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ASSESSMENT OF N_2 FIXATION BY *CASUARINA EQUISETIFOLIA* INOCULATED WITH *FRANKIA* ORS021001 USING ^{15}N METHODS

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Summary—*Casuarina equisetifolia* seedlings, uninoculated or inoculated with *Frankia* strain ORS021001 were grown for 4.5 months in pouches, then transplanted into 1 m^3 concrete containers forming 1 m^2 microplots. Trees were harvested 6.5 months later when they were 11 months old. N_2 fixation was measured using three methods of assessment: the direct isotopic method, the A-value method and the difference method. Estimations of N_2 fixation during the 6.5 months following transplantation were respectively 3.27, 2.31 and 3.07 g N_2 per tree. From these values it was calculated that about 40–60 kg N_2 would be fixed per hectare in a year at normal densities of 10,000 trees ha^{-1}. The results of this experiment confirm that *Frankia* strain ORS021001 can be confidently recommended to inoculate casuarinas in the field. Means to improve nodulation and subsequently N_2 fixation by casuarinas are discussed.

INTRODUCTION

The outstanding ability of casuarinas 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. Because of this characteristic, causuarinas can give a high yield of biomass as well as be exploited as timber, firewood or charcoal in many tropical countries. Furthermore casuarinas are sometimes used in rotational agriculture to improve the N status of soil. Casuarinas are then planted in wasted, N-deficient soils, grown for 5–10 yr, cleared for wood or charcoal, after which the land is planted with various crops, such as yams in New Guinea (Silvester, 1976) or non-N_2-fixing trees such as *Anacardium occidentale* for the production of cashew nuts in India (J.C.G. Ottow, personal communication). The crops or forest plantations following casuarinas benefit from the soil N accretion resulting from the N_2-fixing activity of the actinorhizal tree. Thus it is a primary task to evaluate the amount of N_2 actually fixed by field-grown casuarinas. Up to now, only two estimations have been published. The first one by Hannon, quoted by Silvester (1977), is related to a stand of *Allocasuarina littoralis* near Sydney, Australia. The litter fall was estimated as 29 t ha^{-1} yr^{-1}. Since this litter contained 1%N, the N circulation rate in it was estimated to be 290 k N ha^{-1} yr^{-1}. Taking into account the fact that the N content of all the soils in the region was less than 0.1% it was assumed that at least 75% of this N was recently fixed, thus suggesting a fixation rate of 218 kg N_2 ha^{-1} yr^{-1}.

The second estimation is related to a 13-yr-old *Casuarina equisetifolia* stand established in the sand dunes of the Cap-Vert peninsula 30 km from Dakar (Dommergues, 1963). By comparing the soil and tree N content of a planted plot with the soil N content of an adjacent plot devoid of vegetation, the mean N_2 fixation rate was calculated to be *ca.* 58 kg N_2 ha^{-1} yr^{-1}.

Since the results mentioned above are questionable because the methods of estimation are not the most reliable and since we wanted to check the effectiveness of recently isolated strain of *Frankia* ORS021001 (Diem *et al.*, 1982), we decided to apply the ^{15}N-tracer technique. We report here the results of a study to evaluate N_2 fixation by *Casuarina equisetifolia* inoculated with *Frankia* ORS021001 and growing in conditions very close to those encountered in the open field. This experiment covered a growth period of 6.5 months thus providing a basis for estimation of the annual N_2 fixation. Three different methods for measuring N_2 fixation were compared: the direct isotopic dilution method, the A-value method and the difference method.

It is now well known that casuarina roots have symbioses not only with *Frankia* but also with ecto- (Bamber *et al.*, 1980) or endomycorrhizal fungi (Rose, 1980; Diem *et al.*, 1981) that help the trees scavenge mineral nutrients, especially P, thus enhancing nodulation and N_2 fixation (Diem and Gauthier, 1982). However this response to mycorrhizal infection occurs only when the soil content in available P is low ($\leqslant$ 10 kg P ha^{-1}). Since the aim of the experiment reported here was not to study the effect of mycorrhizal fungi we added to the soil a relatively high level of P (30 kg P ha^{-1}) at the onset of the experiment and later N-free Hewitt (1966) solution so that the plant requirements in elements other than N were largely fulfilled, thus masking a possible effect of any mycorrhizal infection.

