

# Application of $^{15}\text{N}$ natural abundance technique for evaluating biological nitrogen fixation in oil palm ecotypes at nursery stage in pot experiments and at mature plantation sites

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**Abstract** A range of different species of diazotrophic bacteria has been found in tissues and the rhizosphere of oil palm plants, suggesting a potential to benefit from biological nitrogen fixation (BNF). A few studies have confirmed that plantlets at nursery stage can benefit significantly from BNF after inoculation with *Azospirillum* spp. but no data are available regarding the benefit from naturally-occurring diazotrophic bacteria in oil palm. The results described here were derived from two pot trials laid out under controlled conditions with plantlets from two important regions for palm oil production in Brazil, as well as from different field sites of mature oil palm plantations. The  $^{15}\text{N}$  natural abundance technique was employed to estimate plant dependence on BNF (%Ndfa) by the different ecotypes grown in soil and previously characterized as hosting diazotrophic bacteria. From both pot trials it was possible to identify some ecotypes

of high potential for  $\text{N}_2$ -fixation that reached in some cases approximately 50%Ndfa. However, the accuracy of measurement still needs to be improved using more suitable reference plants for pot experiments. Values of  $\delta^{15}\text{N}$  signals from oil palm and reference plants in the field were inconclusive concerning any benefit from BNF to oil palm, owing to apparently high temporal and spatial variability of  $\delta^{15}\text{N}$  of the plant-available N in the heterogeneous soil matrix for the different palm and reference plant tested.

**Keywords** *Elaeis guineensis* · *E. oleifera* ·  $\text{N}_2$ -fixation · Delta  $^{15}\text{N}$  technique

## Introduction

There are approximately 3,000 species of palm tree around the world, of which around 1,600 occur in the American tropics. In relation to the oil palm, the *Elaeis*, *Oenocarpus*, *Attalea*, *Orbignya* and *Syagrus* families include more than half of the species already described and even though they are grown throughout the humid tropics, plantation areas are predominately of *Elaeis guineensis* Jacq., the African oil palm (Hartley 1977). In Brazil, oil palm, or Dendê as it is known locally, originated from Western Africa, Gulf of Guiné (Zeven 1964), although the species *E. oleifera* naturally occurs in the country. In Brazil large areas in the North and Northeast regions are cropped to oil palm making Brazil the third largest

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producer of palm oil in South America following Ecuador and Colombia.

The oil produced by palm trees has many uses such as in culinary, in the industry of inks, wax and soap making, and also in steel manufacturing. Furthermore, besides ethanol from sugarcane, oil palm is considered an important alternative for fossil fuel substitution due to its high oil productivity (3–4 ton/ha/year) and highly positive energy balance (Urquiaga et al 2005).

Intensively managed oil palm crops are frequently supplied with N fertiliser that can reach up more than half of the total production costs of this crop with a negative impact on the energy balance (total energy produced/fossil energy used to grow and process the crop) of the produced oil. Moreover, it is not well established how efficient N fertilizer is utilized by the crop (Balasubramanian et al 2004). Using a  $^{15}\text{N}$ -labelled urea Bah and Rahman (2004) have found that halving the normal N rate recommended to oil palm did not affect dry matter yield which suggests that our understanding of the N economy of oil palm is incomplete.

Biological nitrogen fixation (BNF) is a natural process of N acquisition by plants and the substitution of mostly fossil energy N-fertiliser by BNF inputs would not only reduce production costs but also reduce dependence on fossil energy sources. The African oil palm has been shown to associate with various nitrogen-fixing bacteria (Ferreira et al. 1995; Shamsuddin et al. 1995). These plants have been shown to be colonized by *Azospirillum brasilense*, *A. amazonense*, *Herbaspirillum seropedicae* and other as-yet-unidentified  $\text{N}_2$ -fixing bacteria (Dobereiner et al. 2000). These bacteria are present in roots, stems, leaves and in the endosperm of the fruit. Probably, a new *Herbaspirillum* species is present in roots, stems and leaves of these palm trees (Ferreira et al. 1995).

