PN-AAV-252 45730

Plant and Soil 87, 223–231 (1985). Ms. 6151
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Importance of bacteroid number in nodules and effective nodule mass to dinitrogen fixation by cowpeas

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Received 31 October 1984, Revised February 1985

Key words Nitrogen fixation Rhizobia *Vigna unguiculata*

Summary Biological nitrogen fixation is considered an important trait of cowpeas (*Vigna unguiculata* (L.) Walp. var. 'California Blackeye' No. 5) for economical production yet the process does not alone provide the quantity of nitrogen required by the plant for maximum productivity. Two experiments were undertaken to determine the potential of an increase in nodule mass and number of bacteroids resulting in increased nitrogen fixation. Cowpeas were grown in a glasshouse for 7 weeks under conditions forcing near total dependence on biological nitrogen fixation for growth. Nodule mass on the roots was varied by inoculating seeds with various ratios of effective and ineffective rhizobia that could be identified serologically and by the color of nodule formed. The results of both experiments demonstrated a linear relationship between total nodule mass formed by the effective rhizobia and quantity of nitrogen fixed. The regression coefficients were high in both experiments ($r = 0.99^{**}$ and 0.91^{**}) The relationship between total nitrogen fixed and total number of bacteroids of the effective strain was not consistent. In one experiment the regression coefficient was 0.93^{**} but in the other experiment it was 0.65^{**}. From these results it appears that there is good potential for increasing nitrogen fixation in cowpeas by increasing nodule mass. An increase in nodule mass would also result in an increase in the number of bacteroids.

Introduction

Cowpeas (*Vigna unguiculata* (L.) Walp.) nodulate readily, but do not obtain adequate nitrogen from fixation for maximal plant growth[4]. The stimulation of cowpea growth by addition of combined nitrogen cannot be explained solely on the basis of alleviating nitrogen deficiency while nodules are forming[4]. Apparently, the plant needs more nitrogen than can be provided by the symbiotic system alone. The factors limiting nitrogen fixation in cowpeas are not fully understood but total nodule mass on several cultivars related positively with the quantity of nitrogen fixed[12].

The potential of an increase in nodule mass on cowpeas resulting in higher rates of nitrogen fixation may be revealed by examining the quantitative relationship between total nodule mass and quantity of nitrogen fixed using an individual cultivar. If the relationship is positive and linear then there is potential for increasing nitrogen fixation by increasing nodule mass. However, if a curvilinear relationship exists

then some factor other than nodule mass is likely limiting nitrogen fixation. Another parameter that is related to nodule mass and perhaps more directly related to quantity of nitrogen fixed than nodule mass is the quantity of bacteroids present in nodules. Experimentation was undertaken to examine the relationship of these two parameters to nitrogen fixation. The results indicated that there is potential for increasing nitrogen fixation by increasing nodule mass and number of bacteroids.

Materials and methods

Plant culture

Cowpea (*Vigna unguiculata* (L.) Walp. var. California Blackeye No. 5) seeds were surface disinfected, pregerminated, and sown in modified Leonard jars filled with vermiculite[11]. Four seeds were sown per container and thinned to two after emergence. For Experiments I and II plants were grown in a glasshouse with wet-pad cooling during the day. Ambient temperature was approximately 24°C night and 38°C day. Plants were watered with nitrogen-free plant nutrient solution[11].

Rhizobium culture and inoculation

Effective and ineffective strains of Rhizobium were utilized for these experiments. Strain T1 from our laboratory produced dark colored nodules on cowpea which have been found to be effective in preliminary experiments[5]. Strain CB756, originally from Sydney University, Australia was obtained from the NifTAL culture collection, Paia, Hawaii as strain TAL309. A spontaneous mutant of CB756, obtained as a single colony isolate from an earlier investigation[1] was used as the ineffective strain because it was able to nodulate the plant but did not fix nitrogen.

Inocula were prepared by growing rhizobia in yeast extract broth[11] for 7 to 10 days on a rotary shaker at 28°C. Cell densities in the cultures were determined by direct microscopic counts and diluted to 1×10^7 cells per ml with nitrogen-free plant nutrient solution. The diluted cultures were mixed in appropriate quantities to yield different ratios of T1:CB756. The quantity of effective nodule tissue on plants was varied by inoculating plants with different ratios of effective and ineffective strains of rhizobia. Effective nodule tissue was identified by the method in which an effective strain of rhizobia capable of forming dark colored nodules was utilized[5].

