_2-fixation. By shoot dry weight measurements (Table 2) the strains of *B. japonicum* could be ranked in the following order of decreasing effectiveness: USDA 110 > USDA 123 > USDA 31 > USDA 33 > SM-5. Shoot N measurements showed similar order of ranking. However, the differences in effectiveness between USDA 110 and 123 were not significant for shoot total N or shoot N fixed and % N values for USDA 123 were greater than for USDA 110. This effect can be explained by examining the nodule dry weight and total nitrogenase activity measurements (Table 2). Strain USDA 123 produced significantly more nodule mass to fix the same amount of N_2 as fixed by USDA 110. Considering the dependence of N_2-fixation process on photosynthate supply by the host plant (Finke *et al.,* 1982), soybean plants inoculated with USDA 123, had to divert comparatively larger amounts of carbon fixed to the nodulated root system for the maintenance of less efficient nodules (Table 2).

When the chlorophyll measurements were used to rank the strains of *B. japonicum,* a ranking similar to that obtained by using shoot total N was achieved (USDA 110 = USDA 123 > USDA 31 > USDA 33 > SM-5), indicating the reliability of chlorophyll data for such effectiveness rankings. Although shoot dry weight and total N contents did not differentiate the completely yellow SM-5 and slightly yellow-green USDA 33 inoculated plants, the chlorophyll measurements were significantly different (Table 2), thus indicating sensitivity of the chlorophyll method.

Strain SM-5 was found to be non-fixing as indicated by all tests (Table 2). Also, all tests (Table 2) showed that USDA 110 was more effective on Clark soybeans than was USDA 31. This is the reverse of results with variety Davis (Singleton *et al.,* 1985).

The linear coefficients of correlation for indicators of N_2-fixation capacity in soybean in response to

Table 2. Effect of strains of *B. japonicum* inoculation on chlorophyll contents, growth, nodulation and N_2-fixation characteristics of glasshouse-grown soybean

	Growth characteristics							
B. japonicum	Chlorophyll (a + b) (mg 100 cm^{-2})	Shoot dry weight (g per jar)‡	% N	Shoot total N (mg per jar)‡	Shoot N fixed (mg per jar)‡	Nodule dry weight (mg per jar)‡	SNA*	TNA†
SM 5	0.51d§	1.18d	1.04e	12.27c	0.00	117.e	0.00	0.00
USDA 33	1.60c	1.60d	1.79d	27.74c	16.47c	180d	87.26b	7.83c
USDA 31	3.03b	3.80c	2.15c	82.80b	70.53b	379c	87.53b	16.40b
USDA 123	3.75a	6.55b	3.21a	203.66a	191.39a	974a	46.43c	22.53b
USDA 110	3.69a	7.70a	2.79b	214.64a	202.36a	620b	147.93a	46.28a
cv (%)	11.06	13.36	6.67	15.97	16.23	10.42	23.10	25.68

*Specific nitrogenase activity (SNA), μmol C_2H_4 g nodule dry weight^{-1} h^{-1}.
†Total nitrogenase activity (TNA), μmol C_2H_4 plant^{-1} h^{-1}.
‡Values are per Leonard jar containing 2 plants.
§Means having the same letters within a column were not significantly different by Duncan's Multiple Range test at 5% probability.

Table 3. Coefficients of linear correlation (r) for chlorophyll contents, growth, nodulation and N_2-fixation characteristics of glass-grown soybean as affected by inoculation with strains of *B. japonicum* of varied effectiveness

	Growth characteristics†					
Traits	Shoot dry weight	Shoot total N	Shoot N fixed	Nodule dry wieght	TNA	SNA
Shoot total N	0.98***	—	—	—	—	—
Shoot N fixed	0.98***	1.00***	—	—	—	—
Nodule dry weight	0.87***	0.91***	0.91**	—	—	—
TNA	0.92***	0.86**	0.80***	0.62***	—	—
SNA	0.57**	0.50*	0.17 NS	0.22 NS	0.69***	—
Chlorophyll (a + b)	0.89***	0.88***	0.88***	0.85***	0.79***	0.60**

*, **, ***Indicate simple correlation coefficients significant at 0.05, 0.01 and 0.001 levels of probability.
†Explanation as in Table 2.
NS, not significant.