Plantlets of oil palm inoculated with *Azospirillum brasilense* and *A. lipoferum* had their shoot and root growth stimulated by 30 and 60%, respectively, compared to non-inoculated control plants, and the  $^{15}\text{N}$  analysis indicated that BNF could contribute with up to 40% of the total N accumulated by the plants (Amir et al 2001). More recently, Carvalho et al. (2006) found no differences in population of diazotrophic bacteria isolated from leaves and roots of oil palm plants grown in soil with or without N-fertiliser application. However, N fertilised plants developed much better than the non-fertilised ones, suggesting

that BNF can not provide all the N needs for optimal plant growth.

The results presented here aimed to verify the degree of reliance on BNF by the ecotypes evaluated by Carvalho et al (2006). In addition, we attempted to measure the BNF contributions to adult plants under field conditions using the  $^{15}\text{N}$  natural abundance technique.

## Materials and methods

Investigation of the contribution of BNF to oil palm was carried out in two pot experiments in Seropédica, RJ, with ecotypes from the North and Northeast regions of Brazil. The different ecotypes were provided by Embrapa Western Amazon and CEPLAC (Executive Commission for the Cocoa Crop). A further evaluation was performed with adult plants of an oil palm plantation in the State of Bahia.

### Ecotypes trials

Plastic pots of 1 L volume were filled with a mixture (1:1) of quartz sand and non-sterile subsoil of a Typic Hapludult extremely poor in available nitrogen. Liming and P and K fertilization were performed in order to correct nutritional deficiencies.

A first trial was set up using plantlets of the following ecotypes of *E. guineensis* Jacq.: “401008”, “315116”, “400709”, “401105”, “C-2001”, “C-2301” and “C-2312”, all of them from the Amazon State, in the Northern region of Brazil. In this trial the ecotype “Caiaué” of *E. oleifera* was also included. A second trial used the ecotypes “Deli”, “Tenera”, “Dura Comum”, “Campinhos”, “Rio Pardo”, “Dura Dupey”, “La-Mé”, “Yangambhi” and “C-2501”, from Bahia State, in the Northeast region of Brazil. Six replicates of each oil palm ecotype were used.

Both trials were laid out in a randomized block design in the greenhouse. Pots were watered on a daily basis and left to grow for 1 year. At harvesting, whole plants were sampled and evaluated for total dry mass accumulation after reaching a constant weight in a forced-air oven at 65°C. The dry material was ground to <0.85 mm in a Wiley mill. After milling, sub-samples were analysed for total N following the semi-micro Kjeldahl digestion followed by distillation and titration using an automated system (Urquiaga et al 1992). The BNF dependence (%Ndfa) of oil

palm ecotypes were estimated by the  $^{15}\text{N}$  natural abundance technique as described below.

#### Field sampling of adult plants

Oil palm plantations belonging to the Experimental Station “Lemos Maia” and the Experimental Station of Rio Pardo, municipality of Canavieiras, Bahia State, were sampled. These areas were planted to the ecotypes of *E. guineensis* “Tenera” (11 years old), “Dura comum” and “Nativo” (8 years old).

Index leaves (third leaf) of 12 plants of each ecotype and also from neighboring putative non- $\text{N}_2$ -fixing reference species, including ferns, shrubs and trees were sampled for the estimation of oil palm BNF dependence by the use of the  $^{15}\text{N}$  natural abundance technique. Among the reference plants, well known species were included such as cacao (*Theobroma cacao*), *Citrus* sp. and rubber tree (*Hevea brasiliensis* L.).

#### Plant BNF dependence

The technique is based on the assumption that atmospheric  $\text{N}_2$  shows a natural abundance of 0.3663 atom %  $^{15}\text{N}$  which does not vary perceptibly (Mariotti 1983), but in soil N may be as much as 0.005 atom % higher as this value. To deal with these small values, the units used are called  $\delta^{15}\text{N}$  (delta  $^{15}\text{N}$ ) units expressed in parts per thousand (‰) and determined by the equation:

$$\delta^{15}\text{N} (\text{‰}) = 1000 (\text{atom } \% \text{ } ^{15}\text{N} \text{ sample} - 0.3663)/0.3663$$

such that the  $^{15}\text{N}$  natural abundance of the air is 0.00 ‰.

