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Evaluation of ^{15}N-isotope dilution for measurement of nitrogen fixation in chickpea (*Cicer arietinum* L.)*

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Summary. N accumulation, nodulation, and acetylene reduction activity were measured at frequent intervals during the growth of two chickpea genotypes, and N_2 fixation was estimated by an isotope-dilution method, using safflower as a non-N_2-fixing reference. Safflower was more efficient at N uptake than both the chickpea genotypes for at least the first 50 days and thus could not be used as an accurate reference control. We recommend that further work should employ non-nodulating genotypes of chickpea as reference plants and use slow-release forms of ^{15}N fertilizer. Direct genotype comparison by isotope dilution estimated that genotype K 850 fixed 16–18 kg ha^{-1} more N than G 130, and this difference was supported by the greater nodule mass and acetylene reduction activity in the K 850 cultivar. Inoculation with an ineffective chickpea *Rhizobium* sp. led to 69% nodulation on cultivar K 850 but only 33% on G 130. While nodule weight, N uptake, and acetylene reduction activity decreased with inoculation in K 850, the isotope dilutions were similar for both inoculation treatments. The lack of a significant effect on N_2 fixation was ascribed to the partial success of inoculant establishment.

Key words: N_2-fixation – ^{15}N – *Cicer arietinum* – Isotope-dilution – Acetylene reduction assay

Chickpea (*Cicer arietinum* L.) is a grain legume with a broad base of genetic variability and a wide climatic tolerance, which ranges from Mediterranean to semi-arid tropical environments. In India chickpea is grown mainly on residual moisture, both in the intermittent rainfall season of the north and in the dry season of the semi-arid regions (Van der Maesen 1972). Accurate measurement of N_2 fixation is essential when studying the N economy of cropping systems.

In spite of the extensive literature on the use of the ^{15}N isotope-dilution technique in measuring N_2 fixation, few studies have been concerned with chickpea. The present study was designed to measure N_2 fixation by two chickpea genotypes, with and without inoculation, in soil containing effective rhizobia, using safflower as a non-fixing plant. Problems with the application of the isotope-dilution method have been identified (Witty 1983), particularly the mis-matching of N_2-fixing legumes and reference plants. The present experiment was designed to assess whether the treatments satisfied the assumptions of the isotope-dilution method.

Materials and methods

Cultivars used. Two "Desi" genotypes of chickpea were studied, cultivar K 850 which has a large nodulation capacity and cultivar G 130 which has a small nodulation capacity (Rupela and Dart 1980). Safflower was chosen as a non-N-fixing control plant on the basis of its short stature, particularly under low soil-N conditions, and its ability to grow in the post-rainy season at Hyderabad. Although most of the Vertisols at the ICRISAT centre contain large populations of effective chickpea rhizobia (Toomsan et al. 1982), the crop was grown with and without inoculation using a standard chickpea *Rhizobium* sp. strain IC 2002 (ex Rothamsted 3889). Sequential harvests were taken to examine the growth and N uptake in both crops and nodulation and acetylene reduction activity in the chickpea.

Experimental design. The crops were sown on 27 November 1982 in a deep Vertisol at the ICRISAT Centre, Patancheru, 25 km west of Hyderabad, India. The concentration of available NO_3^- (KCl-ex-

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tractable NO_3^-) was < 4 ppm in soil samples taken from 1.2 m profiles at the beginning of the experiment. The treatments were sown in six replicate blocks of 15 × 3.6 m, each divided into two subblocks of 3 × 3.6 m and 9 × 3.6 m. Labelled fertilizer (10 kg N ha^{-1} as $K^{15}NO_3$, 8.570 atom% ^{15}N excess) was applied in solution to the smaller subblock for isotope-dilution measurements, and the larger subblock received an equivalent addition of unlabelled fertilizer. The four treatments, chickpea cultivars K 850 and G 130, each inoculated and uninoculated, were sown in equal randomized plots containing four rows 30 cm apart with in each subblock. Each subblock of treatments was bordered by two rows of safflower plants, also spaced 30 cm apart. Within the rows, chickpea plants were spaced at 10 cm and safflower at 5 cm.

