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Symbiotic Interactions of *Acacia cyanophylla* with Soil Indigenous Rhizobia in a Semiarid Mediterranean Site: Implications of Intraplant Variation in ^{15}N Natural Abundance on N_2 Fixation Measurements

[1]Hafedh Nasr, [2]Mohamed Habib Ghorbel and [3]Yvon René Dommergues
[1]National Research Institute for Rural Engineering, Water and Forest,
P. O. 2, 2080 Ariana, Tunis, Tunisia
[2]Department of Biology, Faculty of Sciences,
University Tunis-El Manar,
1060 Tunis, Tunisia
[3]Directeur de Recherche au CNRS, 11 rue Maccarani, 06000 Nice, France

Abstract: We mainly aimed at investigating symbiotic interactions of 4-year-old *Acacia cyanophylla* Lindl. (blue-leafed wattle) with soil indigenous rhizobia in a semiarid Mediterranean site, in terms of nodulation and N_2 fixation. Secondary, we measured the density of indigenous *A. cyanophylla*-compatible rhizobia into the soil, in parallel with the biomass, nitrogen and ^{15}N natural abundance ($\delta^{15}N$) in the shoot components. A small indigenous population of *Acacia*-compatible rhizobia was detected. Concurrently, there were scarce perennial nodules on *A. cyanophylla*. The species produced small vegetative biomass and had low N content. The biomass was more allocated to stems than to phyllodes, whereas N content was more allocated to latter component. *Acacia cyanophylla* and its paired non-N_2-fixing *Olea oleaster* Hoffmgg. et Link. (wild olive tree) showed striking intraplant variation in $\delta^{15}N$, which suggested marked isotopic discrimination during N re-allocation among plant components. The measured N_2 fixation in phyllodes of *A. cyanophylla* was low. It was however not possible to measure N_2 fixation in total shoots, because of similar $\delta^{15}N$ values in shoots of *O. oleaster* and in fully N_2-dependent *A. cyanophylla*. Present results indicated no positive symbiotic interactions between *A. cyanophylla* and the indigenous population of rhizobia in semiarid Tunisia.

Key words: Biomass, isotopic discrimination, nitrogen fixing trees, nodules, rhizobium

Introduction

The N_2-fixing tree symbioses are largely used to manage nutrient-stressed soils, mainly in semiarid and arid zones, characterized by sparse and low-productive plant cover. Sustainable use of these symbioses however relies on available information of their actual N_2 fixation capacities (Dommergues *et al*., 1999). The ^{15}N natural abundance ($\delta^{15}N$) method, one of the more reliable techniques for measuring N_2 fixation (Unkovich and Pate, 2001), has been widely used to measure N_2 fixation in acacia symbioses under field conditions. Nevertheless, measurements based on the sampling of whole tree were poorly documented and have been made for only young trees

Corresponding Author: Hafedh Nasr, National Research Institute for Rural Engineering, Water and Forest, P.O. 2, 2080 Ariana, Tunis, Tunisia Tel: +216-71 230039, Fax: +216-71 717951

(Muofhe and Dakora, 1999), because of the huge amount of time and labour required for harvesting aged trees. Alternatively, N_2 fixation in acacia symbioses, as in other N_2-fixing tree symbioses, has generally been measured using foliar $\delta^{15}N$ (Polley *et al*., 1997; Galiana *et al*., 2002; Chikowo *et al*., 2004). The latter approach may provide helpful information on actual N_2 fixation when $\delta^{15}N$ values do not differ between plant components, nevertheless values can be component-linked and thus the sampling should include whole plant or total shoot rather than individual components (Peoples *et al*., 1991).