The proportion of N (%Ndfa) derived from the atmosphere by the different varieties of oil palm was calculated by the equation:

$$\% \text{Ndfa} = 100 (\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{fixing plant}}) / (\delta^{15}\text{N}_{\text{ref}} - B)$$

where  $\delta^{15}\text{N}_{\text{fixing plant}}$  is the  $^{15}\text{N}$  abundance of the putative “ $\text{N}_2$ -fixing” oil palm variety, and  $\delta^{15}\text{N}_{\text{ref}}$  is the  $^{15}\text{N}$  abundance of a suitable non- $\text{N}_2$ -fixing reference plant;  $B$  should be the  $^{15}\text{N}$  abundance value of the same “ $\text{N}_2$ -fixing” oil palm variety when grown with solely atmospheric  $\text{N}_2$  as an N source. Since this has never been achieved the value of  $B$  was assumed to be 0.0 ‰ (Boddey et al 2001). In the case of the pot

trials the oil palm variety with the highest  $^{15}\text{N}$  abundance was utilised as reference plant.

Only seedlings of the ecotypes used in the second pot trial were analyzed for total N content and  $^{15}\text{N}$  natural abundance technique to allow the calculations of the  $^{15}\text{N}$  natural abundance of the N taken up during plant development (Witty 1983).

Isotopic analyses of  $^{15}\text{N}$  natural abundance were performed submitting sub-samples of already dry and milled plant material to a further milling process in order to attain a homogeneous powdered material by using a roller-mill similar to that of Smith and Myung (1990). Aliquots containing between 35 and 70  $\mu\text{g}$  of N were analysed for  $^{15}\text{N}$ -isotope abundance using a continuous flow isotope-ratio spectrometer consisting of an automatic C and N analyser coupled to a mass spectrometer (Finnigan Delta Plus).

#### Statistics

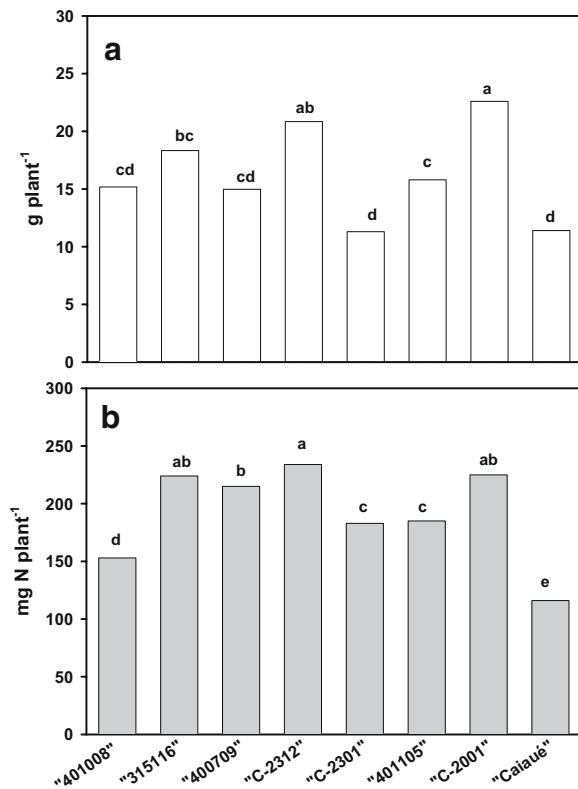
Data of the ecotype pot as well as the plantation field trials were subjected to ANOVA and means were separated by Tukey test at  $p < 0.05$ . Field data were presented as means of replicated collection sites.

## Results

#### Pot experiments

After 1 year of growth in pots the oil palm ecotypes coming from the North region presented no more than 23 g of dry matter in aerial tissues (Fig. 1a), with the ecotype “C-2001” showing the best growth and “Caiaue” the least. Nitrogen in plants at harvest varied from 116 to 234 mg N plant $^{-1}$ , and ecotypes ranked similarly for dry matter and N accumulation as there was only a 30% difference between ecotypes regarding the N content (0.74 to 0.99 mg N g dry matter $^{-1}$ ) (Figure 1a and b). The ecotype “C-2312” accumulated most N although this value was statistically the same as for “C-2001” and “315116”.