Rhizobium inoculation, maintenance, and harvesting of the crop. Each chickpea seed was inoculated at sowing with 5 ml of a suspension in water of peat inoculant of the chickpea *Rhizobium* sp. strain IC 2002 (6.3×10^2 cells ml^{-1}) poured over the seed in the furrow. The inoculant strain used was a subculture of IC 2002 but this has since been shown to be ineffective in N_2 fixation compared with the mother culture and is renamed IC 2094. A post-sowing sprinkler irrigation was given to ensure good germination, crop establishment and movement of the fertilizer into the soil. A further irrigation was applied 41 days after sowing. In order to estimate nodulation, acetylene reduction activity, and N uptake, harvests were taken from the large unlabelled fertilizer plots after 24 days and then at about 10-day intervals. The plants were dug up, the soil shaken off, and roots and shoots separated. All small subblocks to which ^{15}N was applied were harvested when the chickpea pods were full (75 days growth) but before there was any appreciable loss of the lower leaves.

Analytical methods. Acetylene reduction activity was measured over a 30-min incubation period in the field, immediately after the root systems were dug up (Hardy et al. 1973). The dried shoots were ground, the N content was determined by Kjeldahl digestion, and the ammonia in the digests was estimated by an automated indophenol-blue method. The N in the digests was concentrated by a Conway microdiffusion technique (Conway 1939) for ^{15}N analysis, and ^{15}N enrichments were measured with a Micromass 622 mass spectrometer (V.G. Isogas, Northwich, Cheshire, U.K.). N_2 fixation was calculated by the equation (Rennie et al. 1978):

$$\%N \text{ fixed} = 1 - \frac{\text{atom\% } ^{15}N \text{ excess in legume}}{\text{atom\% } ^{15}N \text{ excess in control}} \times 100 .$$

Nodule typing and Rhizobium counts. The proportion of nodules formed by the inoculant strain was determined by enzyme-linked immunosorbent assay (ELISA) (Kishinevsky and Bar-Joseph 1978). Nodules on five plants per plot were dug up, pooled, and stored frozen in 20% glycerol until subsampled. Preparation of antigen and antiserum were carried out as described by Vincent (1970); the agglutination titre of the serum prepared was 1/3200. The number of chickpea rhizobia in the soil at the beginning of the experiment was estimated at five points in the field, using a plant-infection technique (Toomsan et al. 1984).

Results and discussion

Growth, N-uptake, and estimates of N_2 fixation using the non-legume control

The initial rate and total amount of N uptake was much higher in the safflower than in the chickpea, but N uptake in the safflower was retarded after about 50 days when some plants died due to an attack by stem borer (Fig. 1). Chickpea growth was not affected, but chickpea cultivar K 850 had a consistently higher dry-matter and N yield than G 130 ($P < 0.05$). Inoculation gave a consistent reduction in N accumulation in both cultivars.

^{15}N enrichment in the chickpea was lower (0.367–0.498 atom% ^{15}N excess) than in the safflower (0.961 atom% ^{15}N excess), and on this basis chickpea cultivar K 850 appeared to fix substantially more N (36–42 kg N ha^{-1}) than cultivar G 130 which fixed 20 kg N ha^{-1} (Table 1). Although the safflower provided suitable reference values for the N_2-fixation estimates in the chickpea, as expected from the genotypic differences in acetylene reduction activity (Rupela and Dart 1980), it had a very different N-uptake pattern from that of the two chickpea cultivars at least for about the first 50 days (Fig. 1). The atom% ^{15}N of soil N available to a plant may decline rapidly after the addition of labelled nitrate fertilizer (Witty 1983), so that the initial rapid N accumulation in the safflower was likely to have occurred during a period of high ^{15}N enrichment. The effect of this would be to overestimate N_2 fixation in the chickpea if the isotope-dilution method were used. Unfortunately, N uptake by the safflower was seriously checked at the second irrigation. Towards the end of the experiment, the safflower continued to accumulate N rapidly, although uptake had already ceased in the chickpea (Fig. 1). As the surface soil was dry (begining to crack), this N was probably absorbed from soil horizons deeper than those explored by the chickpea. The safflower matured 16 days after the chickpea. Clearly, many influences could have been operating, and more information is required in order to accept these estimates.