For Experiment I the ratios were 100:0, 80:20, 60:40, 40:60, 20:80, 10:90, 5:95, 1:99, 0.1:99.9, and 0:100. For Experiment II the ratios were 100:0, 90:10, 70:30, 50:50, 30:70, 10:90, and 0:100. Seeds were inoculated after sowing by dribbling 1 ml of the appropriate inoculum onto each seed before they were covered.

Processing of shoots and roots

Plants were harvested 42 days after inoculation and planting. The shoots were dried to constant weight at 65°C and ground for nitrogen analysis. Nitrogen content was determined by the Kjeldahl procedure[6].

Roots were washed carefully and all nodules were picked and sorted into two groups by color. Nodule fresh weights were determined and nodules were stored at −20°C until they were processed for analysis of bacteroid content.

Bacteroid identification and enumeration

Nodules were homogenized with a mortar and pestle in phosphate buffered saline to release bacteroids. Suspensions were diluted as required to obtain a cell density that was convenient to count using a Petroff Hauser counting chamber. Four subsamples were utilized in determining the population of each sample.

Fluorescent antibodies were utilized for identification of bacteroidal strains[7]. Rabbits were injected with whole cell suspensions of the required strain of rhizobia. The antibodies were fractionated from whole blood and tagged with fluorescein isothiocyanate[5] (FITC). The conjugated antibody (FA) preparation was tested for specificity against several strains of rhizobia and was found to be specific for the homologous strain.

Individual nodules were dissected aseptically and smears were made on microscope slides. The smears were air dried and bacteroids fixed to the slide by submersing in 95% ethanol for 30 min followed by air drying. They were stained with FA for 30 min at 37°C in a moist chamber. Excess FA was removed by soaking slides for 10 min in two changes of 0.5 M carbonate-bicarbonate buffer, pH 9.5. Slides were mounted with buffered glycerol and stained bacteroids observed using a Zeiss Universal Research microscope equipped for epi illumination with ultraviolet light and suitable excitation filters.

Statistical methods

A completely randomized design was utilized for each experiment. Four replications were used for each inoculation level in Experiment I and six replications were used in Experiment II. The results were analyzed using regression methods.

Results and discussion

The use of easily distinguishable nodule colors to identify nodules formed by strain T1 and CB756 inef. was highly successful. Fluorescent antibody was used to confirm that dark-colored nodules were formed by strain T1 and that light-colored nodules were formed by CB756 inef. When present, 10 dark-colored nodules were sampled from each plant in Experiment I and tested with FA. All of them were confirmed to contain only strain T1. Because of the very high ratio of light-colored nodules to dark-colored nodules in plants with mixed nodulation, approximately 30 light-colored nodules from each plant were tested with FA. Only a total of three ($< 1\%$) light-colored nodules contained only strain T1 and the frequency of this incidence was considered insignificant for the objectives of our investigation. Both rhizobial strains occurred together in less than 1% of the nodules. In Experiment II, nodules were also tested to confirm the nodule-color and bacterial strain relation and results similar to those of Experiment I were obtained.

Because the cowpeas were grown in a nitrogen deficient medium and were watered with nitrogen-free nutrient solution, essentially all of the nitrogen, except for the relatively small quantity of nitrogen initially contained in the seed, was from nitrogen fixation. Because nitrogen was the nutrient limiting plant growth, the relationship between dry matter production of plant shoots and total nitrogen content was linear, and the coefficient of determination was high ($r^2 \geqslant 0.92^{**}$) for both experiments. For purposes of brevity, only the data on total nitrogen accumulated in shoots is presented.

The quantity of nodule tissue formed by the effective rhizobial strain

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in Experiment I was directly related to the quantity of nitrogen accumulated in cowpea shoots (Fig. 1). The relationship between these parameters was linear over the entire range of data and the coefficient of determination was very large ($r^2 = 0.98^{**}$).

The number of bacteroids in the nodules, from the effective rhizobial strain, also related closely to the quantity of nitrogen fixed (Fig. 2). in this case, the fitted line to the data was curvilinear ($r^2 = 0.86^{**}$). The critical portion of the curve, for the purposes of predicting the potential of increasing nitrogen fixation by increasing the number of bacteroids, is the portion near where maximum nitrogen fixation occurred. The scatter of data points in this region of the curve show little relationship to the fitted line (Fig. 2). Evidence is not adequate to suggest that the number of effective bacteroids beyond approximately 25×10^{10} per plant were limiting nitrogen fixation.