The ecotypes from the Northeast region tested in the second trial did not grow at the same rate as the plants of the first trial. The highest plant dry matter was only 9.9 g plant $^{-1}$  and the lowest 4.7 g plant $^{-1}$ , while variation of N in plant tissues ranged from 0.86 to 1.61 mg N g dry matter $^{-1}$ , which led to a high variation in N accumulation between plants, ranging



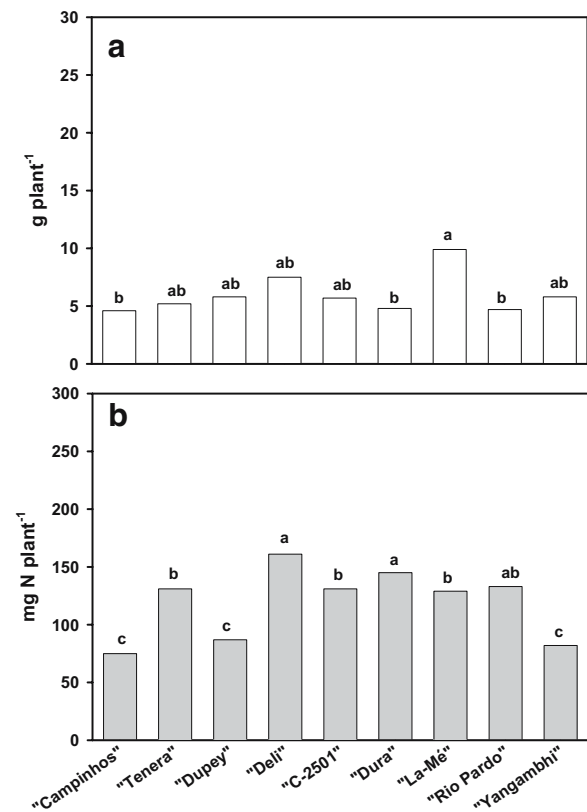
**Fig. 1** Dry matter (a) and total N accumulated (b) in aerial part of different ecotypes of oil palm from the North region of Brazil after 1 year's growth in pots. Means of six replicates. Means followed by the same letter are not significantly different (Tukey HSD test,  $p < 0.05$ )

from 75 to 161 mg plant<sup>-1</sup> (Fig. 2a,b). For this second trial, the ecotypes "Rio Pardo", "Dura" and "Campinhos" differed statistically in plant dry matter from "La-Mé", the latter presenting the highest value. The analysis of total N in plantlets at planting revealed a similarity among ecotypes with an average of  $41.3 \pm 2.4$  mg N plantlet<sup>-1</sup>. The plantlets were also reasonable uniform in <sup>15</sup>N abundance (Table 2) suggesting that the seeds came from a plant collection grown on the same soil. However, the total N in the original seedlings was equivalent to a variable portion of the N in plants at harvest, varying from 21 to 60%, depending on ecotype. Hence, the amount of N accumulated during the experimental period was of the order of 30–118 mg N plant<sup>-1</sup>. This verification could not be done with the ecotypes of the first trial, as there were not enough plantlets available. However, it should be reasonable to consider the amounts of N taken up by the plants in the second trial were 20–30%

lower than in the first, in view of the better growth of plants in the first experiment.

Irrespective of origin, there were significant differences in  $\delta^{15}\text{N}$  among the ecotypes in each trial, suggesting the BNF process benefited the plants at different rates (Tables 1 and 2). For the North region ecotypes, "Caiaue" presented the highest  $\delta^{15}\text{N}$  followed by "C-2001", the former a representative of *E. oleifera* and the latter *E. guineensis*. Taking "Caiaue" as a non-N<sub>2</sub>-fixing reference %Ndfa varied from 14 to 57%, the ecotypes "401008", "315116" and "400709" most benefited from BNF and "401105" and "C-2301" the least. Using "C-2001" as the reference plant %Ndfa decreased a little with estimated contributions varying from 13 to 49% (Table 1).