MATERIALS AND METHODS

The experiment was conducted from July 1982 to May 1983 at the ORSTOM Bel Air Station in Dakar, Senegal, in twelve 1 m^3 concrete containers forming 1 m^2 microplots. The soil used was Bel Air soil, a typical sandy (93% sand), neutral (pH 7.0) soil, with C and N contents of 0.3 and 0.025% respectively (psamment; vernacular name: Dior). The soil was carefully homogenised, introduced into the concrete containers and finally fumigated with methyl bromide.

Seeds of *Casuarina equisetifolia* harvested in the vicinity of Dakar were sown in sterile Bel Air soil. When 1-month-old, the seedlings were planted into 5 × 25 cm polyethylene pouches filled with a mixture of vermiculite and sterile soil (1:5). The seedlings were inoculated by dipping their roots in a suspension of a 2-month-old culture of *Frankia* ORS021001 grown at 28°C in liquid QMOD medium (Lalonde and Calvert, 1979), the amount of *Frankia* inoculum added to each plant being equivalent to 3 μg protein.

Plants were raised in the pouches for 3.5 months and then transplanted into the microplots with four plants per microplot, all of which received PK as K_2HPO_4 at the rate of 17 g m^{-2}.

Treatments

At transplantation time three treatments with four replications each were used as follows:

Treatment 1. No inoculation; application of ^{15}N-labelled NH_4^+-N at the rate of 2 g N m^{-2} (i.e. 0.5 g N $plant^{-1}$) as a solution of $(^{15}NH_4)_2SO_4$ containing 10.5 atom% ^{15}N excess.

Treatment 2. No inoculation; application of ^{15}N-labelled NH_4^+-N at the rate of 10 g N m^{-2} (i.e. 2.5 g N $plant^{-1}$) as a solution of $(^{15}NH_4)_2SO_4$ containing 1.9 atom% ^{15}N excess.

Treatment 3. Inoculation with *Frankia* ORS021001; application of ^{15}N-labelled fertilizer as in treatment 1.

Throughout their growth the plants were carefully irrigated. When the plants were 8 months old, they exhibited symptoms of an undefined nutrient deficiency which was eliminated following application of 11 of Hewitt (1966) N-free nutrient solution to each microplot every 2 weeks. Plants were harvested 6.5 months after transplantation to the microplots when they were 11 months old. Three out of the 16 plants in treatment 1 and three out of 16 plants in treatment 2 were found to bear nodules. These contaminated plants were discarded so that calculations were based on 13 plants instead of 16 in treatments 1 and 2.

Estimation of N_2 fixation

Shoots were divided into three fractions: cladodes, branchlets (dia <4 mm), and stems plus branches (dia >4 mm), dried to a constant weight at 60–70°C. Then each fraction was completely ground into 100 μm powder. Samples of each powdered fraction were analyzed for total N and ^{15}N. ^{15}N analyzes were carried out at the Seibersdorf Laboratory (IAEA) using Dumas' method (the combustion performed in this technique converts total N directly to N_2) and

Table 1. Influence of inoculation with *Frankia* ORS021001 on nodule weight, height, dry weight and N content of 11-month-old *Casuarina equisetifolia*[1]

Treatments[2]	Nodule weight (g $tree^{-1}$)	Height (cm)	Dry weight (g $tree^{-1}$)				N content (%)			
			Cladodes	Branchlets (dia ≤4 mm)	Stems and branches (dia >4 mm)	Total	Cladodes	Branchlets (dia ≤4 mm)	Stems and branches (dia >4 mm)	N total (g $tree^{-1}$)
1	0	170 ± 22[a]	151 ± 32[a]	43 ± 13[a]	100 ± 27[a]	295 ± 70[a]	0.86 ± 0.05[a]	0.36 ± 0.02[a]	0.30 ± 0.01[a]	1.77 ± 0.45[a]
2	0	192 ± 20[ab]	204 ± 58[ab]	66 ± 18[b]	140 ± 34[ab]	409 ± 105[ab]	1.00 ± 0.09[a]	0.39 ± 0.03[a]	0.29 ± 0.04[a]	2.71 ± 0.75[a]
3	17 ± 9	216 ± 21[b]	260 ± 88[b]	76 ± 20[b]	188 ± 58[b]	525 ± 162[b]	1.30 ± 0.14[b]	0.54 ± 0.05[b]	0.40 ± 0.09[b]	4.78 ± 1.90[b]

