Nitrogen Fixation by Various Methods

The highest amount of nitrogen was fixed from the atmosphere by *A. nilotica* followed by other tree species registering 167. 177 and 176 mg/ seedling by % Ndfs, isotope dilution and 'A' value technique respectively (Table.2). *Acacia nilotica* performed better in shoot and root growth, nodulation and in turn in N_2 fixation compared to other three species.

Among the three methods, isotope dilution technique and 'A' value method recorded higher estimate of N_2 fixation followed by the method based on% Ndfs. In most cases, all the three produced statistically similar estimates of N_2 fixation. According to Danso (1991), the isotope dilution and 'A' value methods appear to be the most accurate. The 'A' value technique has more underlying assumptions and is more complicated conceptually and mathematically. The method based on % Ndfs gave little bit lower estimates of N_2 fixation due to the immobilization of initial enrichment in soil (Kadiata *et al.*, 1995).

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(Received: November 1998 Revised : March 2000

Madras Agric. J., 86(7-9): 422 - 425 July - September 1999
https://doi.org/10.29321/MAJ.10.A00635

BIOLOGICAL NITROGEN FIXATION IN *Casuarina equisetifolia* USING NATURAL ^{15}N ISOTOPIC METHOD

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ABSTRACT

Casuarina equisetifolia seedlings, uninoculated or inoculated with *Frankia* strain Ce2 and *Glomus fasciculatum* were grown for 3 months in pots, harvested and N_2 fixation was measured using ^{15}N isotopic method. Maximum Ndfa percent was 83.3 when inoculated with *Frankia* and Vesicular Arbuscular Mycorrhiza at 10kg fertilizer N level. The modest amount of nitrogen fixed was attributed to the low soil fertility and the result of this experiment confirm that *Frankia* strain Ce 2 can be confidently recommended to inoculate *Casuarinas* along with *Glomus fasciculatum* in the field.

KEY WORDS: *Casuarina equisetifolia*, Frankia, N_2 Fixation, ^{15}N, VA-mycorrhiza

Casuarina species are frequently reported to fix large amounts of N, upto 100 kg N ha^{-1} $year^{-1}$, although exact measures are difficult to be obtained owing to (methotodological limitations (Shearer and Kohl, 1986; Sougoufara *et al.*, 1990) *Casuarina equisetifolia* has potential for use in biomass production, land reclamation, and for rotational agriculture to improve the N status in soil. The outstanding ability of *Casuarina equisetifolia* to thrive in poor N deficient soils is due to their association with *Frankia*, the symbiotic N_2 fixing actinomycete forming nodules on their roots.

As the cost of production and application of nitrogenous and phosphatic fertilisers increase, farmers often cannot afford them, even though they are needed for growth. There is a need for investigating alternative means of increasing nitrogen and phosphate availability in N and P deficient soils.

A multisymbiosis located in the roots of *Casuarina equisetifolia* and microorganisms such as *Frankia* capable of fixing dinitrogen and VA-mycorrhizal fungi capable of absorbing phosphorus from the soil may be great advantage to the host plants (Rose, 1980; Such plants develop well in environments in which nitrogen and phosphorus sources for plant nutrition are limited.

The present study was undertaken to determine the role of *Frankia* and VAM fungi in nitrogen accumulation and also to assess the contribution of symbiotic nitrogen fixation on the economy of this nutrient in *Casuarina equisetifolia* seedlings.

MATERIALS AND METHODS

Soil description

The study area was located in the campus of Tamil Nadu Agricultural University, Coimbatore. The soil of the experiment was red sandy loam (pH 6.8) with a good drainage and medium fertility level (N 297 kg/ha, p 17.5 kg/ha, K 160kg/ha)and EC 0.32m Mhos/cm.

Pot establishment and maintenance

Seeds of *Casuarina equisetifolia* harvested from the natural populations growing in the vicinity of Coimbatore were sown in sterile sand. When one month old, the seedlings were planted into pots filled with a mixture of soil, sand and farm yard manure (2:1:1). Dry soil inoculum (3 g/kg soil) from single fungus pot cultures of *Glomus fasciculatum* were mixed. Where appropriate, thoroughly throughout the soil by vigorous shaking. The seedlings were inoculated by dipping their roots in a suspension of a two month old culture of *Frankia* (Ce 2) grown at 28°C in liquid BuCT medium (Malcolm *et al.*, 1985), the amount of Frankia inoculum added to each plant being equivalent to 3 g protein. The inoculated plants were grown in the green house under semi controlled conditions (approximately 15 h light at approximately 28° C). Plants were watered daily with deionized water and once a week with a modified one quarter strength Hoagland's solution without nitrogen (Hoagland and Arnon,1950).