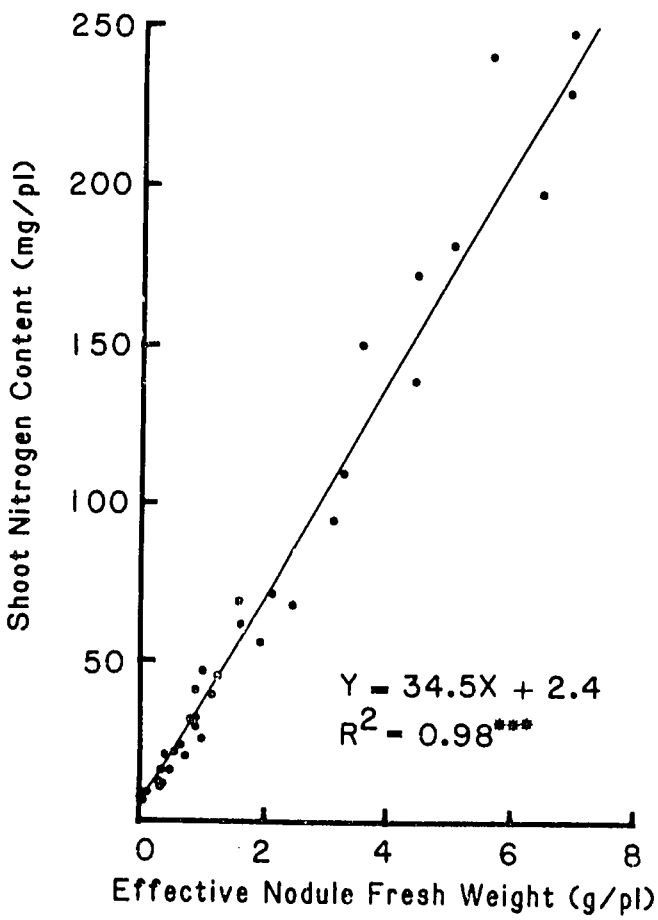

Fig. 1. Linear relationship between shoot nitrogen content and total weight of nodules formed by an effective strain of rhizobia on cowpeas. Each point represents a single plant in Experiment I.

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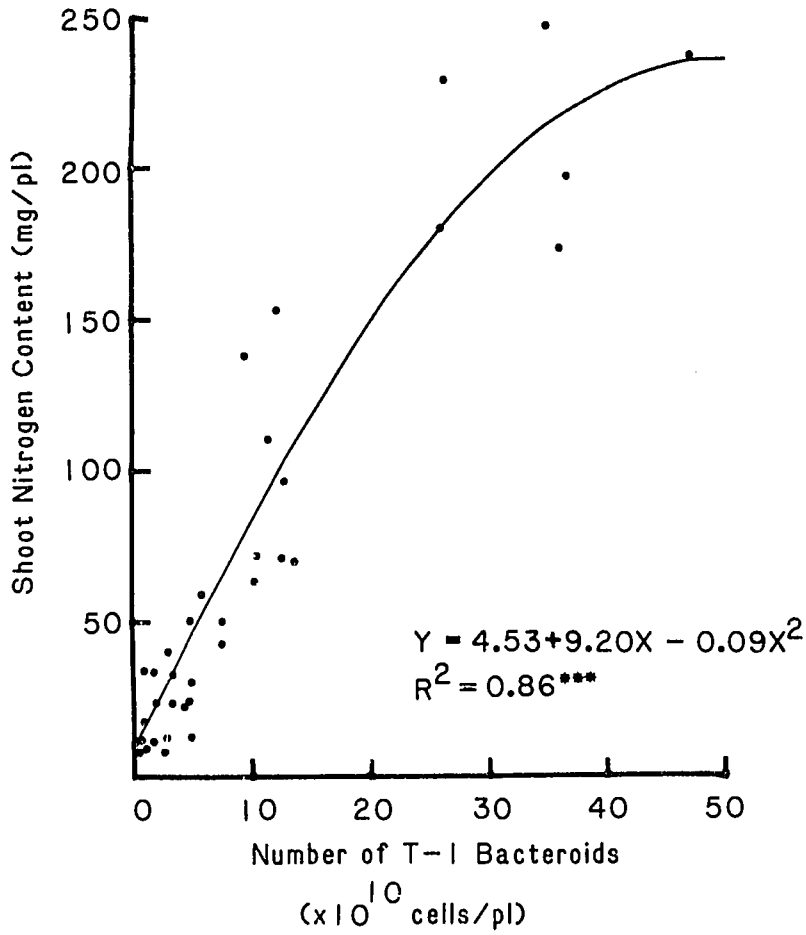

Fig. 2. Curvilinear relationship between shoot nitrogen content and total number of bacteroids of an effective rhizobial strain, in nodules formed on cowpeas. Each point represents a single plant in Experiment I.

