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ORIGINAL PAPER

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Field evaluation of lentil cultivars inoculated with *Rhizobium leguminosarum* bv. *viciae* strains for nitrogen fixation using nitrogen-15 isotope dilution

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Abstract A ^{15}N isotope dilution technique was applied to quantify the extent of N_2 fixation in lentil (*Lens culinaris* Medik.) cultivars as influenced by *Rhizobium leguminosarum* bv. *viciae* strains in a field experiment in Pakistan. The experiment was conducted on a soil with a very small indigenous rhizobial population and where N was a limiting factor for crop production. Significant variations in number of nodules, dry weight of nodules, biomass yield, grain yield, total N yield, proportion of plant N derived from N_2 fixation (P_{fix}) and amount of N derived from the atmosphere (N_{dfa}) were observed among combined treatments of four rhizobial strains and six lentil varieties. In a field previously labelled with ^{15}N, to which a basal dose of 75 kg P_2O_5 ha^{-1} was applied as single super phosphate, N_{dfa} ranged from 15 to 24 kg N ha^{-1} when calculated according to rhizobial strain and from 4 to 38 kg N ha^{-1} when calculated according to lentil variety. Lc 26 was the most effective strain and fixed 243% more N than the indigenous population in the uninoculated control. In treatments with the lentil variety PL-406, N_{dfa} was 38 kg N ha^{-1}, which was 850% higher than with the lentil variety Precoz/F6-20-1 × M-85. Generally, the varieties with greater P_{fix} produced a higher dry matter yield.

Key words Nitrogen-15 isotope dilution · Legumes · *Lens culinaris* · *Rhizobium* · Nitrogen fixation

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Introduction

As a consequence of the persistent energy crisis resulting in higher fertilizer costs, biological N fixation (BNF) has become one of the most attractive strategies for the development of sustainable agricultural systems. The role of BNF, especially in legumes, is well established and documented. However, it has been reported that various varieties or cultivars of grain legumes, namely pigeon pea, faba bean and cowpea, show significant differences regarding their ability to support BNF (Hardarson 1993). The quantification of biologically fixed N by these legume species was carried out using the ^{15}N isotope dilution technique by Hardarson et al. (1993) and McNeill et al. (1998). The influence of the host plant on symbiotic effectiveness, nodulation and N_2 fixation have been reported (Caldwell and Vest 1977; Hardarson et al. 1984) and strong cultivar × strain interactions in soybeans (Israel 1981), beans (Rennie and Kemp 1983; Valverde and Otabbong 1997) and peanuts (Wynne et al. 1980) have been observed. Thus, in BNF not only the micro-symbiont (*Rhizobium*) but also the macro-symbiont (host plant) plays an important role, and their co-selection under a given set of soil and environmental conditions may enhance the amount of fixed N.

Lentil is the second most important grain legume crop in Pakistan and is cultivated on an area of 82,000 hectares (26% of the area under all pulses) with an annual production of 30,000 t grain (Malik et al. 1988). Most of the cultivation of lentil occurs on marginal and less fertile lands with very small indigenous rhizobial populations. Inoculation with superior *Rhizobium* strains is required to increase yields through N_2 fixation (Athar 1998). A number of lentil rhizobial isolates were collected from various agro-climatic zones and screened for their effectiveness in sterilized sand and their competitiveness in unsterilized soil (Shah et al. 1996). Out of these 32 isolates, three promising *Rhizobium* strains, viz., Lc 6, Lc 26 and Lc 33, were selected for this study.

There is little information available about the quantity of N_2 fixation in lentil cultivars as influenced by *Rhizobium* strains, especially when they are grown in a soil with a small native rhizobial population. The present study was undertaken to quantify the amount of N_2 fixed in field-grown lentil under the combined effects of inoculation and varietal diversity.

