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COMPARISON OF DIRECT AND INDIRECT METHODS OF MEASURING NITROGEN FIXATION IN FIELD GROWN CHICKPEA GENOTYPES

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Abstract

Field experiments were conducted to study the nitrogen fixing potential of cultivars and breeding lines of chickpea (*Cicer arietinum*) where 20 chickpea genotypes were compared using the ^{15}N isotope dilution technique, acetylene reduction assay (ARA) and yield parameters such as biomass, grain and total nitrogen. Great differences in nitrogen fixation were observed between and within the experiments. Proportion of nitrogen fixed from air (Pfix) and nitrogen derived from air (Ndfa) ranged from 23-68% and 4-61 kg ha^{-1} for the first year and 37-62% and 24-60 kg ha^{-1} for the second year, respectively. There was highly significant correlation between yield and N_2-fixation but these were non-significantly correlated with nodulation data and ARA. The nodulation and ARA data were not good for field evaluation of chickpea cultivars suggesting that simple yield parameters like grain and total nitrogen yield could be used to screen a large germplasm in field as compared to highly expensive and laborious ^{15}N dilution technique.

Introduction

The ^{15}N isotope dilution technique is considered to be one of the most reliable method for estimation of nitrogen fixation by nodulated legumes in the field (Danso, 1995; Mcneill *et al.*, 1996). The method depends upon differences in the isotopic composition between the sources of N available to the plant i.e., soil N, fertilizer N and atmospheric N (Fried *et al.*, 1983). A special advantage of the technique is that it assesses the integrated amount or proportion of nitrogen derived from atmosphere through N_2 fixation in the field grown legume crops (Reichardt *et al.*, 1987), whereas major limitation of this method in the developing countries is high cost of instruments to measure ^{15}N and the use of expensive ^{15}N-labelled fertilizer (Peoples *et al.*, 1989; Danso, 1995). Experiments were therefore carried out to correlate the simple grain yield and total N data with a rather expensive and more laborious ^{15}N-isotopic methodology and to establish a method applicable for field evaluation of large germplasm in developing countries. The performance of 29 chickpea advanced mutant/ cultivars in the field for better nitrogen fixation based on total N accumulation, ^{15}N isotopic dilution, acetylene reduction, grain yield and nodule mass determinations is presented.

Materials and Methods

Field experiments were conducted at the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, as a part of a FAO/IAEA Coordinated Research Programme.

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Experiment 1: The soil was sandy loam pH 7.6, with initial available NH_4-N and NO_3-N concentrations of 0.98 and 1.54 mg kg^{-1} soil, respectively. The plot was divided into two 20.5X11 m sub-plots. Twenty advanced chickpea mutants/cultivars (Table 1) were selected for screening for N_2 fixation with wheat and barley as reference crops (Herridge *et al.*, 1998). The chickpea genotypes were obtained from Mutation Breeding Division of NIAB. Genotypes C44, CM1918 and CM72 were cultivated varieties while other genotypes were either mutants or hybrids.

Ammonium sulfate labelled with 5.35 % ^{15}N atom excess was applied in one sub-plot @ of 30 kg ha^{-1}. An identical amount of unlabelled ammonium sulfate was added to the other sub-plot. ^{15}N labeled ammonium sulfate solution 80 ml m^{-2}, stock diluted with 5 liters of water was sprayed in field at the time of sowing. A basal dose of 75 kg ha^{-1} P_2O_5 as single super phosphate (SSP) was applied in each sub-plot. The experiment was conducted in a randomized complete block design with 3 replications. In each replicate, there were 3 rows of each chickpea genotype, barley and wheat in 3m rows. Seeds were planted with inter-row and inter-plant spacing of 30 cm. Plants were not inoculated with *Rhizobium* because of the presence of effective indigenous chickpea rhizobial population (Hafeez *et al.*, 1987). The native chickpea rhizobial population was 200 cfu g^{-1} soil as determined by the most probable number plant infection technique (Asad *et al.*, 1991).

Experiment 2: The experiment was performed in the succeeding chickpea growing season in the same field with the same design and layout as in the first experiment. Twenty chickpea genotypes were tested while 9 chickpea genotypes which were attacked by gram blight disease in the first experiment (Table 1) were replaced with disease resistant genotypes (Table 3). Before the experiment, 30Kg N ha^{-1} as ammonium sulfate enriched with 4.69% ^{15}N atom excess was again applied in solution 80 ml m^{-2}, stock diluted with 5 liters of water was sprayed in field at the time of sowing. The indigenous chickpea rhizobial population was 220 cfu g^{-1} soil. The genotypes were not inoculated as good nodulation was observed in the first experiment.