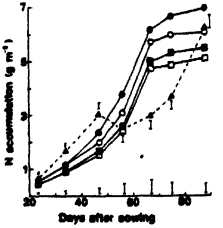
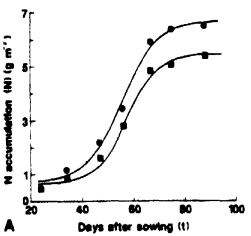


Fig. 1. N accumulation in safflower (▲) and chickpea cultivars K 850 (○,●) and G 130 (□,■) with inoculation (○,□) or without inoculation (●,■) with chickpea *Rhizobium* strain IC 2002. *Vertical bars* indicate SE of safflower means (on graph) and chickpea means (along *x* axis)

Table 1. Dry-matter and N yield, ^{15}N enrichment and values from isotope-dilution calculations for crops harvested after 75 days of growth

Treatment	Shoot dry matter (kg ha^{-1})	Total N uptake (kg ha^{-1})	Atom % ^{15}N excess	Fertilizer recovery[a] (%)	N release from soil (kg ha^{-1})	N fixed			N fixed[b] (%)
						By difference (kg ha^{-1})[b]	By isotope dilution (kg ha^{-1})[b]	By isotope dilution (kg ha^{-1})[c]	
Uninoculated									
K 850	2960	68	0.369	29	23	35	42	18	61
Inoculated									
K 850	2550	59	0.367	25	20	26	36	16	62
Uninoculated									
G 130	1830	41	0.488	22	18	8	20	1	49
Inoculated									
G 130	1880	41	0.498	23	18	8	20	–	48
SE	± 127	± 3.3	± 0.029	± 1.8	± 1.4	–	± 2.7	–	± 3.2
Safflower	3800	33	0.961	37	29				
SE	± 171	± 0.9	± 0.014	± 0.9	± 0.8				

[a] 10 kg N ha^{-1} added as $K^{15}NO_3$ (8.570 atom % ^{15}N excess)
[b] With reference to safflower
[c] With reference to inoculated chickpea



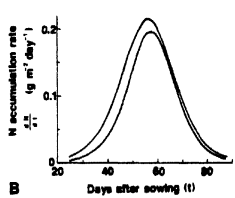
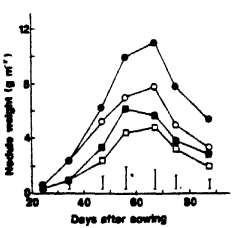


Fig. 2A, B. Mean N accumulation by inoculated and uninoculated chickpea cultivars K 850 (●) and G 130 (■). **A** Fitted logistic curves (K 850: $N = 0.685 + 6.016/[1 + \exp[-0.142\ (t - 55.94)]$; G 130: $N = 0.620 + 4.834/[1 + \exp[-0.162\ (t - 57.45)]$) where t = time in days; **B** N-accumulation rate calculated from differentiation of the logistic curves



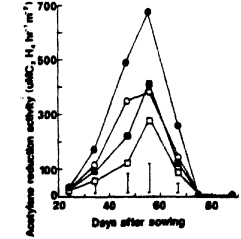


Fig. 3. **A** Nodule weight and **B**, acetylene reduction activity of chickpea cultivars K 850 (○, ●) and G 130 (□, ■) with inoculation (○, □), or without inoculation (●, ■) with chickpea *Rhizobium* strain IC 2002. *Vertical bars* represent SE of means