Within acacias, the blue-leafed wattle *Acacia cyanophylla* Lindl., syn. *A. saligna* (Labill.) H. L. Wendl. (Fabaceae/Mimosoideae) can have great potential in producing biomass in degraded areas (Nasr *et al*., 1986). *A. cyanophylla* is originated from south-western Australia and has been successfully introduced and naturalised in a wide range of contrasting environments, mainly in southern and northern regions in Africa. This is most likely because of its striking symbiotic promiscuity in nodulating and fixing N_2 in symbiosis with either slow or fast-growing rhizobia, as reported by Nasr *et al*. (1999). The N_2-fixing capacity of *A. cyanophylla* is largely improved by dual inoculation with compatible rhizobium and arbuscular-mycorrhizal fungus in addition to appropriate P supply (Nasr and Diem, 1987). The species however has poor growth when unable to fix N_2 (Stock *et al*., 1995). There is little information on the nodulation patterns of *A. cyanophylla* symbiosis in semiarid Mediterranean environments (Nasr *et al*., 1995), whereas there is no available data on its N_2 fixation ability in these environments.

An important consideration in introducing legumes is their symbiotic interaction with soil-resident rhizobium strains (Yates *et al*., 2004) and successful establishment of introduced legumes generally relies on their ability to nodulate with the soil-resident rhizobia (Parker, 1962). In Australia, indigenous rhizobia nodulating acacias are generally widespread in arid zones, where the host plants are commonly nodulated (Beadle, 1964); nodules are however most often ineffective (Lawrie, 1983). This strengthens that acacias symbioses have generally low N_2-fixing capacities (Roughley, 1987; Danso *et al*., 1991). At our knowledge, there is no available information on long-term interactions between *A. cyanophylla* and soil indigenous rhizobia in the Mediterranean region. We carried out an experimental plantation in a semiarid Mediterranean site, to mainly investigate symbiotic interactions of 4-year-old *A. cyanophylla* with putative soil indigenous rhizobia, in terms of nodulation and N_2 fixation. In parallel, we measured the density of indigenous *A. cyanophylla*-compatible rhizobia into the soil and the biomass, nitrogen content and $\delta^{15}N$ in the shoot components. Implications of intraplant variation in ^{15}N natural abundance on N_2 fixation measurements are discussed.

Materials and Methods

Experimental design

The experimental site was located at the Kondar region in the semiarid central Tunisia. The soil had poor natural plant cover and low fertility. Soil characteristics averaged 2 mg C g^{-1} soil, 0.5 mg N g^{-1} soil, 0.9 mg P_2O_5 g^{-1} soil and 0.3 mg K_2O g^{-1} soil. Seeds of *A. cyanophylla* Lindl. (seedlot KL086) were disinfected and scarified with 96% (v/v) H_2SO_4. The disinfected seeds were sown in a tyndallized sandy clay loam soil in plastic growth bags, which were placed in nursery benches and watered daily. No rhizobial inoculum was added to saplings. Three-month-old saplings were outplanted to 0.5 ha plot. Wild olive tree *Olea oleaster* Hoffmgg. et Link. (Oleaceae), one of the dominant native species in the experimental site, was planted between *A. cyanophylla* hedgerows, as a reference plant to measure putative N_2 fixation in *A. cyanophylla*. Planting was carried out at the rainy season (late fall). Spacing between plants was 4.5 m (i.e., 494 plant ha^{-1}). Both species are evergreen woody plants and have a mesic origin.

Plant Sampling

When trees were 4-year-old, six randomly-selected replicates of *A. cyanophylla* were harvested. Phyllodes were mixed and repetitively quartered to generate a representative sample. Branches with different diameters were cut into small portions and pooled in a representative sample of branch diameters. Stems were sawn at different heights to make a composite sawdust sample. Concurrently, the stems, branches and leaves of six *O. oleaster* replicates were randomly selected, harvested and sampled separately. This sampling method can minimize ^{15}N measurement errors due to the nitrogen cycling in woody plants. For each sampled *A. cyanophylla* tree, the ground area of potentially nodulating roots, as defined by Nasr *et al*. (1995), was dug out to 50 cm depth. Then, roots were excavated and examined for the presence of nodules. When present, nodules were excised, gently washed, dried and weighed. Fresh fine root samples were fixed in a solution of formalin-acetic acid-ethanol (65-25-910, v/v/v) for mycorrhizal analysis. The fresh samples of shoot components were separately oven-dried at 70°C to a constant weight, weighed and the total biomass of each component was calculated. Dried samples were finely ground for nitrogen analysis.