In Experiment II, as in Experiment I, the quantity of nodule tissue was directly related to the amount of nitrogen fixed (Fig. 3). The relationship was linear with a coefficient of determination of 0.84**. However, in this experiment, the relationship between the quantity of nitrogen fixed and the number of bacteroids was not very strong and regression only accounted for 43% of the variability (Fig. 4). Apparently, in this experiment some parameter other than the number of bacteroids was influencing the quantity of nitrogen fixed in a major way. Probably the reduced light intensity due to the overcast days was a factor.

The results of Experiments I and II establish that a direct relationship

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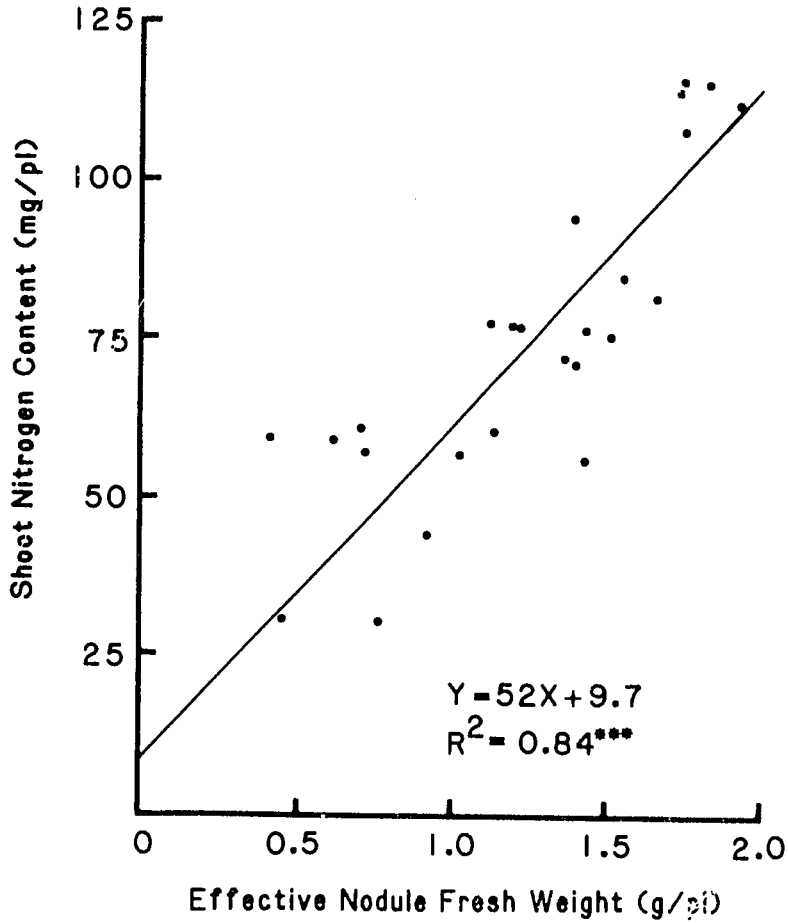

Fig. 3. Linear relationship between shoot nitrogen content and total weight of nodules formed by an effective strain of rhizobia on cowpeas. Each point represents a single plant in Experiment II.

existed between the quantity of effective nodule tissue on cowpeas and the quantity of nitrogen accumulated (Fig. 1 and 3). In these experiments, the quantity of nitrogen accumulated was essentially the same as the quantity of nitrogen fixed since only approximately 5 mg of nitrogen was present in the shoots of uninoculated plants. The quantity of nitrogen fixed by soybeans also relates directly to the quantity of nodule tissue present[2,10]. The coefficient of determination between the quantity of nodule tissue on soybean roots and the amount of nitrogen fixed is high ($r^2 = 0.98^{**}$) and the relationship is linear[11].

Because the plant shoots were dependent on the nodules for their nitrogen, it is reasonable to assume that there was a cause-effect relationship between effective nodule mass and quantity of nitrogen fixed. It appears from the linear relationship that increasing nodule