Sample of 10 plants was collected from the unlabelled area of each genotype at 50% flowering and maturity growth stages to determine nodule number, nitrogenase activity of nodules, shoot and grain dry matter. At physiological maturity, 3 m length of the middle row (equivalent to 0.9m^2) was collected from the labelled area for each genotype, to determine %N and ^{15}N enrichment. ^{15}N analysis of the plant material was done at the FAO/IAEA Agricultural Biotechnology Laboratory in Seibersdorf, Austria on a mass spectrometer (Fiedler & Proksch, 1975). Total N in shoot and grain was estimated by Kjeldahl's method (Bremner, 1965). The results were compared statistically by using Duncan's Multiple Range Test (Duncan, 1955). To calculate Pfix (proportion of N fixed from air) and Ndfa (nitrogen derived from air) the following formulae were used (IAEA, 1983).

$$\text{Pfix (\%)} = \text{(\%Ndfa)} = \left(1 - \frac{\%\,^{15}N \text{ at.excess legume crop}}{\%\,^{15}N \text{ at.excess reference crop}}\right) 100 \qquad (1)$$

$$\text{Ndfa (kg ha}^{-1}) = \frac{\text{Pfix (\%)}}{100} \times \text{Total N yield} \quad (2)$$

Results and Discussion

Experiment 1: Biomass, grain and nitrogen yield varied from 1333 - 5554, 373 - 1890 and 26 - 115 kg ha^{-1}, respectively whereas Pfix and Ndfa ranged from 23-68% and 4-61 kg ha^{-1}. In yield and nitrogen fixation, variety C44 showed better results (Table 2).

Table 1. Chickpea advanced mutants/cultivars tested in the present study.

No.	Genotype	Parentage
1	C-44	Local cultivated variety (Approved variety).
2	CM-1	Mutant of local parent 6153.
3	CM-72	Cultivated mutant of local parent 6153.
4	CH-5*	Hybrid of two local parents Thel white344.
5	CM-1571-12A*	Mutant of exotic ICARDA genotype ILC-195.
6	P12-45F	Hybrid of C44ILC-195.
7	CM-2*	Mutant of local parent 6153.
8	CM-663	Mutant of C727 (hybrid cultivated variety).
9	CH-9	Hybrid of two local parents Thel white344.
10	P8-A	Hybrid of C44 and ILC-195.
11	CM-1918*	Mutant of local parent 6153 (Approved variety).
12	CM-1571-22	Mutant of exotic genotype ILC-195.
13	C-727	Hybrid of F8Punjab 7 (Approved variety).
14	CM-1913*	Mutant of local parent 6153.
15	CM-687	Mutant of C727.
16	CM-2197*	Mutant of CM72.
17	P7-H*	Hybrid of CM72ILC-195.
18	CM-88*	Mutant of C727.
19	CM-1571-12B*	Mutant of exotic genotype ILC-195.
20	P5-B	Single plant selection.
21	MB-75	Hybrid of C44ILC195.
22	50	Single plant selection of C727C141.
23	35	Single plant selection of C727C141.
24	Paidar 91	Hybrid of C-235ILC-191 (Approved variety).
25	Punjab 91	Hybrid of RC-32NEC 138-2 (Approved variety).
26	37	Single plant selection of C727C141.
27	19	Single plant selection of C727C141.
28	MB40	Hybrid of C44ILC195.
29	Noor 91	Selected from Flip81-293C (hybrid of ILC-191ILC-495) (Approved variety).

*Chickpea genotypes infected with Gram Blight disease caused by *Ascochyta rabiei*.

Table 2. Nodulation, ARA, biomass, grain yield, nitrogen yield and N_2 -fixation by various chickpea genotypes (Experiment 1).