Analytical and Calculation Procedures

Density of rhizobia in the soil nearby *A. cyanophylla* was calculated, using the most probable number method (Brockwell, 1963). The fine root samples were cleared with 10% (w/v) KOH, stained with fuchsin-lactic acid solution (Kormanik and McGraw, 1982) and scanned by light microscopy for the presence of arbuscular-mycorrhizal structures.

For each shoot sample, N concentration on a dry matter basis (mg N g^{-1}) and $\delta^{15}N$ expressed in ‰ were measured with a CHN elemental analyser (SCA, CNRS Vernaison, France) connected to a mass spectrometer (Funnigan Mat Delta S, Bremen, Germany).

$$\delta^{15}N‰ = [(\%^{15}N_{sample}/\%^{15}N_{air}) - 1]\times 1000$$

Where,

$$\%^{15}N_{air} = 0.3663$$

The weighted $\delta^{15}N$ in total shoots was calculated using the following equation:

$$Shoot\,\delta^{15}N‰ = [(\delta^{15}N_L\times TN_L) + (\delta^{15}N_B\times TN_B) + (\delta^{15}N_S\times TN_S)]/(TN_L + TN_B + TN_S)$$

Where, TN represents the N content and L, B and S denote photosynthetic components (phyllodes for *A. cyanophylla* and leaves for *O. oleaster*), branches and stems, respectively.

The fraction of plant N derived from atmospheric N_2 (%Ndfa) was measured according to the equation of Shearer and Kohl (1986), as follows:

$$\%Ndfa = [(\delta^{15}N_{NF} - \delta^{15}N_F) / (\delta^{15}N_{NF} - B)]\times 100$$

Where,

$\delta^{15}N_F$ = $\delta^{15}N‰$ in N_2-fixing plant (*A. cyanophylla*)
$\delta^{15}N_{NF}$ = $\delta^{15}N‰$ in non-N_2-fixing reference plant (*O. oleaster*)
B = $\delta^{15}N‰$ during N_2 fixation, also named "B value"

Based on the general equation of the standard error of %Ndfa obtained by Shearer and Kohl (1986), we calculated and used the standard error with null covariance, as follows:

$$SE\ (\%Ndfa) = [(\delta^{15}N_F - B)^2 \times (SE\ \delta^{15}N_{NF})^2/(\delta^{15}N_{NF} - B)^4 + (SE\ \delta^{15}N_F)^2/(\delta^{15}N_{NF} - B)^2 + (\delta^{15}N_{NF} - \delta^{15}N_F)^2 \times (SE\ B)^2/(\delta^{15}N_{NF} - B)^4]^{1/2}$$

Statistical Analysis

A one-way ANOVA with a plant component factor was carried out. When significant differences were found at $p<0.05$, means were compared with Duncan's Multiple-Range Test. The biomass and nitrogen content data were ln-transformed, whilst $\delta^{15}N‰$ data were arcsine-transformed prior to analysis. All data presented are untransformed means.

Results and Discussion

Indigenous Rhizobia and Root Specialisations

The density of indigenous *Acacia*-compatible rhizobia at the experimental site was equal to 65 infective cells g^{-1} soil, indicative of a very small rhizobial population. This density was markedly lower than that nodulating *Acacia* spp. in other African regions (Odee *et al*., 1995). Survival of indigenous rhizobia was likely to be limited by the dry soil conditions, which resulted from long and recurrent drought periods prevailing in the semiarid Mediterranean zones. In parallel, roots of *A. cyanophylla* showed sparse nodules, which averaged 20 g nodule (dry weight) $tree^{-1}$. Root nodules of *A. cyanophylla* were perennials, dichotomously branched and showed a fresh and healthy live component, which covered a dark-coloured and suberized dead component (Fig. 1). This indicated that

