

## Field effect of P fertilization on N<sub>2</sub> fixation rate of *Ulex europaeus*

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**Abstract** – European gorse (*Ulex europaeus* L.) N<sub>2</sub> fixation rate (%Ndfa) was studied in a maritime pine (*Pinus pinaster* Ait.) oligotrophic forest. Fertilization field trials were carried out on 5 sites with various inputs of phosphorus (0–240 kg P<sub>2</sub>O<sub>5</sub>.ha<sup>-1</sup>). Seven to ten years after pine planting, gorse were sampled to evaluate the effect of P fertilization on gorse %Ndfa, determined using the <sup>15</sup>N natural abundance method. One of the prerequisites of this method is the existence of a significant difference between the <sup>15</sup>N/<sup>14</sup>N ratios in the atmospheric N reference and in the stand soil N references. This prerequisite was satisfied for 80 of 120 cases. The average %Ndfa was high (70 ± 3%) but with high local variability. No significant difference in %Ndfa was detected among P treatments. Nitrogen concentration of gorse was significantly higher in the highest dose treatments compared to the control.

*Ulex europaeus* / symbiotic N<sub>2</sub> fixation / <sup>15</sup>N natural abundance / P fertilization / *Pinus pinaster*

**Résumé** – Effet in situ de la fertilisation en phosphore sur le taux de fixation de l'azote atmosphérique d'*Ulex europaeus*. Le taux de fixation de l'azote atmosphérique (%Ndfa) de l'ajonc d'Europe (*Ulex europaeus* L.) a été étudié dans une forêt oligotrophe de pins maritimes. Des essais de fertilisation ont été établis avec plusieurs niveaux d'apport en phosphore (0–240 kg P<sub>2</sub>O<sub>5</sub>.ha<sup>-1</sup>). Sept à dix ans après la plantation de pins, les ajoncs ont été échantillonnés afin d'évaluer l'effet de la fertilisation en phosphore sur le %Ndfa, calculé par la méthode de l'abondance naturelle en <sup>15</sup>N. Cette méthode nécessite notamment une différence significative entre les rapports <sup>15</sup>N/<sup>14</sup>N de la référence atmosphérique et de la référence du sol des peuplements. Cette condition était satisfaite dans 80 cas sur 120. Le %Ndfa moyen était élevé (70 ± 3 %) mais avec une grande variabilité locale. Aucune différence des %Ndfa n'a été détectée entre les traitements. Les teneurs en azote des ajoncs étaient significativement plus élevées pour les doses maximales que pour les témoins.

*Ulex europaeus* / fixation symbiotique de l'azote / abondance naturelle en <sup>15</sup>N / fertilisation en phosphore / *Pinus pinaster*

### 1. INTRODUCTION

Intensively managed forests may suffer in the medium or long-term from nitrogen deficiency [11]. This is particularly true for oligotrophic forests when nitrogen lost by biomass outputs is not offset by N fertilization [16]. This issue has been growing in importance since silvicultural practices have become more and more intensive, notably with rotation lengths getting shorter.

High inputs of nitrogen can be brought naturally into the ecosystem by the presence of N<sub>2</sub>-fixing shrubs [25]. P fertilization, used in maritime pine forests due to its positive effect on pine growth [7,24], may increase these natural inputs in two different ways: (i) by increasing the abundance and biomass of N<sub>2</sub>-fixing shrubs [3]; and (ii) by increasing the N<sub>2</sub> fixation rate [1]. This second point has been mostly developed in laboratory studies that suggest a P effect on N<sub>2</sub> fixation rate. However, these studies conflict with each others, as such an effect is not always detected. Besides, these results appear signifi-

cant mostly when P concentration is either very low or rather high and thus may not be easily transposable to field conditions (e.g. [1, 12, 17, 19]). They nevertheless show that N<sub>2</sub> fixation is not unresponsive to phosphorus availability.