Materials and methods

The experiment was conducted in soil with the following characteristics: texture, loam; pH, 7.8; electrical conductivity, 0.91 dS m^{-1}; organic C, 0.54%; total N, 0.062%; available NH_4-N, 0.90 $\mu g\ g^{-1}$; NO_3-N, 1.32 $\mu g\ g^{-1}$; cation exchange capacity 18.4 mEq 100 g^{-1} dry soil; Olsen P, 9 $\mu g\ g^{-1}$; extractable K, 210 $\mu g\ g^{-1}$; and an indigenous population of *Rhizobium leguminosarum* bv. *viciae*, 36 cells g^{-1} soil. The study took place at the Nuclear Institute for Agriculture and Biology (NIAB; Faisalabad), located in a semi-arid region of the central Punjab, Pakistan, as part of an FAO/IAEA coordinated research programme. The soil was therefore non-saline, low in N and available P and belonged to the Hafizabad series. The experiment was conducted in an organically ^{15}N-labelled field to which a basal dose of P_2O_5 75 kg ha^{-1} was applied as single super phosphate. Before labelling the soil the field was divided into two equal macro-plots. ^{15}N was applied at the rate of 30 kg ha^{-1} in the form of $(NH_4)_2\ SO_4$ enriched with ca 5% ^{15}N atom excess in one macro-plot. An identical amount of unlabelled $(NH_4)_2\ SO_4$ was applied to the other macro-plot; all the other conditions were kept the same in both macro-plots. The ^{15}N-labelled $(NH_4)_2\ SO_4$ was applied in solution (80 ml m^{-2}) at the time of chickpea sowing (in the previous year). After harvesting chickpeas, maize was sown in the same field and the stubble ploughed in after it was harvested. The experiment was laid out in a completely randomized block design with four replicates. Each replicate was divided into five plots.

Four *Rhizobium*-strain treatments (local isolates Lc 6, Lc 26 and Lc 33 and one exotic strain, TAL 1397, obtained from NifTAL, Hawaii, USA) and one uninoculated control were randomly allocated to the plots. Peat-based single-strain inocula were prepared from these strains. Each macro-plot was further divided into seven micro-plots to which six lentil varieties (Precoz, Precoz/F6-20-1 × M-85, Precoz/F6-23-1 × M-85, PL-406/M7-10-62, M-85 and PL-408) and one wheat variety as a reference crop were randomly allocated. The seeds of the lentil cultivars were obtained from the Mutation Breeding Division, NIAB.

The seeds were pelleted with the single-strain inocula with gum arabica as an adhesive. Two seeds $hole^{-1}$ were sown at a plant-to-plant distance of 15 cm and row-to-row distance of 30 cm, and were thinned to one seedling $hole^{-1}$ when the seedlings were 1 week old. When the seedlings were 2 weeks old, a liquid broth culture of the same single strain (2 ml $seedling^{-1}$) was applied directly to soil near the seedling roots in the respective inoculation micro-plots to ensure an inoculation response.

At the 50% flowering stage, ten plants from each treatment were harvested and nodule number and nodule dry weight were recorded. At maturity the plants were harvested, and data for shoot and grain weight were recorded. Total N analyses were done using the Kjeldahl method (Bremner 1965). The $^{15}N/^{14}N$ analysis of leaves and grain was done at the IAEA's Seibersdorf Laboratory (Vienna), using a mass spectrometer (Fiedler and Proksch 1975). The results were compared statistically by using Duncan's multiple range test (Duncan 1955). The proportion of plant N derived from N_2 fixation (P_{fix}) and the amount of N derived from the atmosphere (N_{dfa}) were calculated as described by the International Atomic Energy Agency (1983).