B. japonicum inoculation are shown in Table 3. Chlorophyll measurements were found to be highly correlated with all other indicators of N_2-fixation although again the relationship between chlorophyll content and shoot N appeared curvilinear. This might be expected, since when chlorophyll concentration no longer limits plant growth plants may continue to respond to nitrogen by increasing in size. Of particular interest is its good correlation with total N accumulated in plant tops which is considered to be the most reliable measure of symbiotic performance (Vincent, 1970; Wynne *et al.,* 1980).

Specific nitrogenase activity (SNA) proved to be a poor indicator of N_2-fixation as indicated in Table 3. This supports the findings of Pacovsky *et al.* (1984) and Somasegaran and Martin (1986) who have found similar misleading relationships. Therefore, data on SNA as N_2-fixation indicator in soybean-*Bradyrhizobium* symbiosis should be interpreted with great caution.

To investigate the relationship between total N accumulated in plant tops and chlorophyll in fieldgrown soybean, plants from an already established field experiments were sampled at R6 growth stage (Fehr *et al.,* 1971). The experiment, conducted on a low N, silty clay loam soil at the Kula Agricultural Park on the island of Maui, Hawaii included the treatments shown in Table 4. The results (Table 4) showed that chlorophyll measurements detected treatment effects in the same way as did shoot dry weight and total N measurements. A highly significant coefficient of correlation $(r = 0.92; P < 0.001)$

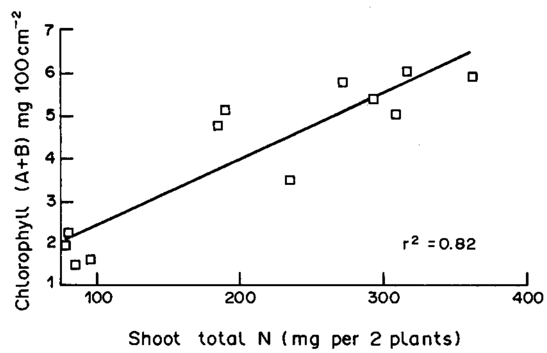

Fig. 3. Relationship between chlorophyll and shoot total N in field-grown soybean plants harvested at R6 growth stages.

between total N and chlorophyll, further justifies the use of chlorophyll as an indirect measure of N status of field-grown soybeans (Fig. 3). About 85% of the variation in chlorophyll can be ascribed to differences in total N accumulated in shoots of field-grown soybeans.

Although no previous attempt has been made to use chlorophyll as an indirect criterion for ranking strains of rhizobia for their effectiveness, Wynne *et al.* (1980) shows significant correlations between plant dry matter, plant total N contents and plant colour, suggesting the use of plant colour for rapid evaluation of rhizobia nodulating peanuts in the greenhouse. However, our results suggest that chlorophyll measurements may be a more sensitive, quantitative and reliable procedure for such effectiveness ratings of rhizobia. It is non-destructive and could be used at various stages of plant development.

Table 4. Effect of mineral N_2 and *B. japonicum* inoculation on field-grown soybean [*Glycine max* (L.) Merr.] growth characteristics

	Growth characteristics*		
Treatments	Chlorophyll (a + b) (mg 100 cm^{-2})	Shoot dry weight (g per plant)	Shoot total N (mg per plant)
Uninoculated (control)	1.81	4.03	88.75
Inoculation† (3 strain mix)	4.62	7.13	237.75
Nitrogen‡ (1000 kg ha^{-1})	5.78	10.03	315.5
LSD (0.05)	0.64	1.44	49.27
cv (%)	12.81	16.39	18.91

*All values are means of 4 replicates.
†Three-strain mixed inoculum contained strains of *B. japonicum* (USDA 138, USDA 136b and USDA 110) mixed in equal proportion in peat before use.
‡In these treatments, non-nodulating isolines of soybeans were used.

Acknowledgements-This work was supported in part by an USAID Cooperative Agreement (NifTAL Project). We thank R. B. Martin for technical assistance, P. L. Nakao for computer analysis; and Susan Hiraoka and Patricia P. Joaquin for manuscript preparation. We also thank Janice Thies for the use of her field-grown soybean experiment for these studies.

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