A previous study tested the field P effect on the fixation rate of leguminous shrubs in a large forest of southwestern France [3]. However, the requested conditions for the used method (<sup>15</sup>N natural abundance method) to be properly applied were not met in the fertilized site. It was thus impossible to address the question of the field P effect on fixation rate, even though other P effects on fixing shrubs were quantified. The natural abundance method also revealed to be usable on another sites of the same area.

The objective of this study is to readdress the field P effect on N<sub>2</sub> fixation rate in the same area and on the same specie, but with a strengthened sampling scheme. It tried to use the <sup>15</sup>N natural abundance method on other fertilization trials than Augusto et al. [3]. It also used the other blocks of the previously studied trial as conditions allowing or forbidding the method are very heterogeneous even on short distances.

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**Table I.** Characteristics of each site. Pines C130: Circumference at 130 cm height. Significant differences are as given by a *t*-test with a 5% error threshold, and confirm the P effect on pine growth [7, 24]. 3 blocks have been sampled at Blagon and 1 for each of the other sites.

Site	Pine density (stems.ha <sup>-1</sup> )	Pines age at sampling (year)	P fertilization dose (kg P <sub>2</sub> O <sub>5</sub> .ha <sup>-1</sup> )	Pines C130 (cm)
Blagon	1530	7	0	24.9 a
			80	29.0 b
			160	30.7 c
			240	30.7 c
Lue	1100	8	0	22.4 a
			40	31.2 b
			80	28.5 b
			120	28.0 b
Caudos	1250	7	0	23.2 a
			40	29.2 b
			80	34.9 c
			120	37.6 c
Clochettes	1666	8	0	34.2 a
			80	38.6 b
Grand Ludee	1250	10	0	31.5 a
			120	31.2 a

## 2. MATERIALS AND METHODS

### 2.1. Experimental sites (Tab. I)

The experiment took place in the “Landes” forest of southwestern France (see [3] and [22] for further details). The N<sub>2</sub>-fixing species studied was European gorse (*Ulex europaeus* L.), a leguminous perennial evergreen spiny shrub found in 60% of the stands of the forest (French Forest Survey). More details on gorse are given by Richardson & Hill [20] and Clements et al. [8].

Five sites were selected: Lue, Caudos, Clochettes, Grand Ludee, and Blagon, the last being the one used in the previous experiment [3]. All the sites were maritime pines (*Pinus pinaster* Ait.) stands established during triple superphosphate fertilization experiments set up between 1994 and 1997. Two to 4 doses of phosphorus (hereafter named Px with *x* = dose of P as kg P<sub>2</sub>O<sub>5</sub>.ha<sup>-1</sup>, P0 being the control) were investigated in each trial (Tab. I). Maximal dose ranged from 80 to 240 kg P<sub>2</sub>O<sub>5</sub>.ha<sup>-1</sup>.

### 2.2. Theory of the <sup>15</sup>N natural abundance method

This method allows estimating the percentage of nitrogen derived from the atmosphere (%Ndfa) in a N<sub>2</sub>-fixing plant. It is based on the comparison of the <sup>15</sup>N abundance of a N<sub>2</sub>-fixing plant to those of a non fixing plant [15]. The <sup>15</sup>N isotopic enrichment (δ<sup>15</sup>N) is calculated as below, defined according to the atmosphere which is considered as the standard:

$$\delta^{15}\text{N} = \frac{[\text{N}^{15}]/[\text{N}^{14}]_{(\text{plant})} - [\text{N}^{15}]/[\text{N}^{14}]_{(\text{atm})}}{[\text{N}^{15}]/[\text{N}^{14}]_{(\text{atm})}} \times 1000.$$