^{15}N isotope dilution for comparative assessment of chickpea genotypes

In view of the problems associated with using non-legume controls, a different approach was adopted, using isotope dilution to compare N_2 fixation in the chickpea genotypes. In order to facilitate comparison of N accumulation in the two chickpea genotypes, logistic curves were fitted to the N-uptake data (Fig. 2A) and the gradient, or instantaneous rate of N up-

take was calculated by differentiation of the logistic equation (Fig. 2B). N accumulation in the two chickpea cultivars was similar in timing and duration, although cultivar K 850 yielded significantly more N (Fig. 2A) and more rapidly than cultivar G 130 (Fig. 2B). However, comparison of acetylene reduction activity and nodule mass (Fig. 3) with N uptake (Fig. 2) suggests that the larger and faster N accumulation in cultivar K 850 was a result of greater N_2 fixation rather than a higher uptake of N from the soil.

When the amount of N_2 fixed in cultivar K 850 was calculated by isotope dilution, using the chickpea genotype with a lower level of N_2 fixation as a reference plant, cultivar K 850 was estimated to fix 16–18 kg N ha^{-1} more than cultivar G 130. This difference estimated between the chickpea genotypes was similar to that obtained using the safflower control. As the uptake of soil and fertilizer N was apparently similar in both chickpea genotypes, it is reasonable to assume that the comparative estimates of N_2 fixation in the chickpea genotypes are reliable.

Inoculation with an ineffective Rhizobium

The population of chickpea rhizobia in the soil at the beginning of the experiment was estimated to be 1.2×10^3 g^{-1} soil. In spite of this the ineffective inoculant strain formed 69% of the nodules recovered from cultivar K 850 and 33% of those from cultivar G 130. Though the amount is negligible, about 2% of the nodules from the uninoculated plots reacted with the IC 2002 antiserum. Inoculation with ineffective *Rhizobium* reduced nodule mass, acetylene reduction activity (Fig. 3), N accumulation (Fig. 1), and specific acetylene reduction activity (reduced by 25.0% to 28.5%) in both the cultivars.

This inoculation treatment allowed us to test the proposition that ineffective strains can be used to produce non-N-fixing reference plants for isotope-dilution studies (Chalk 1985), a suggestion not previously evaluated experimentally in the presence of native rhizobia. The fact that the success rate of inoculation can vary with different genotypes (33% in cultivar G 130 and 69% in cultivar K 850 in this experiment) limits the use of a plant that is nodulated with ineffective rhizobia as a reference. Further, the ultimate N accumulation in such a host would depend on the functioning of the nodules formed by the effective native population (67% in cultivar G 130 and 31% in cultivar K 850, in this trial), which could confound the effect of the ineffective nodules, as perhaps happened in the present experiment.

Establishment of an introduced inoculant strain in the presence of a large population of effective native strains requires large inoculant populations in relation to the soil population (Weaver and Frederick 1974). The success of the inoculation can be increased by careful selection of the host-strain combination (Kvien et al. 1981), but complete exclusion of effective native rhizobia throughout the growth of a legume is improbable. Thus, as previously indicated (Rennie 1982), it is unlikely that inoculation with ineffective rhizobia will provide control plants that do not fix N where there are effective native rhizobia.

Matching of N-uptake patterns between the test and reference crops was definitely poor for the first 50 days of plant growth and was unreliable later on, due to insect damage and the long duration of the safflower N uptake. However, the data on genotype comparisons seem realistic. While the use of slow-release forms of ^{15}N fertilizer may reduce errors due to the poor matching of N-uptake patterns between chickpea and safflower (Giller and Witty 1987), there is a clear need for a more suitable non-N-fixing control plant for use with chickpea, such as the non-nodulating genotypes which are now available (Davis et al. 1985; Rupela and Sudarshana 1986).

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