Symbiotic Interactions of *Acacia cyanophylla* with Soil Indigenous Rhizobia in a Semi-arid Mediterranean Site: Implications of Intraplant Variation in $^{15}$N Natural Abundance on N$_2$ Fixation Measurements

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Abstract: We mainly aimed at investigating symbiotic interactions of 4-year-old *Acacia cyanophylla* Lindl. (blue-leaved wattle) with soil indigenous rhizobia in a semi-arid 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 ($^{15}$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* Hoffmg. & Link. (wild olive tree) showed striking intraplant variation in $^{15}$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 $^{15}$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 semi-arid 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 semi-arid 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 ($^{15}$N) method, one of the more reliable techniques for measuring N$_2$ fixation (Unkovich and Patc, 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.

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(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 \( ^{15}N \) (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 \( ^{15}N \) 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-leaved wattle \textit{Acacia cyanophylla} Lindl., syn. \textit{A. saligna} (Labill.) H. L. Wendl. (Fabaceae/Mimosoideae) can have great potential in producing biomass in degraded areas (Nasr et al., 1986). \textit{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 \textit{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 \textit{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 and 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 \textit{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 \textit{A. cyanophylla} with putative soil indigenous rhizobia, in terms of nodulation and \( N_2 \) fixation. In parallel, we measured the density of indigenous \textit{A. cyanophylla}-compatible rhizobia into the soil and the biomass, nitrogen content and \( ^{15}N \) 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\(_2\)O\(_5\) g\(^{-1}\) soil and 0.3 mg K\(_2\)O g\(^{-1}\) soil. Seeds of \textit{A. cyanophylla} Lindl. (seedlot KL086) were disinfected and scarified with 96% (v/v) H\(_2\)SO\(_4\). The disinfected seeds were sown in a tyndalized 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 \textit{Olea oleaster} Hoffm. et Link (Oleaceae), one of the dominant native species in the experimental site, was planted between \textit{A. cyanophylla} hedgerows, as a reference plant to measure putative \( N_2 \) fixation in \textit{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 %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-lactate acid solution (Kormann 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 %N were measured with a CHN elemental analyser (SCA, CNRS Vernaison, France) connected to a mass spectrometer (Finnigan Mat Delta S, Bremen, Germany).

\[ ^{15}N\% = \left( \frac{\% ^{15}N_{\text{excte}}}{\% ^{15}N_{\text{at}}} - 1 \right) \times 1000 \]

Where,

\[ \% ^{15}N_{\text{at}} = 0.3663 \]

The weighted %N in total shoots was calculated using the following equation:

\[ \text{Shoot} \%N = \left( ^{15}N_L \times \text{TN}_L + \left( ^{15}N_B \times \text{TN}_B \right) + \left( ^{15}N_S \times \text{TN}_S \right) \right) / \left( \text{TN}_L + \text{TN}_B + \text{TN}_S \right) \]

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 N₂ (%Ndfa) was measured according to the equation of Shearer and Kohl (1986), as follows:

\[ \% \text{Ndfa} = \left( ^{15}N_{\text{exc.}} - ^{15}N_2 \right) / \left( ^{15}N_{\text{exc.}} - B \right) \times 100 \]

Where,

\[ ^{15}N_2 = ^{15}N\% \text{ in } N_2 \text{-fixing plant (A. cyanophylla)} \]

\[ ^{15}N_{\text{exc.}} = ^{15}N\% \text{ in non-N}_2 \text{-fixing reference plant (O. oleaster)} \]

\[ B = ^{15}N\% \text{ during } N_2 \text{ fixation, also named "B value"} \]
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:

\[
\text{SE}(\%\text{Ndfa}) = \left[ (\text{SE}^{13}\text{N}_{\text{rf}} - B)^2 \times (\text{SE}^{15}\text{N}_{\text{rf}}) \right]^{1/2} + (\text{SE}^{15}\text{N}_{\text{rf}} - B)^2 \times (\text{SE}^{13}\text{N}_{\text{rf}} - B)^2 \times (\text{SE}^{15}\text{N}_{\text{rf}} - B)^2 \times (\text{SE}^{13}\text{N}_{\text{rf}} - B)^{10}
\]

**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 %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

![Image of root nodule]

**Fig. 1:** Root nodule on *Acacia cyanophylla* with a Dead Component (DC) covered by a Live Component (LC). Bar is 1 cm
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 $^{15}$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.

*Biomass, Nitrogen Content and 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 (Sharmanughavel 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 $^{15}$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 $^{15}$N, as expressed by differences in $^{15}$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 (Pe) (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 $^{15}$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 $^{15}$N between phyllodes of *A. cyanophylla* and leaves of *O. oleaster* (Fig. 4), suggesting little or no contribution of N$_2$ fixation to phylloide N. For measuring %Ndfa, Unkovich and Pati (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 (Galina 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 $^{15}$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 $^{15}$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 $^{15}$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 $^{15}$N method.

Present results showed that *A. cyanophylla* and its neighbouring non-N$_2$-fixing *O. oleaster* had negative shoot $^{15}$N values, whereas in contrast, Nast et al. (2005) reported that *Casuarina glauca* Sieber ex. Sprung, and its neighbouring non-N$_2$-fixing *Stipa tenacissima* L., in plantations in another experimental site located at the study area, had positive shoot $^{15}$N values. The $^{15}$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 $^{15}$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 $^{15}$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 $^{15}$N. Nevertheless, we suggested at least two possible explanations for such differences in $^{15}$N between the two plant pairs. Firstly, these differences may reflect horizontal variation in $^{15}$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 $^{15}$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 $^{15}$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 N₂ 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.

Acknowledgements

We thank Dr. Murray Unkovich (Soil and Land Systems, School of Earth and Environmental Sciences, University of Adelaide, Australia) for critical review of the manuscript. We also thank Dr. David Carty (NyPa-Greenbridge, Flemington, El Dorado, USA) for helpful comments on an earlier draft of the manuscript. Thanks to Hervé Casabianca (CNRS, Solaize, France) for assistance on mass spectrometric analysis and to the Regional Department of Agriculture in Sousse (Tunisia) for field support.

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