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

K.E. Giller1,**, M.R. Sudarshana2, J.A. Thompson2,*** and O.P. Rupela2

1 Soils Division, Rothamsted Experimental Station, Harpenden, Herts, AL5 2JQ, UK
2 International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru P.O., A.P. 502324, India

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-
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 $15 \times 3.6$ m, each divided into two subblocks of $3 \times 3.6$ m and $9 \times 3.6$ 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 ($6.3 \times 10^{9}$ 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):

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

**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 was 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 (beginning 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.

![Figure 1](https://example.com/figure1.png)

**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).