Results and discussion

The data (Table 1) showed that inoculation had a significant effect on nodulation, biomass, grain yield, N yield, P_{fix} and N_{dfa} in lentil. In treatments with the locally isolated strain, Lc 26, the highest number of nodules, nodule dry weight, biomass, grain yield, total N and N_{dfa} occurred, which were 200%, 200%, 78%, 57%, 76% and 243% higher, respectively, than in the uninoculated control. The second most effective strain was the standard exotic strain, TAL 1397; lentils inoculated with this strain produced 442 kg grains ha^{-1}. The highest P_{fix} (33%) occurred in the treatments with Lc 33. The locally isolated strain, Lc 26, out-classed the exotic TAL 1397 strain in terms of nodulation, biomass and grain yield of lentils, and gave statistically similar results to those of the TAL 1397 treatments with respect to amount of N_2 fixed. The strain associated with the highest lentil biomass was also associated with the highest grain yield, N yield and N_{dfa}. The uninoculated control (native population) gave the poorest performance with respect to all the parameters studied. These results were in accordance with those of Herrera and Longeri (1985), who reported that inoculation alone increased nodulation 85-fold, plant dry weight by 341% and seed yield by 114% in lentil. Bremer et al. (1990) also observed that inoculation increased the yield of lentil by up to 135%, while P_{fix} and total N_2 fixed ranged from 0 to 76 kg ha^{-1} and 0 to 105 kg ha^{-1}, respectively. The response to inoculation was significant due to the very small indigenous *Rhizobium* population (36 cells g^{-1} soil) in the experimental field. Many workers similarly reported that strains used as inocula were able to perform better than the indigenous population of N_2–fixing symbionts, especially when the native rhizobial population was very small (Gibson 1968; Singleton and Tavares 1986; Danso and Owiredu 1988). The mean %P_{fix} and N_{dfa} were 63% and 243% higher in Lc 26 than in the uninoculated control, showing that the quantity of N_{dfa} was significantly higher in the soil where N was a limiting factor for crop production. This was in agreement with the results of Bremer et al. (1990), who observed that higher NO_3 levels resulted in less N_2 fixation and vice versa. There were statistically significant differences amongst the strains with respect to nodule number, nodule dry weight, biomass, grain yield, N yield, P_{fix} and N_{dfa}. Similar differences in N_2 fixation by *Rhizobium* strains were also reported by Date and Roughley (1977).

The data in Table 1 showed that there were significant differences amongst various lentil cultivars in response to inoculation. Although all the six lentil cultivars were statistically similar with respect to number of nodules, the nodule dry weight differed significantly. M-85 produced the highest nodule dry weight while the variety Precoz/F6-20-1 × M-85 gave the lowest nodule dry weight, which was statistically similar to those in the Precoz and Precoz/F6-23-1 × M-85 treatments. The

Table 1 Response of six lentil cultivars to inoculation with *Rhizobium leguminosarum* bv. *viciae* strains. Means followed by *same letter(s)* in a column are not statistically different ($P>0.05$) according to Duncan's multiple range test. *UN* Uninoculated (grown in presence of native population of rhizobia), *LSD* least significant difference

	Nodule no.	Nodule dry weight (mg)	Biomass yield	Grain yield	Total N yield	N_{dfa}	P_{fix}
	Plant^{-1}		(kg ha^{-1})				(%)
Strains[a]							
Lc 6	5 b	7.3 b	2389 c	432 bc	52 c	15 b	26 ab
Lc 26	6 a	8.7 a	2849 a	483 a	67 a	24 a	31 a
Lc 33	4 c	6.6 bc	2502 bc	416 c	57 bc	22 a	33 a
TAL 1397	4 c	6.1 c	2533 b	442 b	63 ab	21 a	30 a
UN	2 d	2.9 d	1603 d	307 d	38 d	7 c	19 b
Lentil cultivars[b]							
Precoz	4 b	5.9 c	1831 c	358 b	40 c	4 b	9 c
Precoz/F6-20-1 × M-85	4 b	5.5 c	1837 c	326 b	47 c	4 b	9 c
Precoz/F6-23-1 × M-85	4 b	6.0 c	1915 c	328 b	46 c	10 b	23 b
PL-406/M7-10-62	5 b	8.0 b	3119 b	521 a	69 b	32 a	46 a
M-85	6 a	9.7 a	3347 a	546 a	79 a	37 a	46 a
PL-406	5 ab	8.0 a	3258 ab	537 a	78 a	38 a	48 a
LSD (strains × cultivars) 5%	1.3	1.3	324.5	41.2	12.9	18.3	5.9