In the case of the trial (2) of Northeast-region ecotypes it was possible to estimate the % Ndfa based on  $\delta^{15}\text{N}$  values of the N acquired by the plants after discounting the contribution of the <sup>15</sup>N natural abun-



**Fig. 2** Dry matter (a) and total N accumulated (b) in aerial part of different ecotypes of oil palm from the Northeast region of Brazil after 1 year's growth in pots. Means of six replicates. Means followed by the same letter are not significantly different (Tukey HSD test,  $p < 0.05$ )

**Table 1** Natural  $^{15}\text{N}$  abundance of the different oil palm ecotypes and their BNF dependence (%Ndfa) using “Caiuaé” and “C-2001” as references (highest  $^{15}\text{N}$  values) after 363 days of growth

Ecotypes	$\delta^{15}\text{N}$	%Ndfa	
		“Caiuaé” <sup>a</sup>	“C-2001” <sup>b</sup>
401008	+2.81 f <sup>4</sup>	56.6	49.3
315116	+3.40 ef	47.4	38.6
400709	+3.46 ef	46.5	37.5
C-2312	+4.05 de	37.4	26.9
C-2301	+4.43 cd	31.5	20.0
401105	+4.83 c	25.3	12.8
C-2001	+5.54 b	14.4	–
Caiuaé	+6.47 a	–	–
CV (%)	7.26	–	–

<sup>a</sup> %Ndfa using “Caiuaé” as reference plant.

<sup>b</sup> %Ndfa using “C-2001” as reference plant.

<sup>c</sup> Means followed by the same letter are not significantly different (Tukey HSD test,  $p < 0.05$ ).

Mean of six replicates.

dance of the planted plantlets from the  $\delta^{15}\text{N}$  signals obtained at harvest. The very similar  $\delta^{15}\text{N}$  signals of plantlets of the ecotypes used did not change the ranking order based on  $\delta^{15}\text{N}$  found in harvested plants

**Table 2** Natural  $^{15}\text{N}$  abundance in plantlets at planting, at harvest and estimated for the N acquired during growth of the oil palm ecotypes and their BNF dependence (%Ndfa) using “Yangambhi” as the reference (highest  $^{15}\text{N}$  value) after 363 days of growth

Ecotypes	$\delta^{15}\text{N}$			% Ndfa
	Plantlets	Harvested plant N	N acquired during growth <sup>a</sup>	
Campinhos	+5.4	+4.0	+2.0 e <sup>b</sup>	76
Tenera	+4.8	+4.5	+4.4 d	47
Dupey	+5.5	+5.1	+4.7 d	43
Deli	+5.6	+5.1	+5.0 cd	40
C-2501	+5.1	+5.3	+5.3 c	36
Dura	+5.8	+5.5	+5.4 c	34
La-Mé	+5.1	+6.2	+6.4 b	22
Rio Pardo	+5.3	+6.6	+7.2 ab	13
Yangambhi	+5.7	+6.8	+8.3 a	–
CV (%)	–	–	6.8	–

<sup>a</sup> Estimated  $\delta^{15}\text{N}$  of the N acquired by the plant assuming 100% of plantlet N was part of the whole plant N at harvest.

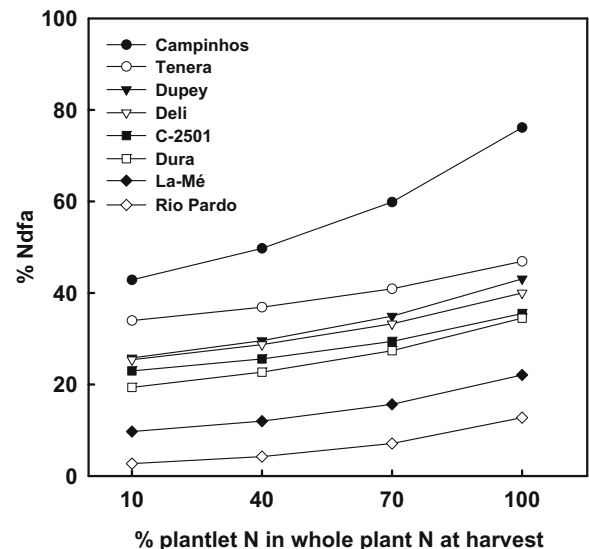
<sup>b</sup> Means followed by the same letter are not significantly different (Tukey test,  $p < 0.05$ ).