Addition of ^{15}N urea (10% atom excess)

The ^{15}N urea equivalent to 10, 40 and 70 kg/ha soil weight basis was applied to each pot after the establishment of seedlings. The fertiliser applied to each pot on fifth,tenth and fifteenth day as 3 splits.

Treatments

At transplantation time, nine treatments with three replications each were used Treatments 1-3, no inoculation: application of ^{15}N labelled fertilizer at the rate of 10; 40 and 70 kg N/ha as a solution of ^{15}N urea containing 10% atom excess. Treatments 4-6, inoculation with *Frankia* Ce 2: application of ^{15}N labelled fertilizer at the rate of 10,40 and 70 kg N/ha.

Treatments 7-9, inoculation with *Frankia* Ce 2 and *Glomus fasciculatum;* application of ^{15}N labelled fertiliser at the rate of 10,40 and 70kg N/ ha.

Measurement of plant nitrogen status

Plants were harvested at 60 and 90 days after transplanting, oven dried at 80°C for 96 hours and weighed. Each sample was ground to pass through a 40 mesh sieve and analyzed for total nitrogen content by the standard micro-kjeldahl technique.

Non-fixing system (nfs)

The non-fixing control seedlings were grown in the green house and watered daily with deionized water. At 60 and 90 days, the plants were harvested, oven dried at 80 C For 70 hours and ground to 40 mesh and the non fixing capacity was compared with other treatments.

^{15}N determination

Plant samples were digested using the micro-kjeldhal technique, modified to include nitrite and nitrate with the salicylic acid pretreatment. Stream distillation with NaOH (40%) was performed in a glass distillation unit with 250 ml sample flasks. Ammonia was recovered in 3 ml of 4% boric acid without indicator over the 40 ml of a 100 ml beaker.

Cross contamination was avoided by distilling an additional 20ml of 95 percent ethanol between samples. Distillates were immediately acidified with 2 drops of 1 N H_2SO_4 and dried in a ventilated oven at 85°C. They were then transferred with 2 ml of distilled water in a disposable culture tube and dried again to be analyzed by a mass spectrometer. The percent N in the plant derived from the atmosphere (%Ndfa) was assessed by the natural ^{15}N dilution technique (Kohl and Shearer, 1980) and calculated as follows.

$$\% \text{ Ndfa} = 1 - \left(\frac{\text{at \% } ^{15}\text{N ex (fs)}}{\text{at \% } ^{15}\text{N ex (nfs)}}\right) \times 100$$

Where fs is the fixing system and nfs is the non fixing system.

$$\% \text{ Ndfa} = 1 - \left(\frac{\text{at \% } ^{15}\text{N ex (plants)}}{\text{at \% } ^{15}\text{N ex (fertilizer)}}\right) \times 100$$

% Ndfs = 100 - (% Ndfa - % Ndff)

Total N content per plant, Ndfa, N in the plant derived from the soil (Ndfs), and fertilizer (Ndff) were compared using a one way analysis of variance.

RESULTS AND DISCUSSION

Total nitrogen content in *Casuarina equisetifolia*

Maximum N contents of 0.114 and 0.232 g/plant were recorded with the combined application of *Frankia* and VAM at 70 kg N/ha on 60 and 90 days after planting respectively, but was decreased with the application of *Frankia* alone at 70 kg N/ha accounting to 0.091 and 0.184 g/plant at 60 and 90 days after planting. Rates of N accumulation decreased with the decreasing levels N application

Table 1. N_2-fixation by *Casuarina equisetifolia* inoculated with *Frankia* and VA mycorrhizal fungi as estimated by ^{15}N isotope dilution technique.