Three δ<sup>15</sup>N are used to estimate the %Ndfa: that of the leguminous plant studied (N<sub>2</sub>-fixing species, δ<sup>15</sup>N<sub>leg</sub>), that of a reference plant (non N<sub>2</sub>-fixing species, δ<sup>15</sup>N<sub>ref</sub>), and that of a leguminous plant with a %Ndfa equal to 100% (same N<sub>2</sub>-fixing species, δ<sup>15</sup>N<sub>fix</sub>):

$$\% \text{Ndfa} = \frac{(\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{leg}})}{(\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{fix}})} \times 100.$$

It should be noted that the δ<sup>15</sup>N of the bulk soil greatly differs from the pool of nitrogen available to plant nutrition [15, 26]. Thus, using δ<sup>15</sup>N<sub>soil</sub> rather than δ<sup>15</sup>N<sub>ref</sub> would have lead to errors in %Ndfa estimations.

The <sup>15</sup>N natural abundance method needs to satisfy several conditions in order to be applicable: (i) a significant difference between δ<sup>15</sup>N<sub>ref</sub> and δ<sup>15</sup>N<sub>fix</sub> must exist (ii) the reference species absorbs the mineral nitrogen in the same soil volume and during the same periods as the N<sub>2</sub>-fixing species. These conditions have been previously tested in the ‘Landes’ forest [3]. It appeared that (i) the significant difference between δ<sup>15</sup>N<sub>ref</sub> and δ<sup>15</sup>N<sub>fix</sub> exists in some sites but not in the northern blocks of Blagon, which forbade the authors to answer the question of the P effect (ii) usable reference species are *Erica scoparia* and *Calluna vulgaris*, the first being the best as its morphology is closer to that of *Ulex europaeus* and (iii) some variability occurred in δ<sup>15</sup>N<sub>ref</sub> at a local scale, so that there could be a significant difference between δ<sup>15</sup>N<sub>ref</sub> and δ<sup>15</sup>N<sub>fix</sub> in other (southern) blocks of Blagon, and/or in other sites.

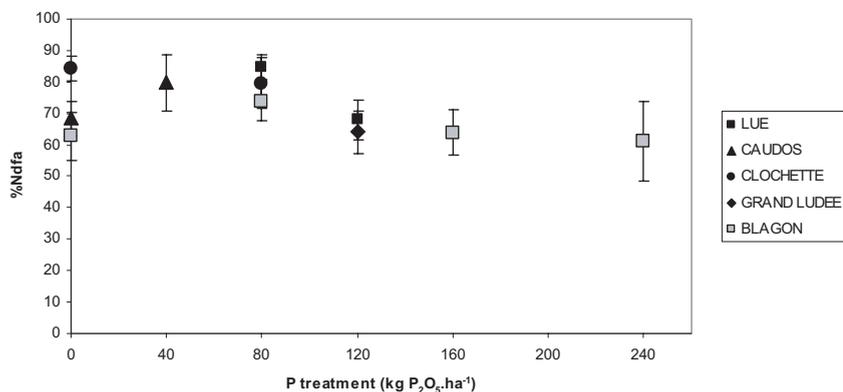
### 2.3. δ<sup>15</sup>N<sub>fix</sub> determination

δ<sup>15</sup>N<sub>fix</sub> determination occurred in the same manner than in Augusto et al. [3], but with one more sampling year (2006), resulting in a slightly different mean δ<sup>15</sup>N<sub>fix</sub> value (-0.55‰ with *n* = 14 versus -0.50‰ in [3]).

### 2.4. Sampling and analyses

#### 2.4.1. N content and fixation rate determination

Lue, Caudos, Clochettes and Grand Ludee trials were sampled in February and March 2005. Blagon was sampled in July 2005. In Blagon, 4 treatments (0, 80, 160 and 240 kg P<sub>2</sub>O<sub>5</sub>.ha<sup>-1</sup>) were sampled in the 3 southern blocks (different from those previously sampled by [3]). For each of the 4 other sites, only one block was used per site, with one sampling area in each treatment. The sampling areas were located near the center of the treated plots to avoid edge effects.