Means of six replicates.

but did alter the magnitude of the dilution (Table 2). The BNF contribution to the ecotypes ranged from 13 to 76% (Table 2) taking “Yangambhi” as reference plant, which presented, in absolute terms, the highest  $\delta^{15}\text{N}$  among the ecotypes. The ecotype “Campinhos” most benefited from the process followed by “Tenera”. These %Ndfa estimates were optimistic as it was assumed 100% of the N in plantlets remained in harvested plants. Reducing the participation of plantlet N in harvested plants decreased %Ndfa estimates especially in the case of ecotypes presenting the lowest  $\delta^{15}\text{N}$  values (Fig. 3).

Field experiment

Results of  $\delta^{15}\text{N}$  in index leaves of adult ecotypes of oil palm and in several supposedly non- $\text{N}_2$ -fixing species were inconclusive (Table 3). In the “Dura” plantation,  $\delta^{15}\text{N}$  in oil palm plants were +5.84‰ whilst reference species ranging from +2.97 to +7.16‰. Only the samples of flame vine [*Pyrostegia venusta* (Ker.) Miers], a creeping plant, had a  $\delta^{15}\text{N}$  value above that for oil palm. This scenario was the same for the area planted to “Tenera” that presented +4.60 ‰ against a range of +1.83 to +3.13‰ found in three reference



**Fig. 3** Changes in oil palm ecotypes BNF dependence (% Ndfa) as a function of the assumed proportion of the plantlet N as part of the harvested plant N. Estimates of %Ndfa are derived from the resulting  $\delta^{15}\text{N}$  of the N effectively acquired by the plants during the growth experimental period calculated according to Witty (1983)

**Table 3** Data of  $^{15}\text{N}$  natural abundance for three adult oil palm ecotypes and various reference species collected from the area of each ecotype plantation

Oil palm ecotype	Reference plants	$\delta^{15}\text{N}$
Dura		<b>+5.8</b>
	“Fidalgo” ( <i>Aegiphila sellowiana</i> Cham.)	+3.0
	“Pindoba” ( <i>Attalea compta</i> Mart.)	+4.3
	Flame vine [ <i>Pyrostegia venusta</i> (Ker.) Miers]	+7.2
	Rubber tree [ <i>Hevea brasiliensis</i> (Willd. ex A. Juss.) Müll. Arg.]	+4.7
Tenera		<b>+4.6</b>
	Fern [ <i>Thelypteris dentata</i> (Forsk.) E. St. John]	+2.5
	Citrus ( <i>Citrus</i> sp.)	+3.1
	“Arnica” ( <i>Lychnophora ericoides</i> Mart.)	+1.8
Nativo		<b>+7.2</b>
	Cacao ( <i>Theobroma cacao</i> L.)	+8.5
	True myrtle ( <i>Myrtus communis</i> L.)	+11.4
	“Capim-de-cheiro” ( <i>Cyperus ferax</i> L.C. Rich.)	+8.6
	“Abiu” ( <i>Pouteria caimito</i> Radlk.)	+7.0
	Star fruit ( <i>Averrhoa carambola</i> L.)	+9.0

plants. Only in the area of “Nativo” a potential contribution of BNF could be assigned to oil palm. Samples taken from adults of “Nativo” presented +7.15‰ and four of five reference plants presented a higher  $\delta^{15}\text{N}$  value among them woody, shrubby and grass species. In this case, only the “Abiu” tree (*Pouteria caimito* Radlk.) samples averaged a  $\delta^{15}\text{N}$  value similar to oil palm but slightly lower.