[a] Means calculated across treatments with all lentil cultivars (means of 18 observations)

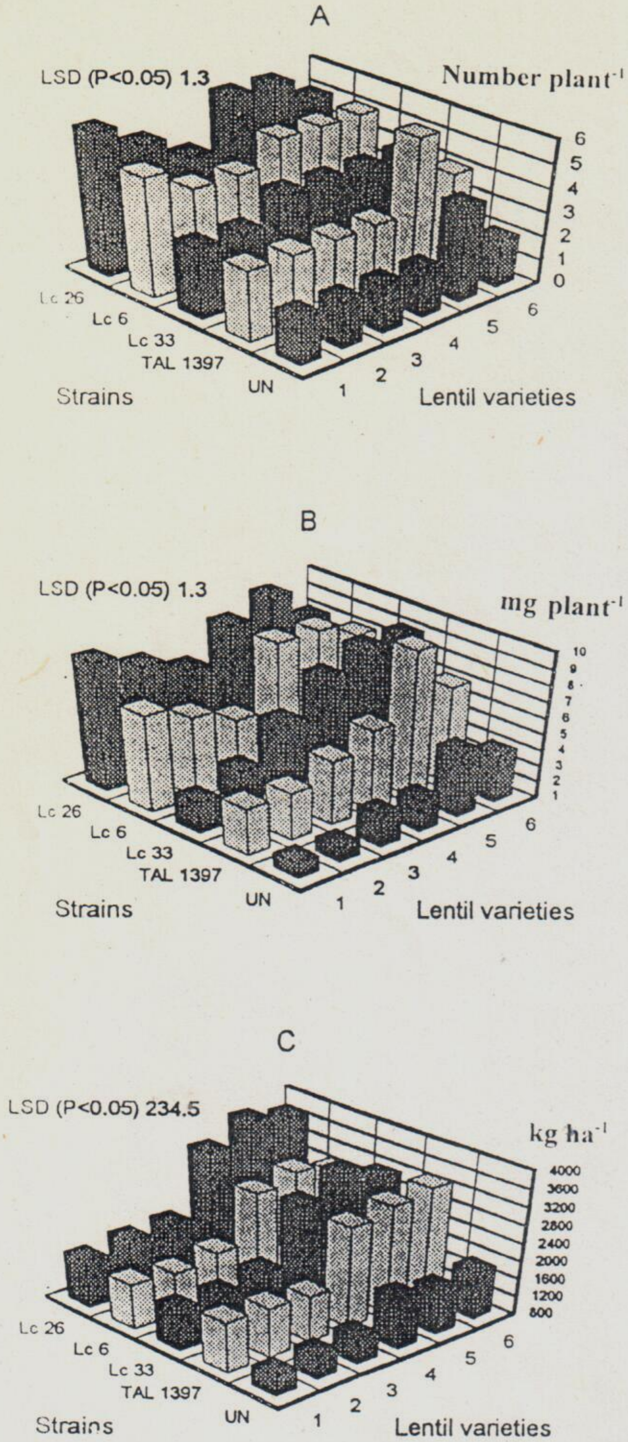
[b] Means calculated across treatments with all rhizobial strains (means of 12 observations)