Fig. 1: Root nodule on *Acacia cyanophylla* with a Dead Component (DC) covered by a Live Component (LC). Bar is 1 cm

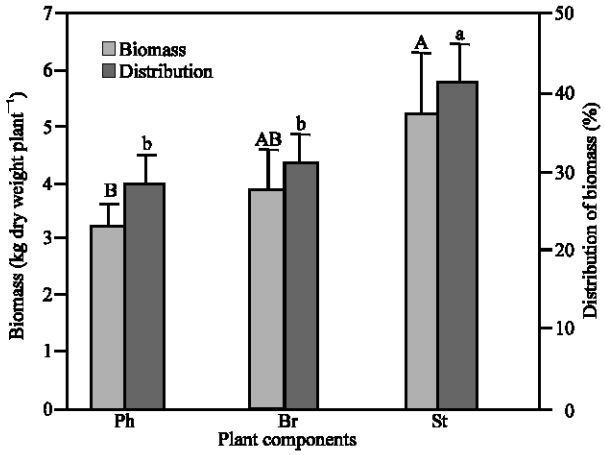

Fig. 2: Biomass and its distribution in Phyllodes (Ph), Branches (Br) and Stems (St) of *Acacia cyanophylla*. Error bars indicate SE of the mean; n = 6. Bars with the same case letter are not significantly different at $p<0.05$

nodule growth was indeterminate and cyclic with a senescence phase, most probably at the dry season, followed by a regrowth phase during the relatively less dry season; thus underlying that nodule growth can cease during dry periods and resume at relatively moist periods. At senescence phase, nodules can release large numbers of viable cells, which constitute an inoculant source of new roots (Brockwell *et al*., 2005). Thus renewed nodule growth with seasonal mortality may be a strategy that helps nodule persistence and expansion on roots of perennial N_2-fixing plants in dry environments. Analysis of the collected fine-root samples showed that both *A. cyanophylla* and *O. oleaster* were devoid of arbuscular mycorrhizal structures, which may decrease plant $\delta^{15}N$ (Spriggs *et al*., 2003). Absence of arbuscular mycorrhizal fungi could be attributed to the lack of appropriate soil moisture, concomitant to poor native-plant cover at the study site.

Distribution of Biomass, Nitrogen Content and $\delta^{15}N$

Biomass of stems, expressed as dry weight, of *A. cyanophylla* was significantly higher than each of the two other components (Fig. 2). Within shoots, biomass was similarly distributed between phyllodes and branches. Total biomass, on per hectare basis, was equal to 6 Mg ha^{-1}, strongly lower than that reported for *Acacia* spp. growing in other regions (Shanmughavel and Francis, 2001; Harmand *et al*., 2004), probably because performance of the nodulating rhizobium strains and environmental conditions differed. The N content in phyllodes was low (Fig. 3) and in the range of non-nodulated woody legumes growing on low-fertile Sahelian soils (Breman and Kessler, 1995), a fact most likely due to very low available soil N concomitant with no or low N_2 fixation. In contrast to biomass partitioning, N content was more allocated to phyllodes than to stems. Total N accumulated by *A. cyanophylla* plantation was equal to 57 kg N ha^{-1}. The $\delta^{15}N$ values in phyllodes of *A. cyanophylla* and in leaves of *O. oleaster* were positive, whereas those in branches and stems were negative (Fig. 4). The magnitude of intraplant variation in $\delta^{15}N$, as expressed by differences in $\delta^{15}N$ between shoot components, varied from 1.27 to 2.81‰ for *A. cyanophylla* and from 0.31 to 3.39‰ for *O. oleaster*. This intraplant variation was comparable to that obtained for other woody N_2-fixing and non-N_2-fixing trees (Yoneyama, 1984; Boddey *et al*., 2000;