**Figure 1.** Average N<sub>2</sub> fixation rate (%Ndfa) of *Ulex europaeus* according to sites and P fertilization.

In each sampling area, green twigs from 5 pairs *Ulex europaeus*/reference plant (*Erica scoparia* or *Calluna vulgaris*) were collected. Pairs were selected so that the two plants and their sizes were as close as possible. The distance between the two plants, their respective heights as well as the species of the reference (*Erica scoparia* or *Calluna vulgaris*) were systematically recorded in Blagon. The green twigs were then dried at 65 °C for 48 h, coarsely ground (Willey-ED5 grinder) then finely ground in a ball mill (Retsch PM4 planetary grinder) before N content and δ<sup>15</sup>N determination by spectrometry ('sector field' ICP-MS). In the previous study of Blagon, repeats were bulked together before δ<sup>15</sup>N determination leading to a unique pair of δ<sup>15</sup>N values (δ<sup>15</sup>N<sub>ref</sub> and δ<sup>15</sup>N<sub>leg</sub>) per sampling area. Here, all individual samples were analyzed independently.

#### 2.4.2. Growth determination

Except in Blagon, all European gorse stems in the sampling plots were cut and then brought to the laboratory. Stems were sorted along diameter at 10 cm, and then 10 of them were selected according to a systematic sub-sampling based on the frequency distribution of stem diameters. The 5 remaining biggest stems were then added to the sub-sample. The selected stems were cut at 10 cm shortly after sampling, and the growth rings immediately numerized for measurement with the ImageTool software (UTHSCSA).

#### 2.5. Mathematical and statistical data analysis

According to Watt et al. [25], it is acceptable to calculate %Ndfa when the difference between δ<sup>15</sup>N<sub>fix</sub> and δ<sup>15</sup>N<sub>ref</sub> is 1‰ or higher, provided the soil has been homogenized by ploughing before stand installation, which is the case on all of our sites. We therefore discarded the samples who did not exhibit such a difference. We did the same for negative values of %Ndfa, while %Ndfa values slightly higher than 100 were assumed to be equal to 100.

Statistical analyses were performed either with the STATISTICA software v6.0 (StatSoft Inc., 1984–2001) or with the SAS/STAT software (SAS Institute Inc. 1999). Kruskal-Wallis ANOVA were used to assess differences between treatments, as well as Mann-Whitney U tests whenever ANOVA showed significant differences. Growth rings differences between treatments were tested per year with Bonferroni *t* tests. All significant differences were determined for a 5% error.

### 3. RESULTS AND DISCUSSION

#### 3.1. Effect of P fertilization on gorse growth and nitrogen concentration

Individual growth of gorse was significantly higher only for the higher doses treatments (P80 and P120) in Caudos. A similar effect had been previously shown in Blagon for the P160 and P240 treatments [3]. It thus seems like gorse growth is positively affected only for very high P doses (P120 being the maximum currently used by local foresters).

The N concentration of gorse increased gradually with P doses (mean [N] across all sites: P0 = 11.5 ± 0.2; P40 = 11.9 ± 0.4; P80 = 12.4 ± 0.3; P120 = 12.6 ± 0.4; P160 = 13.9 ± 0.5; P240 = 14.0 ± 0.6). This result was observed in all sites but it was significant only for the higher doses in Lue (P80 and P120) and Blagon (P160 and P240). Again, an individual response of gorse seems to be more likely to occur for high or very high P doses.

#### 3.2. *Ulex europaeus* fixation rate (Fig. 1; Appendix I)

Augusto et al. [3] showed that most of the conditions required for use of the natural abundance method according to Högberg [15] and Boddey et al. [5] were satisfied in our context, except for the difference between δ<sup>15</sup>N<sub>fix</sub> and δ<sup>15</sup>N<sub>ref</sub> in some cases. The same problem occurred here in a less dramatic manner, as the absolute difference between δ<sup>15</sup>N<sub>fix</sub> and δ<sup>15</sup>N<sub>ref</sub> was low as well as being highly variable. However, following the 1‰ minimum difference preconized by Watt et al. [25] we still retained a sufficient number of %Ndfa values (80 out of 120).