highest biomass, grain yield and N yield were produced by M-85; the values of these parameters were statistically similar to those in the PL-406 and PL-406/M7-10-62 treatments. The highest $\%P_{fix}$ and N_{dfa} occurred in the PL-406 treatment and were statistically similar to those in the M-85 and PL-406/M7-10-62 treatments. The six lentil cultivars could be divided into two distinct groups, i.e. Precoz, Precoz/F6-20-1 × M-85 and Precoz/F6-23-1 × M-85 forming one group and M-85, PL-406 and PL-406/M7-10-62 the other group. In the treatments with Precoz and its hybrids, nodule dry weight, biomass, grain yield, N yield, $\%P_{fix}$ and N_{dfa} were statistically lower than the other group; the only exception was nodule number. Precoz is a large-seeded variety from Argentina and is not well adapted to Pakistan; even its hybrids with M-85 were suboptimal in terms of nodulation, biomass and N_2 fixation. These results showed that the various cultivars varied with respect to N_2 fixation, which was in agreement with the results of Date and Roughley (1977), who observed differences in N_2 fixation among various host plants. Caldwell and Vest (1977) also reported the influence of the host plant on symbiotic effectiveness, nodulation and N_2 fixation.

The effects of the legume host-*Rhizobium* interaction on nodule number, nodule dry weight, biomass, grain yield, N yield, P_{fix} and N_{dfa} are shown in Fig. 1. It was observed that the inoculation response was statistically significant in all the cultivar × *Rhizobium* treatments. The highest number of nodules was observed in the treatments with Lc 26 × M-85 (Fig. 1A), and did not differ statistically from those in the treatments with Lc 26 × Precoz, Lc 26 × PL-406/M7-10-62, and TAL 1397 × M-85. All the cultivars when uninoculated, except M-85, had the lowest number of nodules. The highest nodule dry weight was observed in Lc 26 × M-85, which was statistically similar to that in the Lc 26 × PL-406/M7-10-62, Lc 33 × M-85, Lc 26 × Precoz and Lc 6 × M-85 treatments (Fig. 1B). The lowest nodule dry weight was obtained in the uninoculated Precoz treatment. Nodule numbers were positively correlated ($r=0.80$) with nodule dry weights. The highest biomass, grain yield, N yield and N_{dfa} were produced in the Lc 26 × M-85 treatments (Fig. 1C–G). Nodule dry weight was positively correlated with biomass ($r=0.90$), grain yield ($r=0.78$) and N yield ($r=0.74$). The highest P_{fix} was observed in the Lc 26 × PL-406 treatments.

Many other researchers (Bello et al. 1980; Rennie and Kemp 1983; Skot 1983) have shown interactions between *Rhizobium* strains and host plants. Several reports have shown that the amount of N_2 fixed by a *Rhizobium* strain is strongly influenced by the host plant (Graham and Rosas 1977; Ruschel et al. 1982; Hardarson et al. 1984). It was also clear from the results presented here that the inoculated cultivars produced a higher biomass, grain yield, N yield, P_{fix} and N_{dfa} than the uninoculated cultivars. There was a strong correlation between biomass and N yield ($r=0.96$) and biomass and N_{dfa} ($r=0.92$). These findings were in agreement with those of Danso et al. (1987) who observed that, in general, the varieties or treatments with a higher dry matter yield supported greater fixation (high N_{dfa} show high $\%P_{fix}$) and vice versa.

It was apparent from the results that the various rhizobial strains and cultivars examined have a different inherent potential for Nfda. The range of $\%P_{fix}$ obtained for the various cultivars- in combination with different strains was 3–52%, which was comparable to the range of 0–76% observed by Bremer et al. (1990) in a similar study. This means that one should be very