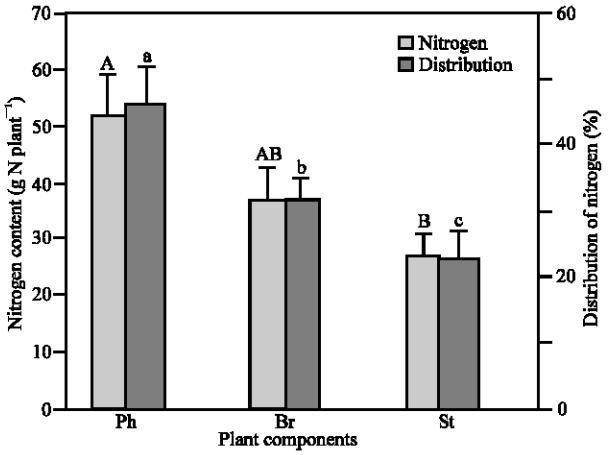

Fig. 3: Nitrogen content and its distribution in Phyllodes (Ph), Branches (Br) and Stems (St) of *Acacia cyanophylla*. Error bars indicate SE of the mean; n = 6. Bars with the same case letter are not significantly different at $p<0.05$

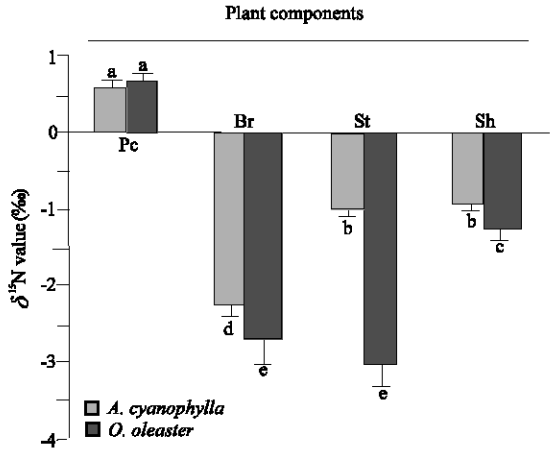

Fig. 4: ^{15}N natural abundance (^{15}N) in Photosynthetic components (Pc) (i.e., phyllodes for *Acacia cyanophylla* and leaves for *Olea oleaster*), Branches (Br), Stems (St) and Shoots (Sh) of *A. cyanophylla* and *O. oleaster*. Error bars indicate SE of the mean; n = 6. Bars with the same letter are not significantly different at $p<0.05$

Schmidt and Stewart, 2003) and indicated that ^{14}N was preferentially re-allocated from N-enriched photosynthetic components to woody components. This is commonly attributed to the mobilization of N from young leaves to later-formed woody tissues (Unkovich *et al*., 2000). Intraplant variation in ^{15}N obviously indicated that ^{15}N in phyllodes of *A. cyanophylla* and that in leaves of *O. oleaster* were not representative of the other plant components. In spite that roots were not investigated here, ideally, wherever feasible, ^{15}N in roots should also be measured to get further information on the distribution of ^{15}N within the whole plant.