From the 60 %Ndfa values calculated in Blagon, 18 were discarded (P0 = 0; P80 = 9; P160 = 1; P240 = 8). The absolute differences between δ<sup>15</sup>N<sub>fix</sub> and δ<sup>15</sup>N<sub>ref</sub> were on average 1.94 ± 0.19‰ for Blagon. In the control treatment, where no value was discarded, there was no significant difference among blocks. Consequently, values of the three blocks were merged per treatment. No significant difference was then detected between the treatments. Including the discarded values in the data analysis did not change this result. Across all treatments, the average value of nitrogen fixation rate was 63% with a standard error of 4%.

Similarly, 22 %Nd<sub>fa</sub> values were discarded from the 60 calculated values in the four other sites. The absolute difference between  $\delta^{15}\text{N}_{\text{fix}}$  and  $\delta^{15}\text{N}_{\text{ref}}$  was on average  $1.49 \pm 0.99\%$ . We calculated the mean %Nd<sub>fa</sub> value of a sampling plot only if at least 3 from the 5 %Nd<sub>fa</sub> values of this plot were satisfying the 1‰ difference criteria. Thus we could not calculate the mean for the following plots: P0 and P40 of Lue, P80 and P120 of Caudos and the P0 of Grand Ludee.

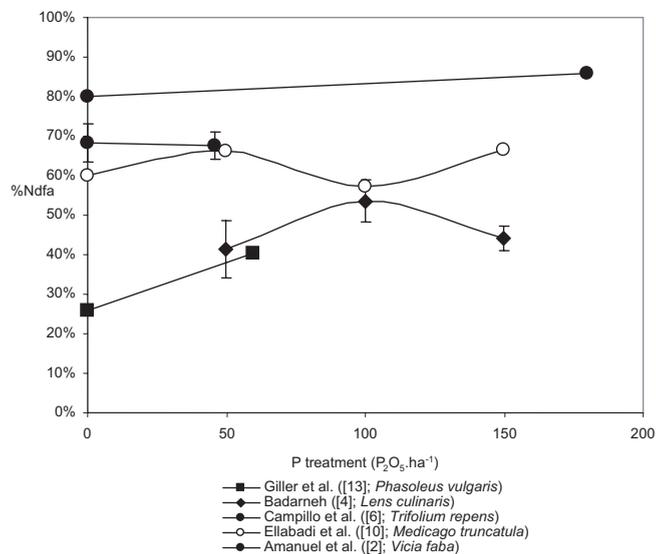
It was assumed that gorse was growing in similar conditions in the five sites and therefore the fixation rates per treatment were globally compared (Fig. 1). Across all sites and treatments, the average nitrogen fixation rate was 70% with a standard error of 3% (standard deviation = 28%). No significant difference was detected among the treatments of the five sites.

### 3.3. Relevance of the $^{15}\text{N}$ natural abundance method in our context

Some authors such as Högberg [15] preconized a minimum difference of 5‰ between  $\delta^{15}\text{N}_{\text{fix}}$  and  $\delta^{15}\text{N}_{\text{ref}}$ . Our values concerning the fixation rate could therefore be considered as low confidence level results. Despite this limitation, the absence of any effect of in situ P fertilization seems quite robust, as it emerged from 80 individuals and is stable across all sites and treatments. Because of the variability of the rejected values, some treatment means were more reliable than others. In Blagon, almost all the values for the P0 and P160 treatments were retained and their values show reasonable standard errors as well as remarkably close means. Moreover, there was no significant difference between %Nd<sub>fa</sub> values calculated with a difference of 3‰ or more between  $\delta^{15}\text{N}_{\text{fix}}$  and  $\delta^{15}\text{N}_{\text{ref}}$  (%Nd<sub>fa</sub> =  $79 \pm 6\%$ ;  $n = 14$ ) compared to those calculated with less than 3‰ of difference (%Nd<sub>fa</sub> =  $71 \pm 5\%$ ;  $n = 66$ ). Finally, Danso et al. [9] showed that the reliability of the fixation rate calculation increases with increasing rate, and our %Nd<sub>fa</sub> values were rather high. Therefore, we assumed that the  $^{15}\text{N}$  natural abundance method gave here results with an acceptable level of confidence.