Sr. No.	Genotypes	Nodule dry wt. mg plant^{-1}	Nodule	ARA	Biomass	Grain	Nitrogen	Pfix	Ndfa
				yield kg ha^{-1}			%	kg ha^{-1}	
1	C-44	19	173	16.7	5554	1890	115	68	61
2	CM-1	10	63	5.7	3944	1397	82	56	36
3	CM-72	13	77	6.1	2706	1062	55	49	20
4	CH-5	14	197	19.1	4105	1140	78	45	25
5	CM-1571-12A	14	120	20.4	4056	1286	81	44	29
6	P12-45F	14	130	24.7	3829	925	79	40	21
7	CM-2	15	197	29.7	3018	674	60	36	17
8	CM-663	20	140	22.3	3665	1389	76	34	17
9	CH-9	20	153	8.7	3695	1411	74	36	20
10	P8-A	18	127	20.5	4041	1470	89	38	24
11	CM-1918	10	60	8.9	2137	745	40	39	12
12	CM-1571-22	14	170	27.9	4192	1531	86	32	21
13	C-727	29	150	17.6	4047	1401	84	32	24
14	CM-1913	19	87	10.4	4174	1167	83	36	19
15	CM-687	21	73	6.8	3768	1212	71	32	17
16	CM-2197	15	140	17.3	2831	985	56	29	18
17	P7-H	16	87	3.4	1333	373	26	23	4
18	CM-88	18	103	13.3	2598	1061	50	27	12
19	CM-1571-12B	21	197	56.1	2770	1337	62	23	20
20	P5-B	14	107	6.1	3627	998	71	24	15
	LSD	5.0	26.1	5.7	2145.0	910.0	24.4	7.5	6.3

Pfix = Proportion of plant N fixed from the atmosphere.

Ndfa = Nitrogen derived from the atmosphere,

ARA = Acetylene reduction assay (μmol h^{-1} plant^{-1}).

The values in the columns are the averages of 3 replicates compared at $P<0.05$ with DMRT

Table 3. Nodulation, biomass, grain yield, nitrogen yield and N-fixation by various chickpea genotypes (Experiment 2).

Sr. No.	Genotypes	Nodule dry wt. mg plant^{-1}	Nodule	Biomass	Grain	Nitrogen	Pfix	Ndfa
			----------yield kg ha^{-1}----------			%	kg ha^{-1}	
1	MB-75	14	440	5693	2400	98	62	60
2	CM-687	29	150	4036	1867	77	57	45
3	P8-A	15	390	5567	2578	92	56	53
4	P12-45F	11	400	5340	2222	94	53	50
5	C-44	19	490	5275	2644	92	52	49
6	50 29	470	4610	2256	92	52	47	
7	35 19	270	3463	1589	54	52	29	
8	P5-B	14	220	4393	2044	74	51	38
9	Paidar 91	14	300	3797	1656	66	51	34
10	C-727	17	430	4786	2344	87	50	45
11	CM-1571-22	15	340	4562	2133	77	50	39
12	CM-72	13	220	3391	1567	66	50	31
13	CM-1	15	270	4747	2133	86	49	43
14	Punjab 91	38	340	4430	2256	68	47	32
15	37 22	280	3771	2011	78	47	36	
16	19 25	420	3904	1844	65	43	26	
17	MB40	16	440	4170	1533	75	43	32
18	CM-663	30	200	4202	2044	72	41	30
19	CH-9	17	310	4448	1933	77	41	31
20	Noor 91	11	310	3952	1644	64	37	24
	LSD	15.2	183.6	1446.0	667.5	27.6	17.2	18.6

Pfix = Proportion of plant N fixed from the atmosphere.

Ndfa = Nitrogen derived from the atmosphere.

The values in the columns are the averages of 3 replicates compared at $P<0.05$ with DMRT.

Pilbeam *et al.*, (1998) have also reported 16-48 kg ha^{-1} nitrogen fixed by chickpea, depending on the season.

There was a non-significant correlation between nodule number and nodule dry weight (Table 4) with maximum nodule number observed in C727 and maximum nodule dry weight was in CH5, CM2 and CM1571-12B. The highest acetylene reduction activity (ARA) was found in genotype CM1571-12B which had the highest nodule dry weight. There was a highly significant correlation between nodule dry weight and ARA indicating that nodule dry weight could be a better indicator of differences in acetylene reduction activity of chickpea genotypes than nodule number. On the other hand the genotype CM1571-12B, with significantly higher nodule dry weight and ARA, produced lower biomass and grain yield and poor nitrogen fixation. These observations suggest that nodule number, nodule dry weight and ARA were not the only criterion for the screening of chickpea genotypes for N_2-fixation capability in the field. Bremer *et al.*, (1990) also reported that the poor correlation between ARA and nitrogen yield may be due to the fact that ARA is a point measurement and sampling at one point in time may not be very effective in determining nitrogen fixing potential. The ARA technique is not a method of choice for measuring BNF (Danso, 1995). Minchin *et al.*, (1994) have suggested that ARA is of very limited application even for pot-grown legumes and would not recommend the use of uncalibrated ARA for field studies. Ideal N_2 fixation will be those that combine high yield with high N_2 fixation capability, and this has been an important aim in many programmes specifically directed towards high N_2 fixation. In the present study, there was a significant correlation between biomass and grain yield and biomass and total nitrogen which showed that the genotypes with higher biomass produced higher grain and nitrogen yield. Similarly, Danso *et al.*, (1987) observed that, in general, varieties or treatments with high dry matter yield supported greater nitrogen yields. Lalande *et al.*, (1990) also found that there was significant correlation (0.96) between shoot dry matter and total nitrogen content. In the present study, the genotypes C44 and CM1 showed high grain yield with good nitrogen fixing potential.