[1]Mean values ± confidence interval ($P = 0.05$).
[2]Treatment 1: uninoculated trees with application of ^{15}N-labelled fertilizer, 0.5 g N $tree^{-1}$; Treatment 2: uninoculated trees with application of ^{15}N-labelled fertilizer, 2.5 g N $tree^{-1}$; Treatment 3: inoculated trees with application of ^{15}N-labelled fertilizer, 0.5 g N $tree^{-1}$. For each experiment, numbers in column with same letter do not differ significantly, $P = 0.05$ (Duncan, 1955).

emission spectrometry. For each individual tree N and ^{15}N values were calculated taking into account the weight, N and ^{15}N contents of the different fractions of the tree (cladodes, branchlets, stems and branches).

As already indicated in the Introduction N_2 fixation was assessed using three methods: the direct isotope dilution method (Bremner, 1977; Fried and Middleboe, 1977; Vose *et al.*, 1982) the A-value method proposed by Fried and Broeschart (1975) and the difference method (Williams *et al.*, 1977).

(*a*) *Use of the direct isotope dilution method.* The percentage y of the plant N derived from N_2 fixation was calculated according to the formula:

$$y = 1 - \frac{en}{eo}$$

eo and *en* being the atom% ^{15}N excess in non-N_2-fixing and N_2-fixing plants respectively.

The individual y values for each of the 16 N_2-fixing plants were calculated taking into account each of the *en* values whereas *eo* was the average value for the non-N_2-fixing trees.

If N was the total content of each N_2-fixing tree, N_2 fixed per tree was:

$$Y = \frac{y \times N}{100}$$

(*b*) *Use of the A-value method.* In this modification of the isotope dilution method proposed by Fried and Broeschart (1975), the ^{15}N-labelled fertilizer is applied at a low rate to the N_2-fixing plant but at a normal (higher) rate to the non-N_2-fixing plant.

The A-value method involves the assumption that, when confronted with different sources of N, the plant uptake is directly proportional to the amount of N available in each source, provided that this amount is measured in the same unit, designated A. This unit is expressed as fertilizer N equivalent:

$$\frac{\%\text{ N derived from fertilizer}}{\text{A-value of fertilizer}} = \frac{\%\text{ N derived from } N_2 \text{ fixation } (y)}{\text{A-value of } N_2 \text{ fixation}} = \frac{\%\text{ N derived from soil}}{\text{A-value of soil}}$$

The available amount of soil plus fixed N is determined using the N_2-fixing plants:

$$\text{A "soil + fix"} = \frac{(100 - fn) \times \text{fertilizer rate } (N_2\text{-fixing plants})}{fn}$$

The available amount of soil N is determined using the non-N_2-fixing plants:

$$\text{A "soil"} = \frac{(100 - fo) \times \text{fertilizer rate (non-}N_2\text{-fixing plants)}}{fo}$$

and the A-value for fixed N_2 is:

$$\text{A "fix"} = \text{A"soil + fix"} - \text{A"soil"}$$

fn and *fo* being the percentages of N derived from fertilizer in N_2-fixing and non-N_2-fixing plants respectively:

$$fn = \frac{en}{efn} \quad \text{and} \quad fo = \frac{eo}{efo}$$

en and *eo* being the atom% ^{15}N excess in N_2-fixing and non-N_2-fixing plants respectively and *efn* and *efo* the atom% ^{15}N excess in the fertilizer applied on N_2-fixing and non-N_2-fixing plants respectively.

The percentage y of the total plant N derived from N_2 fixation is calculated using the formula:

$$y = \frac{\text{A "fix"} \times fn}{\text{fertilizer rate } (N_2\text{-fixing plants})}$$

We calculated the average A "soil" value which was considered as a constant in our experiment. For each individual N_2-fixing (inoculated) plant, we calculated A "soil + fix", A "fix.", y and finally the amount of N_2 fixed per plant:

$$Y = \frac{y \times N}{100} \qquad (5)$$

where N was the total N content of each plant.