### 3.4. Nitrogen fixation rate in response to P doses

No response of the N fixation rate to increasing doses of P fertilizer was detected, whatever the site or treatment considered. While this is in contradiction with some laboratory results [1, 12, 17, 19] which mostly showed some effect of phosphorus on nitrogen fixation characteristics (i.e. number and growth of nodules, nodule activity measured by acetylene reduction assays, and fixation rate measured by  $^{15}\text{N}$  isotopic dilution), it is not very surprising. As previously stated, these laboratory results generally showed an effect of phosphorus when it was added in high concentrations or when it ended a severe deprivation of this nutrient. These kind of severe conditions were unlikely to happen in situ, as ecosystems are generally naturally buffered by a number of factors (e.g. soil characteristics, leeching, competition...). Even if the Landes soils are quite poor, notably in phosphorus [22], gorse is considered



**Figure 2.** Nitrogen fixation rate as reported by crop studies. Closed symbol: field experiment; open symbol: pot experiment. [4] and [2]: means of 2 and 3 sites, respectively.

to be an oligotrophic species well adapted to these conditions [8, 20].

The N content of gorse is sometimes nevertheless higher for high doses, and this could be interpreted as a physiological response of gorse to high P doses which may be thought not entirely compatible with the absence of effect on fixation rate. We suggest two hypotheses to explain this apparent contradiction (i) The individual growth increase for high doses is responsible for a larger soil exploration as root growth is stimulated as well as aboveground one (root/shoot ratio not being significantly affected by fertilization: control =  $0.50 \pm 0.13$ ; fertilized =  $0.57 \pm 0.07$ ; Cavard and Augusto, unpublished data), increasing both soil N uptake and N fixation flux without modifying the balance between them (ii) Shadowing due to bigger tree canopies in the fertilization treatments [23] overbalance the potential effect on N fixation rate, as Rastetter et al. [18] predicted a decrease in N fixation rate with decreasing light availability.

Whatever the reasons may be, it nevertheless seems that for these conditions and for the P doses likely to be used in the field, gorse N fixation rate do not respond to P fertilization. Even though our results may be considered as frail because of the small differences between  $\delta^{15}\text{N}_{\text{fix}}$  and  $\delta^{15}\text{N}_{\text{ref}}$ , previously published results of in situ P fertilization trials of annual crops showed very similar trends (Fig. 2; see also e.g. [14] or [21]), which strengthen the likeliness of such a conclusion. Of course, P fertilization could nevertheless increase total N<sub>2</sub> fixation by increasing gorse biomass, but our results concerning P effect on gorse individual growth are not very conclusive under 120 kg P<sub>2</sub>O<sub>5</sub>.ha<sup>-1</sup>.

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**Appendix I.** Retained  $\delta^{15}\text{N}$  values, with a minimum absolute difference of 1‰ between  $\delta^{15}\text{N}_{\text{ref}}$  and  $\delta^{15}\text{N}_{\text{fix}}$  ( $-0.55\text{‰}$ ).