Nitrogen Fixation

It is noteworthy that there was no significant difference in $\delta^{15}N$ between phyllodes of *A. cyanophylla* and leaves of *O. oleaster* (Fig. 4), suggesting little or no contribution of N_2 fixation to phyllode N. For measuring %Ndfa, Unkovich and Pate (2001) proposed to derive "B value" from plants established under similar conditions to those of the investigated ones, because several factors other than N_2 fixation *per se* can discriminate against ^{15}N under fully symbiotic conditions. Nevertheless, B values derived from host plants grown under controlled and optimal conditions have been commonly used to measure field N_2 fixation in acacias (Galiana *et al*., 2002; May and Attiwill, 2003; Chikowo *et al*., 2004). Moreover, B values of *Prosopis* sp. have been widely used to measure N_2 fixation in field-growing acacias (Shearer *et al*., 1983; Shearer and Kohl, 1986; Yoneyama *et al*., 1990; Schulze *et al*., 1991; Handley *et al*., 1994; Polley *et al*., 1997). We however used the B value derived from *Acacia saligna* (syn. *A. cyanophylla*), as reported by Stock *et al*. (1995), because at low %Ndfa values, errors associated with an inaccurate B value are small (Unkovich *et al*., 1994). As expected, measured %Ndfa in phyllodes of *A. cyanophylla*, using leaves of *O. oleaster* as a reference, resulted in a low value, which was equal to 6.2±0.1% (Mean±SE). This value was however in the range reported for *Acacia* spp. growing in other African dry zones (Schulze *et al*., 1991; Handley *et al*., 1994; Stock *et al*., 1995; Lehmann *et al*., 2002), thus supporting little contribution of soil-resident rhizobia in such zones to N nutrition of acacias. The weighted $\delta^{15}N$ in total shoots of *A. cyanophylla* was equal to -0.69±0.13‰ (Mean±SE), whereas that in total shoots of *O. oleaster* was equal to -1.25±0.14‰. Magnitude of difference in $\delta^{15}N$ between shoots (0. 65‰) of the two paired plants was higher than that between photosynthetic components (0.12‰), underlying that %Ndfa value may be higher when pairing shoots relative to photosynthetic components. Interestingly, there was no significant difference ($p<0.05$) in $\delta^{15}N$ between shoots of *O. oleaster*, which is fully soil-N-dependent and those of fully N_2-dependent *A. cyanophylla* (-1.27±0.10‰). Thus, it is not possible to measure N_2 fixation in total shoots of *A. cyanophylla* based on the $\delta^{15}N$ method.

Present results showed that *A. cyanophylla* and its neighbouring non-N_2-fixing *O. oleaster* had negative shoot $\delta^{15}N$ values, whereas in contrast, Nasr *et al*. (2005) reported that *Casuarina glauca* Sieber ex. Spreng. and its neighbouring non-N_2-fixing *Stipa tenacissima* L., in plantations in an another experimental site located at the study area, had positive shoot $\delta^{15}N$ values. The $\delta^{15}N$ values in the two plant pairs were however in the same range as reported in other N-limited areas (Bustamante *et al*., 2004). The $\delta^{15}N$ in a particular species of plant reflects interaction of many soil and plant processes (Stewart, 2001); we however assumed that particularly root patterns should have no significant implications on $\delta^{15}N$ of the two plant pairs because N leaching is trivial in soils in Mediterranean-type climate (Fillery, 2001) and additionally roots of all studied species lacked mycorrhizal structures, which may differently affect plant $\delta^{15}N$. Nevertheless, we suggested at least two possible explanations for such differences in $\delta^{15}N$ between the two plant pairs. Firstly, these differences may reflect horizontal variation in $\delta^{15}N$ of plant-available soil N between the two experimental sites. Secondly, each of the two plant pairs may have same patterns in N physiology, which differed from the other pair. Implications of horizontal variation in $\delta^{15}N$ on the measurements of N_2 fixation can be alleviated in planting the reference plant adjacent to its paired N_2-fixing plant (Shearer and Kohl, 1986; Peoples *et al*., 2001), as the hedgerow planting system we used. Further detailed investigations are needed to precisely identify factors that triggered differences in $\delta^{15}N$ between *A. cyanophylla* and *O. oleaster* pair and *C. glauca* and *S. tenacissima* pair.

It is concluded that trivial and sparse root nodulation, concurrent with low N_2 fixation rate and poor plant growth indicated no positive symbiotic interactions between *A. cyanophylla* and the indigenous rhizobium strains. However, small population of indigenous strains in the study site commonly implies successful establishment of introduced strains. Thus, inoculating saplings with selected host-compatible strains, prior to be outplanted, can potentially stimulate growth of *A. cyanophylla* in semiarid Tunisia.

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