(*c*) *Use of the difference method.* For each individual N_2-fixing plant (treatment 3), N_2 fixation was estimated to be the difference between the total N content of the harvested portion of each of these plants and the average total N content of harvested portion of uninoculated ones.

RESULTS

N_2 fixation by Casuarina equisetifolia

When they were transplanted the seedlings were 4.5 months old, the height of inoculated seedlings was *ca.* 30 cm and that of uninoculated ones was *ca.* 25 cm. The mean N content of inoculated seedlings was $\leqslant 30$ mg plant^{-1}. Thus N_2 fixation before transplantation was $\leqslant 30$ mg plant^{-1}, which is negligible in comparison to the amount of N_2 fixed after transplantation. Therefore N_2 fixation values presented hereafter are related to the 6.5 months following transplantation to the field. During this period N_2 fixation was 2.31–3.27 g N_2 fixed tree^{-1}, depending on the method of assessment (Table 2). If we assume that N_2 fixation would have been stabilized at this rate during the whole year following transplantation, extrapolation would give figures in the range of 4.26–6.04 g N_2 fixed plant^{-1} yr^{-1}.

Table 2 indicates that in 95 out of 100 times we can expect a large variation in the estimation of N_2 fixation since the related values lie within the range of 0.84–4.93 N_2 fixed plant^{-1} if we take into account the highest and the lowest figures calculated from the three methods.

Table 2. N_2 fixation by 11-month-old *Casuarina equisetifolia* as estimated by three different methods[1]

Methods	N_2 fixation[2] expressed as: Per cent N derived from N_2 fixation (y)	N_2 fixation[2] expressed as: g N_2 fixed tree^{-1} (Y)
Direct isotope dilution	55.0 ± 11.0	3.27 ± 1.60
A-value	39.1 ± 11.9	2.31 ± 1.47
Difference	49.0 ± 14.8	3.07 ± 1.86

[1]Mean values ± confidence interval ($P = 0.05$).
[2]For the period of 6.5 months following transplantation to the field.

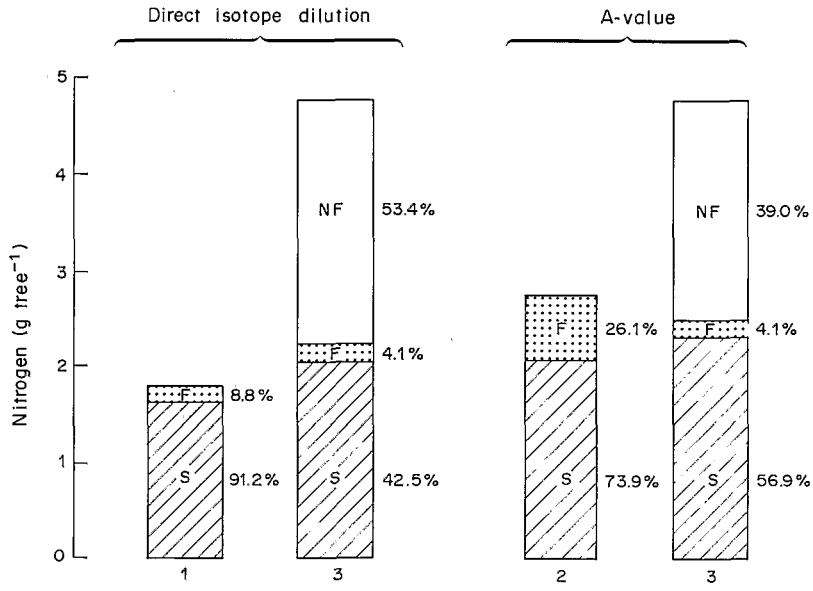

Fig. 1. Contribution of soil (S), fertilizer (F) and N_2 fixation (NF) to the N nutrition of *Casuarina equisetifolia* as assessed using direct isotope (left) and A-value (right) methods. Results expressed as g of N tree^{-1} or as percentage of total N content of the trees. Numbers 1, 2 and 3 refer to the treatments as defined in the text.

Nitrogen uptake from soil and fertilizer

The percentage of plant N derived from soil by N_2-fixing plants was 42.5 or 56.9% according to the method of assessment used (Fig. 1). The percentage of plant N derived from fertilizer was 4.1–8.8% (lower application of fertilizer) and 26.1% (higher application of fertilizer). The contribution of N fertilizer to plant nutrition was small probably because the percentage of utilization of N fertilizer remained low (27.12–31.26%), a situation comparable to that reported for soybean and millet in other Senegalese soils (F. Ganry, unpublished data).