## Discussion

The development of oil palm plants is dependent on several factors and the nutrition at the nursery stage is of paramount importance for the field establishment of a healthy and productive plantation (Wahid et al. 2005). Nitrogen is one of the most expensive nutrients and its rational use is not only worthwhile for economic reasons, but is aligned to the strategy of reducing fossil fuel use in agriculture especially when the crop product is suitable for biofuel programs (Döbereiner et al. 2000).

Biological nitrogen fixation is the most viable source of N to replace N fertilisers based on fossil fuel energy. In the case of most leguminous crops the

importance of BNF inputs is firmly established, but there is also some evidence that some non-leguminous crops such as sugarcane, rice, wheat and maize may also benefit from  $\text{N}_2$  fixation (Boddey et al. 1998; Garcia Salomone et al. 1996; Okon and Labandera-Gonzalez 1994). The occurrence of diazotrophic bacteria in oil palm raised the possibility that this crop could also benefit from BNF (Döbereiner et al. 2000). Furthermore, the inoculation of *Azospirillum* spp. in oil palm plantlets resulted in enhanced growth and a BNF contribution to the plants up to 40% of the accumulated N (Amir et al. 2001). Our results also lead to the same conclusion. At the nursery stage the differences in  $\delta^{15}\text{N}$  among the ecotypes suggest that some of them could be benefiting from BNF with N inputs from air corresponding to something close to 50% of the accumulated N, which was observed in both trials.

Notwithstanding, it must be considered the influence of the plantlet  $^{15}\text{N}$  before planting on the estimates of %Ndfa. When the  $\delta^{15}\text{N}$  technique is used it is important to discount the contribution of  $^{15}\text{N}$  natural abundance of the reproductive organ (seeds, stems, plantlets etc) to the final  $\delta^{15}\text{N}$  of the harvested plant in order to get reliable %Ndfa estimates (Witty 1983). In the case of the second trial, when this analysis was performed the N already present in plantlets represented a large share of the harvested N and the  $\delta^{15}\text{N}$  of plantlets. This had a strong influence on the final  $\delta^{15}\text{N}$  of the ecotypes at harvest, which brought about a great uncertainty in the %Ndfa estimates (Fig. 3). There is no information available regarding how much N from plantlets are actually used for plant development but it seems reasonable to assume that the most is utilised as only residues of the endocarp are left behind. Even though the %Ndfa estimates carry a degree of uncertainty the results presented hereby add more evidences to that from the existing literature (Amir et al. 2000, 2001) that the diazotrophic bacterial populations associated to oil palm at nursery stage are able to obtain significant contributions from BNF.

Irrespective of region different degrees of BNF dependence by the ecotypes were found, and “Caiuae”, the only representative of *E. oleifera*, presented the highest  $\delta^{15}\text{N}$  value or the least dependence on BNF. It would be expected N accumulation by the plants was accompanied by the degree of BNF input, but there were some exceptions. For example the ecotype “C-2001” was among the ecotypes of largest N accumu-

lation while stood out as a potential non-N<sub>2</sub>-fixing reference plant. As there was no appropriate reference plant used in either trial, it would be premature to describe any of the oil palm ecotypes as unable to obtain N from BNF. Moreover, diazotrophic bacteria were verified in leaves and roots of all of the ecotypes (Carvalho et al. 2006), which is strongly indicative that any of the ecotypes could be candidates as “N<sub>2</sub>-fixers”.

Ecotypes of *E. oleifera* and *E. guineensis* are utilised to produce interspecific hybrids targeting short stature, oil quality (mostly unsaturated oil) and disease resistance, including lethal yellowing, the major problem of oil palm cultivation in America (Bergamin Filho et al. 1998) as well *Fusarium* wilt, the major oil palm disease in Africa (Hardon et al. 1985). In this case there are strong chances for gathering together different traits besides the BNF potential. Hybrids selected by breeding programs in Brazil, mostly at the North region evaluated in the first trial of this work presented variable degrees on BNF, which could be better explored regarding the influence of the crossings performed.