Treatment	Total N content (g/plant)		Atom % ^{15}N excess		% Ndfa		% Ndff		% Ndfs		N_2 fixed / seedling (g)	
	60 DAP	90 DAP	60 DAP	90 DAP	60 DAP	90 DAP	60 DAP	90 DAP	60 DAP	90 DAP	60 DAP	90 DAP
Control + 10 kg N/ha	0.0354	0.0540	0.4967	0.3569	-	-	8.7071	6.2564	91.2920	93.7436	-	-
Control + 40 kg N/ha	0.0468	0.1140	0.6745	0.5475	-	-	11.8245	9.5974	88.1755	90.4026	-	-
Control + 70 kg N/ha	0.0655	0.1286	1.3201	0.8274	-	-	23.1413	14.5052	76.8586	85.4948	-	-
Frankia + 10 kg N/ha	0.0583	0.0971	0.2285	0.0716	54.0015	79.9285	4.0050	1.2558	41.9935	18.8157	0.0315	0.0776
Frankia + 40 kg N/ha	0.0717	0.1674	0.3569	0.1939	47.0950	65.5885	6.2557	3.3985	46.6493	31.0129	0.0338	0.1098
Frankia + 70 kg N/ha	0.0917	0.1845	0.7082	0.3305	46.3495	60.0616	12.4154	5.7931	41.2364	34.1453	0.0250	0.1108
Frankia + *Glomus* + 10 kg N/ha	0.0704	0.1321	0.2121	0.0594	57.3000	83.3705	3.7178	1.0404	38.9822	15.5891	0.0403	0.1101
Frankia + *Glomus* + 40 kg N/ha	0.0998	0.2015	0.3035	0.1765	55.0020	68.2710	5.3208	3.0932	39.6770	28.6358	0.0549	0.1376
Frankia + *Glomus* + 70 kg N/ha	0.1145	0.2344	0.6356	0.2864	51.8550	65.3946	11.1413	5.0194	37.0037	29.1176	0.0593	0.1553
CD (0.05%)	0.015	0.034	-	-	2.30	2.75	2.05	2.30	3.10	3.28	0.0055	0.0200

Values represents the mean of three replications

applied either with *Frankia* alone or with *Frankia* and VAM combination (Table 1).

Significant increases in total N content is indicative of the high N requirements of *Casuarina equisetifolia* to realize its growth potential and of the capacity of *Casuarina equisetifolia* to symbiotically fix dinitrogen. The symbiotic N fixation could possibly explain the highest N accumulation/plant obtained in the present experiment.

Symbiotic dinitrogen fixation (Ndfa)

The ^{15}N based estimates should be interpreted with caution since the method used involve not only the assumption that the N_2 fixing plant takes up N from soil and added ^{15}N labelled fertiliser in the same ratio, but also that the time courses of declining ^{15}N enrichment in N and of assimilation of N for the nodulated and non-nodulated treatments are the same. Significant differences were observed between *Frankia* + ^{15}N labelled fertilizer application and Frankia and VAM +^{15}N labelled fertilizer application treatments in dinitrogen fixation (%Ndfa) by *Casuarina equisetifolia* as determined by natural ^{15}N dilution technique, we assessed the average percent Ndfa to 85% (Table 1) at 90 days after planting at lower level of 10kg fertilizer N application/ha along with Frankia and VA mycorrhizal fungi and that enhanced nodulation and nitrogen uptake by the host plant (Diem and Gauthier, 1982). The percent Ndfa was reduced with increase in N level. This showed the inhibitory effect of higher doses of nitrogen on the activities of *Frankia* fixing atmospheric nitrogen.

The percentage of plant N derived from soil by N_2 fixing plants was 38.9-41.9% (lower application of 10 kg N/ha) and 37.0 - 46.6% (higher application of 40 kg N/ha). Increase in percent Ndfs was noticed with the increase in N level and percent Ndfs was higher at 60 days after planting compared to 90 days after planting. The percentage of plant N derived from fertiliser was 3.7-4.0 (lower application of 10kg N/ha) and 11.1-12.4 percent (higher application of 70 kg N/ha) at 60 days after planting but was reduced at 90 days after planting. Values of percent Ndfs was more with single inoculation of Frankia when compared to the combined inoculation of *Frankia* and VA mycorrhizal fungi in the present investigation. However, further experiments are required to confirm these trends under field condition.

The results indicated that inoculation with *Frankia* and VAM fungi significantly increased the growth and N_2 fixation of *Casuarina equisetifolia* seedlings. Application of N fertilizer at a rates of 40 and 70 kg/ha considered a higher rates, thus inoculation appears to be a more efficient way to improve the growth of *Casuarina equisetifolia* than N fertilisation whenever the soil is devoid of *Frankia*, a situation which is most often encountered in India where Casuarinas have not yet been introduced.

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(Received : March 1999 Revised : February 2000)