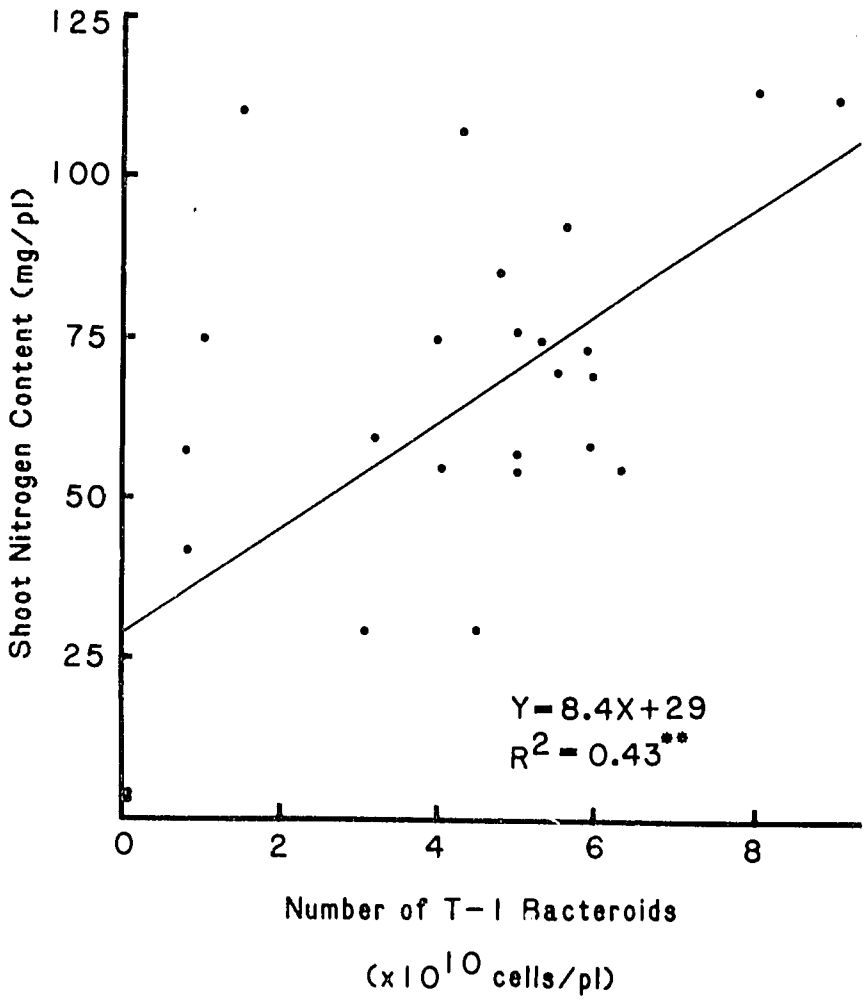

Fig. 4. Linear relationship between shoot nitrogen content and total number of bacteroids of an effective rhizobial strain, in nodules formed on cowpeas. Each point represents a single plant in Experiment II.

mass of cowpeas could potentially increase nitrogen fixation. Perhaps inadequate nodule mass is the reason cowpeas have not demonstrated their full potential of benefiting from nitrogen fixation and require mineral nitrogen for maximal yields[4].

The difference in nodule mass between plants grown in Experiments I and II demonstrate the importance of environmental conditions in allowing the plant to express its genetic potential in formation of nodules (Fig. 1 and 3). Weather conditions during Experiment I were favorable for plant growth but Experiment II was conducted during a season when many days were overcast and rains were frequent. The plants were grown for the same length of time but the maximum

quantity of nodule tissue formed in Experiment I was approximately three times larger than in Experiment II.

In either experiment, the number of bacteroids of the effective strain was not as good of a parameter as effective nodule weight in relating to nitrogen fixation. In Experiment I the shoot nitrogen content had essentially culminated with the number of bacteroids that were present yet the results for effective nodule weight indicate that more nodule mass would result in increased nitrogen fixation. The results of Experiment II in particular cast doubt on the usefulness of the number of T1 bacteroids in predicting nitrogen fixation since the correlation between these parameters was low ($r^2 = 0.43^{**}$). Intuitively, it would seem that the number of T1 bacteroids would relate more closely to nitrogen fixation than the quantity of nodule mass since the bacteroids provide the nitrogen fixing enzyme nitrogenase. Nevertheless, the nodule provides the environment that is essential for nitrogen fixation to occur at high rates. In other research it has been demonstrated that the rate of nitrogen fixation in plants is largely controlled by the nodule environment because isolated bacteroids may fix similar quantities of nitrogen but differ greatly while in the nodules[9].

Future research needs to be undertaken that provides more data of importance in determining the plant characteristics related to the symbiotic system that are limiting biological nitrogen fixation. Our results provide encouragement that increasing nodule mass on cowpeas may result in increased nitrogen fixation.

Acknowledgements The technical assistance of Lisa Mathis Lucia during these investigations is appreciated. Also gratefully acknowledged is the financial assistance provided by Grant No. 59-2481-0-5-018-0 which was funded by the USAID program on factors limiting symbiotic nitrogen fixation for crop production in developing countries and was administered through CSRS and USDA.

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