DISCUSSION

N_2 fixation by Casuarina equisetifolia

From the values on Table 2 it can be calculated that at normal planting densities for *Casuarina equisetifolia*, i.e. 10,000 trees ha^{-1} (Kondas, 1983), in the presence of 20 kg fertilizer N ha^{-1}, N_2 fixation would be in the range of 40–60 kg N_2 fixed ha^{-1} yr^{-1}. The three methods used led to N_2 fixation estimates not very different from each other. The ^{15}N based estimates should be interpreted with caution since the methods used involve not only the assumption that the N_2-fixing plant takes up N from soil and added ^{15}N-labelled fertilizer 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 (Witty, 1983). The difference based estimates differ somewhat from the ^{15}N estimates probably for the reason, suggested by Witty (1983), that the relative fertilizer uptake of non-N_2-fixing and N_2-fixing casuarinas differ, being 8.8 and 4.1% respectively (Fig. 1).

Further field experiments are required to improve the accuracy of the ^{15}N method used, especially by, attempting to get more stable soil enrichment throughout the experiment (Witty, 1983). In addition it would be desirable (1) to study more extensively the influence of plantation density on N_2 fixation taking into account the fact that in semi-arid conditions many plantations do not contain more than 2000 trees ha^{-1} (Andeke-Lengui and Dommergues, 1983; Ataia, 1983); (2) to follow up the N_2-fixing activity of aging plantations, this activity supposedly increasing up to 5–10 yr of age, then decreasing with the progressive accumulation of N in the forest litter.

Variation in the N_2-fixing potential of individual trees

This variation could not be attributed to the endophyte, since trees were inoculated with a pure strain of *Frankia* and contamination by external strains was unlikely. Nor could the variation be due to soil heterogeneity, because the soil was carefully homogenized before filling the microplots. Since Table 1 shows that the total dry weight of trees was in the range of 363–687 g ($P = 0.05$), which indicates a heterogenous growth, we assume that a large part of the variation in N_2 fixation was due to differences in individual tree growth. These differences were probably related (i) to the intrinsic genetic variability of the trees, which were all obtained from seed and exhibited conspicuous differences in shape and colour; (ii) to variations in the physiological state of the seedlings at the time of transplantation, the effect of these variations being intensified by competitive interactions between the trees growing in the same microplot and (iii) to the fact that we used a minimal inoculum, which may not have allowed a homogeneous infection of the root systems.

Improving the growth of casuarinas by proper inoculation

We have seen (Table 1) that inoculation significantly increased the yield of the casuarina trees expressed as total N g tree^{-1} ($\times 2.70$) whereas the

application of N fertilizer at a rate of 2.5 g tree^{-1}, considered a high rate by silvicultural standards, induced a much lower, and non-significant, yield increase ($\times 1.53$). Thus inoculation appears to be a more efficient way to improve the growth of casuarinas than N fertilization whenever the soil is devoid of *Frankia*, a situation which is most often encountered in tropical countries where casuarinas have not yet been introduced. In addition, the result of the experiment reported here together with unpublished experiments using non-sterile soils devoid of *Frankia* confirm that strain ORS021001 can be confidently recommended to inoculate casuarinas in the field.

Preliminary work in field-simulating conditions has shown that by applying a heavy inoculum (equivalent to 30 μg *Frankia* proteins per plant) instead of a sparing inoculum (3 μg *Frankia* proteins), nodulation was more regular and the nodule dry weight expressed per 9-month-old tree nearly doubled (27 ± 5 g nodule tree^{-1}). Since we have been able to improve the culture of *Frankia* strains specific to casuarinas, we can now envisage massive inoculation of these trees, thus hopefully inducing a homogeneous and more abundant nodulation, which is the prerequisite to high N_2 fixation.

Improving the nodulating ability of the host-plant

We have already discussed the origin of the large variation in the N_2-fixing capacity of the casuarina tree. This variation can be partly related to the nodulating ability of the host-plant genotype. Attempting to explore this variability would probably be a fruitful approach to increasing N_2 fixation of casuarina stands.

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