Site	P fertilization dose (kg $\text{P}_2\text{O}_5\cdot\text{ha}^{-1}$ )	$\delta^{15}\text{N}_{\text{leg}}$ (‰)	$\delta^{15}\text{N}_{\text{ref}}$ (‰)	$\delta^{15}\text{N}_{\text{ref}}-\delta^{15}\text{N}_{\text{fix}}$ absolute difference (‰)	%Ndfa
Blagon	0	-1.4	-1.8	1.29	33
		-2.5	-2.7	2.14	10
		-2.5	-4.8	4.25	54
		-0.2	-3.7	3.15	100
		0.0	-3.7	3.11	100
		0.6	-3.3	2.95	100
		-1.0	-3.3	2.22	83
		-1.9	-4.2	2.84	63
		-1.3	-1.5	1.36	20
		-0.8	-4.9	1.71	93
		-1.6	-2.3	2.16	39
		-1.7	-3.1	4.27	56
		-1.3	-4.7	1.97	82
		-2.4	-4.6	2.98	55
		-2.5	-4.6	2.78	53
		80	-1.0	-3.5	2.72
	-1.1		-2.8	3.62	76
	-0.9		-1.3	4.33	49
	-0.8		-2.0	2.04	80
	-1.6		-2.7	1.44	51
	-1.3		-2.7	1.09	64
	160	-0.4	-1.9	2.51	65
		-1.1	-2.3	3.32	70
		-2.2	-2.7	1.72	25
		-1.2	-4.8	2.54	86
		-1.6	-2.5	4.20	46
		-0.5	-2.6	4.07	100
		-1.2	-2.0	4.06	57
		-1.4	-1.6	1.43	26
		-1.1	-3.1	2.16	76
		-1.3	-3.6	2.18	75
		-1.3	-2.3	3.04	58
		-1.3	-3.2	1.75	71
		-0.7	-2.6	2.61	91
		-1.7	-1.9	2.09	17
	240	-0.7	-3.5	1.37	93
-0.7		-3.9	1.42	94	
-1.6		-2.0	2.28	29	
-0.9		-2.8	1.51	82	
-1.7		-2.1	1.43	25	
-0.8		-2.0	1.09	82	
-1.4		-1.6	1.29	23	
0	0.4	-1.8	1.25	100	
	0.6	1.0	1.52	25	
40	1.3	-3.4	2.82	100	
	0.1	-1.7	1.13	100	
80	0.0	-2.9	2.30	100	
	0.8	-1.8	1.26	100	
	-1.0	-2.8	2.24	82	
	-1.3	-2.1	1.50	52	
	-0.7	-2.2	1.61	90	
	-0.1	-1.6	1.05	100	

## Appendix I. Continued.

Site	P fertilization dose (kg P <sub>2</sub> O <sub>5</sub> .ha <sup>-1</sup> )	δ <sup>15</sup> N <sub>leg</sub> (‰)	δ <sup>15</sup> N <sub>ref</sub> (‰)	δ <sup>15</sup> N <sub>ref</sub> -δ <sup>15</sup> N <sub>fix</sub> absolute difference (‰)	%Ndfa	
Caudos	120	-1.7	-2.0	1.48	20	
		0.2	-3.0	2.41	100	
		-1.7	-3.1	2.54	56	
		-0.9	-1.9	1.34	71	
		-0.8	-3.5	2.94	92	
	0	0.1	-2.2	1.70	100	
		-1.0	-1.8	1.27	61	
		-1.1	-2.1	1.56	63	
		40	-0.2	-2.2	1.64	100
			-0.5	0.5	1.04	92
Clochettes	0	-0.6	-4.0	3.48	98	
		-0.6	-3.6	3.08	99	
		-1.1	-1.9	1.35	57	
		-1.3	-2.8	2.28	67	
		0.4	-2.7	2.12	100	
	80	-1.8	-1.8	1.28	4	
		-0.8	-2.3	1.73	87	
		-0.5	-2.1	1.56	100	
		-0.9	-3.2	2.69	86	
		-0.3	-2.6	2.10	100	
Grand Ludee	0	-1.0	-3.5	2.96	86	
		-1.8	-2.6	2.08	38	
	120	-2.0	-2.7	2.11	32	
		-2.6	-4.7	4.20	50	
		0.2	-2.2	1.61	100	
		0.9	-3.0	2.47	100	