Experiment 2: In the second year's experiment, 9 varieties which were attacked by gram blight (Table 1) were replaced with blight resistant varieties. These 9 disease-resistant varieties and the varieties repeated from the previous year's experiment showed better performance. This may be attributed to the cultivation of chickpea in the previous season that helped to maintain a higher number of rhizobia in the soil ($2.2X10^2$ cfu g^{-1} soil) as it has already been reported that rhizobia proliferate better in the rhizosphere of their host plant (Reyes & Schmidt, 1979; Toomsan *et al.*, 1983; Rupela *et al.*, 1987).

Several factors that affect plant growth, such as disease, would indirectly influence N_2 fixation. The disease resistant variety MB75 gave the highest biomass yield, nitrogen yield, Pfix and Ndfa. It was statistically similar to C44, the best genotype of the previous year's experiment. Regarding nodulation, biomass yield, grain yield and nitrogen fixation, there was again significant correlation between biomass and grain yield, biomass and total nitrogen, grain yield and total nitrogen and Ndfa and total nitrogen (Table 4), confirming the previous year's results. Galal (1997) also reported a significant correlation between N_2-fixation, N-uptake and biomass. ARA was not conducted during second year because of its non-significant contribution in screening of genotypes

Table 4. Correlation of various measured parameters in experiment 1 and 2

Nodule dry wt.	Nodule no.	Biomass yield	Grain yield	Nitrogen yield	Pfix	Ndfa
Experiment 1						
Nodule no.	0.31^{NS}					
Biomass yield	0.33^{NS}	0.23^{NS}				
Grain yield	0.31^{NS}	0.37^{NS}	0.82^{***}			
Nitrogen yield	0.36^{NS}	0.26^{NS}	0.99^{***}	0.86^{***}		
pfix 0.07^{NS}	0.07^{NS}	0.58^{**}	0.47^{*}	0.57^{**}		
Ndfa	0.27^{NS}	0.14^{NS}	0.79^{***}	0.75^{***}	0.81^{***}	0.84^{***}
ARA	0.73^{***}	0.23^{NS}	0.17^{NS}	0.22^{NS}	0.17^{NS}	-0.20^{NS} 0.09^{NS}
Experiment 2						
Nodule no.	-0.10^{NS}					
Biomass yield	0.58^{**}	-0.18^{NS}				
Grain yield	0.49^{*}	0.14^{NS}	0.87^{***}			
Nitrogen yield	0.53^{*}	-0.16^{NS}	0.89^{***}	0.80^{***}		
Pfix 0.13^{NS}	-0.09^{NS}	0.49^{*}	0.47^{*}	0.53^{*}		
Ndfa	0.40^{NS}	-0.16^{NS}	0.84^{***}	0.77^{***}	0.91^{***}	0.83^{***}

^{NS}: Non-Significant; *, **, ***: Significant at $p<0.05$, $p<0.01$ and $p<0.001$ levels, respectively.

(Table 4). Such similar reports have been made by Ruschel *et al.*, (1979) where some varieties, very well nodulated and with a high level of nitrogenase activity, do not show a yield response linked to nodulation. It would suggest that nodulation data and ARA are not suitable screening tools especially under field conditions.

^{15}N dilution methodology is laborious and rather expensive, hence can not be employed for large field areas (Amarger *et al.*, 1979). The correlation data given in Table 4 for the two years show highly significant correlation between N_2-fixing (Pfix and Ndfa) and yield (grain and nitrogen) parameters indicating that simple parameters like grain and nitrogen yield can be effectively used for the screening of different genotypes for better nitrogen fixation. Similar findings were reported by Ruschel *et al.*, (1982) who found that the ranking of the cultivars for their N_2-fixing efficiency was the same whether derived from grain yield, nitrogen yield or ^{15}N dilution technique. Minchin *et al.*, (1994) reported that under resource-poor situation where ^{15}N- based methods cannot be used, simple parameters such as dry mass yield and total N-harvest under N-limiting soils could be considered more dependable as also observed in the present study.

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