Although very promising results were observed from the pot trials with young plants, field evaluations of adult plants has resulted no clues regarded to the role of BNF to oil palm. The variation in  $\delta^{15}\text{N}$  signals among oil palm ecotypes was not an indication of BNF potential as the  $\delta^{15}\text{N}$  values of each area were not comparable, partially because of the distance and also different management histories. Several of the reference plants presented natural  $^{15}\text{N}$  abundance lower than that observed in the oil palm tissues (Table 3), suggesting the reference plants and ecotypes of oil palm explored very differently specific pools of soil N. From the review of Boddey et al. (2000) the variation in  $\delta^{15}\text{N}$  signatures of soil N pools can negate any possibility of using the isotopic technique if these different N pools are relatively important in size and differently available to the N<sub>2</sub>-fixing and reference species. These authors also mentioned that in the case of perennials, litter recycling through senesced aerial tissues and roots can change the  $\delta^{15}\text{N}$  labelling of the portion of the soil surrounding the plants, which is another drawback of using natural  $^{15}\text{N}$  abundance technique for BNF studies of a perennial plantation. In this case, it would be recommended for similar studies with adult oil palm the adoption of the strategy followed by Urquiaga et al (1992) who grew sugarcane in a large

concrete tank filled with a soil previously labelled with  $^{15}\text{N}$  fertiliser, which allowed good uniformity of  $^{15}\text{N}$  enrichment in depth and time.

Gehring and Vlek (2004) also failed to quantify the BNF contribution to legume trees in both native and secondary forest in Amazonia owing to the high variability in  $\delta^{15}\text{N}$  signals among available non-N<sub>2</sub>-fixing references. The best estimates were achieved after grouping the potential reference plants into species types when they found non-N<sub>2</sub>-fixing legumes would be most suitable. Similarity in growth and the exploitation of different N-pools seems to be essential when investigations are carried out in more complex environments such as those formed by perennial species. In our study the strategy was to sample available plants of different families with very different growth patterns. This would allow checking the uniformity of the  $\delta^{15}\text{N}$  signal of the most important available soil N pools in space and time. The reference plants of the plantations of the oil palm “Dura” and “Tenera” presented  $\delta^{15}\text{N}$  signals well below the observed in the index leaves of the oil palms, with the sole exception of the creeping species, flame vine. On the other hand, the reference plants in the “Nativo” plantation area indicated a less variable soil  $\delta^{15}\text{N}$ . The area of “Dura” and “Tenera” presented averaged  $\delta^{15}\text{N}$  soil available N signals with 18 and 15% variation, based on the values presented by each reference plant. In the case of “Nativo” the variation was halved.

The lower variation in  $\delta^{15}\text{N}$  signal in “Nativo” plantation is an indication of greater spatial and temporal evenness of this signal in different N pools. In addition the most (four out of five) reference plants at the “Nativo” area presented values of  $\delta^{15}\text{N}$  higher than that of this oil palm ecotype so that according to the assumptions of the  $^{15}\text{N}$  natural abundance technique it could be estimated the oil palm was obtaining approximately 19% of its N from BNF. Clearly, this estimate is just a first attempt to extrapolate to adult plants the observed benefit of diazotrophic bacteria at the nursery stage since those bacteria are still present in adult plants (Carvalho 1997).

## Conclusions

The use of the  $^{15}\text{N}$  natural abundance technique allowed confirmation that some ecotypes of oil palm at nursery stage are able to obtain significant con-

tributions from plant-associated  $N_2$  fixation. However, the lack of a suitable reference plant of the soil-derived  $\delta^{15}N$ -signal makes it difficult to make accurate estimates of the magnitude of these contributions.

When attempts were made to quantify BNF contributions to mature oil palms trees using the  $^{15}N$  natural abundance technique, the reference species sampled to estimate the  $^{15}N$  abundance of soil-derived N showed large differences between species, some showing significantly higher, and some lower,  $^{15}N$  abundance than the oil palm varieties. For this reason the data provide no evidence for or against the hypothesis that the adult oil palm varieties in the plantations were able to significantly benefit from associated BNF.

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