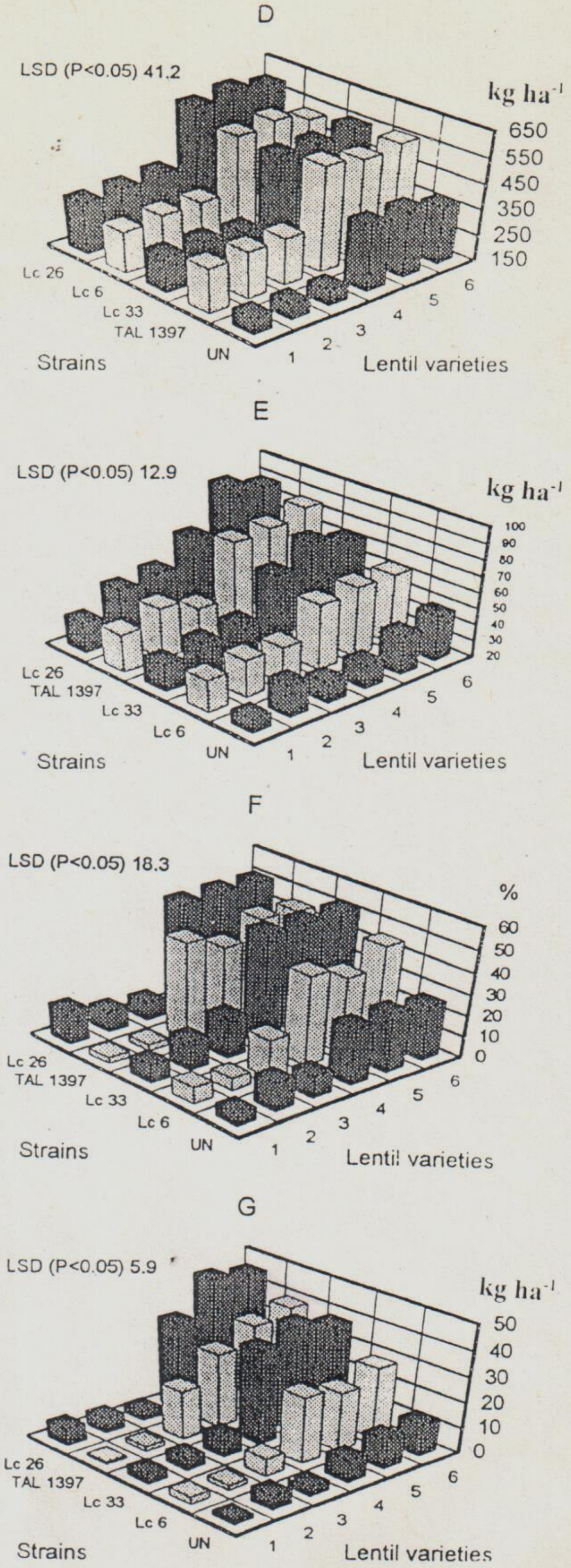


Fig. 1 Lentil cultivar-*Rhizobium* strain interactions: **A** nodule number, **B** nodule dry weight, **C** biomass, **D** grain yield, **E** N yield, **F** proportion of plant N derived from N_2 fixation, **G** amount of N derived from the atmosphere. *1* Precoz, *2* Precoz/F6-20-1 × M-85, *3* Precoz/F6-23-1 × M-85, *4* PL-406/M7-10-62, *5* M-85, *6* PL-406, *LSD* least significant difference

careful when selecting cultivars for high rates of N_2 fixation. It was found in a previous study (unpublished data) that the cultivar M-85 produced 124 kg N ha^{-1} in response to the application of 150 kg N ha^{-1}, whereas in the same experimental field in the present study, the same cultivar fixed 26–48 kg N ha^{-1} from the atmospheric N_2 (Fig. 1G).

The results presented here indicated that there is a wide gap between the potential of lentil cultivars with respect to total N yield and the actual quantity of N fixed. This indicates that it is still possible to further improve the efficiency of N_{dfa} in lentil crops through a more critical selection of both *Rhizobium* strains and lentil cultivars. Thus, in order to increase grain yields through higher N_2 fixation, not only is the selection of the most efficient *Rhizobium* strains important, but co-selection of the most appropriate legume hosts equally as important.

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